CONSERVATION OF *CRINUM MALABARICUM* LEKHAK & S.R.YADAV (AMARYLLIDACEAE): A CRITICALLY ENDANGERED AQUATIC ANGIOSPERM

Thesis submitted to
the University of Calicut in partial fulfilment of
the requirement for the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

By

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CERTIFICATE

This is to certify that the thesis entitled **"Conservation of *Crinum malabaricum* Lekhak & S.R.Yadav (Amaryllidaceae): a critically endangered aquatic angiosperm"** submitted to the University of Calicut by **Mr. Harshid P.,** in partial fulfilment for the award of the degree of Doctor of Philosophy in Botany is a bonafide record of the research work carried out by him under my supervision and guidance. No part of the present work has previously formed the basis for the award of any other degree or diploma.

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DECLARATION

I hereby declare that the work presented in the thesis entitled **"Conservation of *Crinum malabaricum* Lekhak & S.R. Yadav (Amaryllidaceae): a critically endangered aquatic angiosperm"** is based on the original work done by me under the guidance of **Dr. N. S. Pradeep** and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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Abstract

Conserving critically endangered plant species requires a multifaceted approach combining ecological, molecular, *in situ*, and *ex situ* strategies. *Crinum malabaricum* Lekhak & S.R.Yadav, a rare and endemic aquatic plant found only in seasonal laterite pools of Northern Kerala, faces significant threats due to habitat fragmentation, changes in hydrology, and human disturbances. This study aims to develop a comprehensive conservation framework by assessing the species' ecological requirements, identifying key threats, exploring its evolutionary lineage, and implementing integrated conservation measures.

Ecological and phenological assessments were conducted to understand the habitat requirements and reproductive biology of *C. malabaricum.* Field studies confirmed that its life cycle is closely linked to seasonal monsoon patterns, and its limited dispersal mechanism further restricts its range. Habitat suitability assessments revealed that, despite identifying new potential sites since 2016, the area of occupancy (AOO) of the species remains critically low. This reinforces its Critically Endangered (CR) classification under IUCN Red List criteria.

Phytochemical analyses were carried out to determine the bioactive properties of *C. malabaricum*, revealing significant alkaloid content with notable antioxidant and acetylcholinesterase inhibitory activities. These findings underscore the pharmacological potential of the species, which highlights the importance of conservation initiatives that incorporate sustainable utilization strategies.

Molecular phylogenetic analysis utilizing ITS and *trnL-F* markers has shed light on the evolutionary lineage of the species, confirming its close genetic relationship with *Crinum thaianum* from Southeast Asia. The study indicates that *C. malabaricum* likely evolved as a neo-endemic species due to the unique climatic and geographical conditions of the Western Ghats, diverging from its ancestral lineages traced to Southeast Asia and Pacific Island biotas. This genetic distinctiveness highlights the need for targeted conservation efforts.

To support species recovery, Ecological Niche Modeling (ENM) was employed to identify suitable sites for translocation. This method successfully guided the reintroduction of *C. malabaricum* to selected locations within the Malabar Plain, serving as a pilot initiative for long-term conservation. Additionally, *ex situ* conservation efforts were implemented, including cryopreservation techniques and the maintenance of live collections at the Malabar Botanical Garden and Institute for Plant Sciences. A novel potting method was developed to ensure the species' survival under controlled conditions, thereby securing a genetic reservoir for future restoration efforts.

Additionally, habitat restoration and protection measures were undertaken to mitigate threats and promote community-led conservation initiatives. A formal proposal was submitted to the Kerala State Biodiversity Board to designate the natural habitat of *C. malabaricum* as a Local Biodiversity Heritage Site (LBHS) under the Biological Diversity Act, 2002 (Amendment act, 2023).

This study serves as a model for developing species-specific conservation strategies by integrating ecological research, molecular insights, and conservation technologies. It emphasises the importance of long-term monitoring, habitat protection, and sustainable use of resources. By connecting scientific research with conservation efforts, this study contributes to the broader goal of preserving endangered aquatic flora and other biodiversity in the laterite plateaus of the Western Ghats and beyond.

Key words: *Crinum malabaricum*; Critically endangered species; Laterite plateaus; Aquatic endemic plants; Ecological niche modelling; Molecular phylogeny; Species conservation; Cryopreservation; Species translocation.

സംഗ്രഹം

വംശനാശ ഭീഷണി നേരിടുന്ന സസ്യങ്ങളുടെ സംരക്ഷണത്തിനായി പരിസ്ഥിതി ശാസ്ത്രം, പരിണാമ പഠനം, സംരക്ഷണ ശാസ്ത്രം എന്നിവ സംയോജിപ്പിച്ചുള്ള ശാസ്ത്രീയ പ്രയത്നം ആവശ്യമാണ്. വടക്കൻ കേരളത്തിലെ ചെങ്കൽ തോടുകളിൽ മാത്രം കണ്ടുവരുന്ന അപൂർവ്വ ജലസസ്യമാണ് *ക്രൈനം മലബാറിക്കം* (*Crinum malabaricum*). പൂത്താലി, പോളതാളി എന്നീ പ്രാദേശിക നാമങ്ങളിൽ അറിയപ്പെടുന്ന ഇവ മഴക്കാലങ്ങളിൽ മാത്രമാണ് വളരുന്നത്.

നിലവിൽ ഈ സസ്യം ആവാസവ്യവസ്ഥയുടെ തകർച്ച, ജലചംക്രമണ വ്യതിയാനങ്ങൾ, മനുഷ്യഇടപെടലുകൾ എന്നിവ മൂലമുള്ള ഗുരുതരമായ ഭീഷണികൾ നേരിടുന്നു. ഈ സസ്യത്തിന്റെ സംരക്ഷണത്തിനായി, പാരിസ്ഥിതിക ആവശ്യങ്ങൾ വിലയിരുത്തി, പ്രധാന ഭീഷണികളെ തിരിച്ചറിഞ്ഞ്, അതിന്റെ പരിണാമാപരമായ ചരിത്രം മനസിലാക്കി, ഇൻസിറ്റു (*in situ*) എക്സ്സിറ്റു (*ex situ*) എന്നീ മാർഗങ്ങൾ സംയോജിപിച്ചുള്ള ഒരു സമഗ്രമായ സംരക്ഷണ പദ്ധതി വികസിപ്പിക്കുകയെന്ന ലക്ഷ്യത്തോടെയാണ് ഈ പഠനം നടപ്പാക്കിയിരിക്കുന്നത്.

ക്രൈനം മലബാറിക്കത്തിന്റെ ആവാസവ്യവസ്ഥയുടെ ഘടനാപരവും പാരിസ്ഥിതികവുമായ പ്രത്യേകതകളും അവയിൽ സംഭവിക്കുന്ന കാലിക മാറ്റങ്ങളും സംബന്ധിച്ചുള്ള പഠനങ്ങളിലൂടെ, ഈ സസ്യത്തിന്റെ ആവാസപരമായ ആവശ്യങ്ങളും പ്രതുല്പാദന രീതികളും മനസ്സിലാക്കാൻ സാധിച്ചു. ഫീൽഡ് പഠനങ്ങൾ പ്രകാരം, ഈ സസ്യത്തിന്റെ ജീവചക്രത്തിൽ കാലാവസ്ഥാ വ്യതിയാനം, പ്രത്യേകിച്ച് മൺസൂൺ കാലത്തെ മഴയുടെ വ്യത്യാസങ്ങൾ, പ്രധാനമായ സ്വാധീനം ചെലുത്തുന്നതായി കണ്ടെത്തിയിട്ടുണ്ട്. അതു കൂടാതെ ഇവയിൽ വിജയകരമായ സസ്യ വിതരണ രീതി ഇല്ലാത്തതും ഇവയുടെ വിന്യാസ പരിധിയെ നിയന്ത്രിക്കുന്നു. 2016-ൽ ക്രൈനം മലബാറിക്കത്തിന്റെ പുതിയ ആവാസവ്യവസ്ഥകൾ തിരിച്ചറിഞ്ഞിട്ടുണ്ടെങ്കിലും, ഈ സ്പീഷീസിന്റെ ആവാസ പ്രദേശം (ഏരിയ ഓഫ് ഒക്ക്യുപെൻസി) 10 ചതുരശ്ര കിലോമീറ്ററിനും താഴെയായുള്ള പ്രദേശത്തേക്ക് പരിമിതമാണ്, അതിനാൽ ഇവയെ ഐ. യൂ. സി. എൻ. മാനദണ്ഡങ്ങൾ പ്രകാരം വംശനാശ ഭീഷണി നേരിടുന്ന ജീവജാലങ്ങളുടെ പട്ടികയിൽ 'അതി തീവ്ര അപകടനിലയിലായ' (Critically Endangered)' വിഭാഗത്തിൽ നിലനിർത്തേണ്ടതുണ്ടെന്ന് ഈ പഠനം നിർദേശിക്കുന്നു.

ക്രൈനം മലബാറിക്കത്തിന്റെ സസ്യരാസഘടനയെ കുറിച്ചുള്ള വിശകലനത്തിലൂടെ, ഔഷധഗുണമുള്ള ആൽക്കലോയിഡുകൾ ഈ സസ്യത്തിൽ ധാരാളമായി അടങ്ങിയിരിക്കുന്നതായി കണ്ടെത്തിയിട്ടുണ്ട്. കൂടാതെ, ശക്തമായ ആന്റിഓക്സിഡന്റ് ഗുണങ്ങളും അസെറ്റിൽകൊളിൻ എസ്റ്ററേസ് ഇൻഹിബിഷൻ (acetylcholinesterase inhibition) പ്രവർത്തനങ്ങളും കാണപ്പെടുന്നുണ്ട്, ഇത് മറവി സംബന്ധമായ രോഗങ്ങളുടെ ചികിത്സയ്ക്ക് സഹായകരമാകാം. ഈ കണ്ടെത്തലുകൾ, സസ്യത്തിന്റെ ഔഷധപരവും

സാമ്പത്തിക മൂല്യവുമുള്ള സാധ്യതകൾ തെളിയിക്കുകയും, ഇവയുടെ സംരക്ഷണ പ്രയത്നങ്ങൾക്ക് ഊർജം പകരുകയും ചെയ്യുന്നു.

ITS, *trnL-F* മാർക്കറുകൾ ഉപയോഗിച്ചുള്ള പരിണാമ പരമായ പഠനങ്ങൾ (Molecular Phylogenetic studies) ഈ സസ്യതിന്റെ പരിണാമ ചരിത്രം വെളിപ്പെടുത്തുന്നതിനും മറ്റു സസ്യങ്ങളുമായുള്ള ബന്ധങ്ങൾ മനസിലാക്കുന്നതിനും സഹായകരമായി. ക്രൈനം *തായിയാനം* (*Crinum thaianum*) എന്ന തെക്കുകിഴക്കൻ ഏഷ്യൻ സ്പീഷീസുമായുള്ള ക്രൈനം മലബാറിക്കത്തിന്റെ അടുത്ത ബന്ധം ഈ പഠനത്തിലൂടെ സ്ഥിതീകരിച്ചു. ക്രൈനം മലബാറിക്കം ഒരു പുതുതായി പരിണമിക്കപ്പെട്ട ‘നിയോ എൻഡമിക്ക്’ (neo-endemic) സ്പീഷിസാണെന്നും, അതിന്റെ ഉത്ഭവം പശ്ചിമഘട്ടത്തിലെ പരിസ്ഥിതി പരമായ പ്രത്യേകതകളുടേയും കാലാവസ്ഥാ വ്യത്യാസങ്ങളുടേയും ഫലമായി സംഭവിച്ചതാകാമെന്നും ഈ പഠനം സൂചിപ്പിക്കുന്നു.

ക്രൈനം മലബാറിക്കത്തിന്റെ പുനസ്ഥാപനം ഉറപ്പാക്കുന്നതിനായി, ആവാസ വ്യവസ്ഥ മോഡലിംഗ് (Ecological Niche Modeling - ENM) സാങ്കേതികവിദ്യ പ്രയോജനപെടുത്തി അനുയോജ്യമായ ആവാസവ്യവസ്ഥകൾ തിരിച്ചറിഞ്ഞു. തിരഞ്ഞെടുക്കപ്പെട്ട സ്ഥലങ്ങളിൽ ഈ സസ്യം വിജയകരമായി വളർത്തുകയുണ്ടായി. ഇത് ഇവയുടെ ദീർഘകാല സംരക്ഷണ പദ്ധതികളുടെ ആദ്യ ചുവടുവെപ്പായി. കൂടാതെ, എക്സ്സിറ്റു സംരക്ഷണം നടപ്പിലാക്കുനതിനായി ഇവയെ സംരക്ഷിത സാഹചര്യത്തിൽ വളർത്തുകയും ക്രയോപ്രിസർവേഷൻ സാങ്കേതിക വിദ്യകൾ ഉപയോഗിച്ച് ഇതിന്റെ ജനിതക വിഭവങ്ങൾ സംരക്ഷിക്കുകയും ചെയ്തു. ഇതോടൊപ്പം ക്രൈനം മലബാറിക്കത്തിന്റെ പ്രകൃതിദത്ത ആവാസവ്യവസ്ഥകളെ ജൈവ വൈവിധ്യ നിയമം, 2002 (ഭേദഗതി നിയമം, 2023) ന്റെ അടിസ്ഥാനത്തിൽ പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക കേന്ദ്രങ്ങളായി (LBHS) പ്രഖ്യാപിക്കുന്നതിനായി കേരള സംസ്ഥാന ജൈവവൈവിധ്യ ബോർഡിന് ഔദ്യോഗിക നിർദ്ദേശം സമർപ്പിച്ചു.

ഈ പഠനം, പരിസ്ഥിതി-ഗവേഷണം, പരിണാമ-പഠനങ്ങൾ, സംരക്ഷണ സാങ്കേതികവിദ്യകൾ എന്നിവ സംയോജിപ്പിച്ചുള്ള ജൈവവൈവിധ്യ സംരക്ഷണത്തിന്റെ മാതൃകയായി നിലകൊള്ളുന്നു. ശാസ്ത്രീയ ഗവേഷണവും സംരക്ഷണ പ്രവർത്തനങ്ങളും തമ്മിലുള്ള ബന്ധം ഊട്ടിയുറപ്പിച്ചു പ്രവർത്തിക്കുന്ന ഈ പഠനം, പശ്ചിമഘട്ടത്തിലെ അപൂർവ്വ ജലസസ്യങ്ങളുടെയും വിശിഷ്യ ചെങ്കൽ കുന്നുകളിലെ മറ്റു ജൈവ വൈവിധ്യങ്ങളുടെയും സംരക്ഷണം ഉറപ്പാക്കുന്നതിന് നിർണ്ണായക സംഭാവന നൽകുന്നു.

സൂചന പദങ്ങൾ: *ക്രൈനം മലബാറിക്കം;* അതി തീവ്ര അപകടനിലയിലായ ജീവികൾ; ചെങ്കൽ കുന്നുകൾ; ജല സസ്യങ്ങൾ; ആവാസ വ്യവസ്ഥ മോഡലിംഗ്; ജീവി സംരക്ഷണം; പരിണാമ പഠനം: ക്രയോ പ്രിസേർവേഷൻ; ജീവി പുനസ്ഥാപനം

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPHS

LIST OF ABBREVIATIONS

%	:	Percentage
µg	:	Microgram
µM	:	Micro molar
µm	:	Micrometer
µmol	:	Micromole
^{0}	:	Degree
^{0}C	:	Centigrade
"N	:	North Latitude
$<, >, =, \leq, \geq$	:	Mathematical symbols indicating less than, greater than, equal to, less than or equal to, and greater than or equal to.
"E	:	East Longitude
ABTS	:	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Antioxidant assay)
AChE	:	Acetylcholinesterase
ACN	:	Acetonitrile
AIC	:	Akaike Information Criterion
$AlCl_3$	:	Aluminium Chloride
AM	:	Ante Meridiem
ANOVA	:	Analysis of Variance
AOO	:	Area of Occupancy
APG	:	Angiosperm Phylogeny Group
Au/Pd	:	Gold-Palladium coating
B & K salts	:	Brewbaker and Kwack salts
BBCH	:	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (scale for phenological growth stages)
BHT	:	Butylated Hydroxytoluene
Bioclim	:	Bioclimatic Modeling
BRT	:	Boosted Regression Trees
cpDNA	:	Chloroplast DNA
CR	:	Critically Endangered
CRD	:	Completely Randomized Design
DEA	:	Diethanolamine

DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
DPPH	2,2-Diphenyl-1-picrylhydrazyl (Antioxidant assay)
DTNB	Di-thiobis nitro benzoic acid (Ellman's Reagent)
ENM	Ecological Niche Modeling
EOO	Extent of Occurrence
eV	Electron Volt
$g\ L^{-1}$	Grams per Liter
GA	Gallic Acid
GAL	Galanthamine (alkaloid)
GAM	Generalized Additive Model
GARP	Genetic Algorithm for Rule-set Prediction
GC-MS	Gas Chromatography-Mass Spectrometry
GelDoc	Gel Documentation System
GeoCAT	Geospatial Conservation Assessment Tool
GIS	Geographic Information System
GLM	Generalized Linear Model
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HPLC	High-Performance Liquid Chromatography
IBM	International Business Machines Corporation
IMD	India Meteorological Department
ITS	Internal Transcribed Spacer
IUCN	International Union for Conservation of Nature
IUCN SSC	IUCN Species Survival Commission
KDE	Kernel Density Estimation
km	Kilometer
km^2	Square kilometers
LC-MS	Liquid Chromatography-Mass Spectrometry
LINEs	Long Interspersed Nuclear Elements
LY	Luteolin-7-O-glucoside (plant compound)
m	Meter
M	Molar Concentration
MA	Model Accuracy

MatK	: Maturase K gene
Maxent	: Maximum Entropy Model
MBGH	: Malabar Botanical Garden Herbarium
MBGIPS	: Malabar Botanical Garden and Institute for Plant Sciences
MEGA	: Molecular Evolutionary Genetics Analysis
mg	: Milligram
mg GAE/g E	: Milligrams of Gallic Acid Equivalent per Gram of Extract
mg LE/g E	: Milligrams of Lycorine Equivalent per Gram of Extract
mg QE/g E	: Milligrams of Quercetin Equivalent per Gram of Extract
mg/mL	: Milligrams per Milliliter
ml	: Milliliter
mm	: Millimeter
MS	: Mass Spectrometry
n	: Sample size
N	: Normality (concentration unit)
NAChRs	: Nicotinic Acetylcholine Receptors
$NaNO_2$	: Sodium Nitrite
NaOH	: Sodium Hydroxide
NbClust	: R package for clustering analysis
NGD	: Normalized Gap Distance
NIST	: National Institute of Standards and Technology
nm	: Nanometer
no.	: Number
NPC	: Non-Protein Coding DNA
NTFPs	: Non-Timber Forest Products
PCA	: Principal Component Analysis
PCR	: Polymerase Chain Reaction
PDA	: Photodiode Array Detector
PEG	: Polyethylene Glycol
PGM	: Plant Growth Medium
pH	: Acidity or Basicity of a Solution
PM	Post Meridiem
POWO	: Plants of the World Online
QGIS	: Quantum Geographic Information System software)
r^2	: Coefficient of Determination (Regression)

RAPD	Random Amplified Polymorphic DNA
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene
RET	Rare, Endangered, and Threatened species
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme
SDMs	Species Distribution Models
Sec.	Seconds
SEM	Scanning Electron Microscopy
SINEs	Short Interspersed Nuclear Elements
spp.	Species (plural)
SPSS	Statistical Package for the Social Sciences
SVM	Support Vector Machine
TAC	Total Alkaloid Content
TE	Tris-EDTA Buffer
TFA	Trifluoroacetic Acid
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
trnL-F	Chloroplast trnL intron and trnL-F intergenic spacer
TTC	2,3,5-Triphenyltetrazolium chloride
USA-NPN	USA National Phenology Network
USGS	United States Geological Survey
UV-Vis	Ultraviolet-Visible Spectroscopy
VI-S	Visible Spectroscopy
VU	Vulnerable
ρ-	Spearman's Rank Correlation Coefficient

Chapter 1

General Introduction

Chapter 1

General Introduction

1.1. Aquatic Biodiversity in India

Biodiversity encompasses the variety of all life forms on Earth, including the communities they form and the habitats in which they thrive. The Convention on Biological Diversity defines biodiversity as "the variability among living organisms from all sources, including terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part; this includes diversity within species, between species, and of ecosystems" (Glowka, 1994). India is rich in biodiversity and cultural diversity, harbours over 7% of the world's biodiversity on merely 2.5% of the Earth's surface. Biodiversity is among the most critical natural resources for sustaining livelihoods and fostering development. In developing nations like India, the conservation and sustainable utilization of its resources are imperative.

Aquatic ecosystems, encompassing rivers, lakes, ponds, wetlands, and other freshwater habitats, play a crucial role in maintaining ecological balance. These ecosystems provide numerous services, such as water storage, carbon sequestration, and habitat for a diverse range of flora and fauna. They also support the livelihoods of millions who depend on these resources for food, water, and cultural practices. Historically, aquatic plants have played a vital role in human life, serving as food sources, fodder, and medicinal resources. However, with changing lifestyles and economic priorities, many aquatic plants have been neglected, often dismissed as weeds, while freshwater ecosystems face degradation.

India is recognized as a megadiverse nation with extensive aquatic biodiversity, supporting the livelihoods of over 1.4 billion people. The country is home to 730 wetland flowering plants, including 114 endemic species (Ansari *et al.,* 2016). Aquatic plants in India possess significant medicinal value, comparable to that of terrestrial herbs. Historically, these plants have been used to treat severe diseases, including cancer and genetic disorders. Unfortunately, the medicinal applications of aquatic plants are now primarily overlooked (Swapna *et al.,* 2011).

The rapid pace of India's economic growth and urbanization poses significant challenges to the conservation of its diverse aquatic ecosystems. This is exacerbated by a lack of information on the distribution, status, and threats aquatic species face. Anthropogenic activities, such as infrastructure development and industrial expansion, place these ecosystems under immense pressure, threatening their ecological integrity. This degradation risks the loss of lesser-known biodiversity, including ecologically, economically, and medicinally valuable plants. Addressing these threats is crucial to preserving India's aquatic biodiversity and the invaluable resources it provides.

1.2. Aquatic Floral Diversity in the Laterite Plateaus of Malabar Plains

The Malabar Plains of the Western Ghats are characterized by a unique phytogeography influenced by the region's monsoon climate. This area experiences high seasonal rainfall, resulting in diverse vegetation types. The plains primarily comprise red soils, laterites, and black soils, each supporting distinct plant communities (Subramanyam & Nayar, 1974).

The laterite plateaus of the Malabar Plains are significant geological formations characterized by their unique composition and structure. Laterite plateaus are distinctive ecosystems due to their unique substratum and extreme environmental conditions. The hard crusts of laterite are primarily distributed along the western coast, extending from Dapoli in Ratnagiri district of Maharashtra, to Malappuram district in Kerala (Balakrishnan *et al.,* 2011). These plateaus are known for their seasonal dynamics, which significantly impact their biodiversity.

The rock pools of the Western Ghats exhibit varying water quality, shaped by factors such as bedrock type, precipitation, and seasonal variations, contributing to a high level of endemic biodiversity (Kulkarni *et al.*, 2021). The aquatic plant diversity in the lateritic plateaus of the Malabar Plains is particularly notable, characterized by a rich array of endemic species. However, this biodiversity faces challenges from anthropogenic activities and the spread of invasive aquatic weeds.

Due to their harsh physical environment, the laterite plateaus in northern Kerala represent a unique ecosystem. These plateaus support specialized plant communities, including many endemic and habitat-specific species. During the monsoon, many seasonal streams and ephemeral ponds form on these plateaus, which host a diverse range of hydrophytes, including endemic species of varying rarity. During post-monsoon seasons, these water bodies dry up, leaving the plateaus arid until the pre-monsoon period (Pramod & Pradeep, 2021).

Despite their ecological importance, laterite plateaus are often mischaracterized as "wastelands," making them highly susceptible to developmental activities, including infrastructure expansion, waste dumping, and mining (Mudke *et al.*, 2020). Large-scale mining for bricks and clay poses one of the most severe threats to the biodiversity of these plateaus. Such activities have profound impacts, both topographically and biologically, endangering these unique ecosystems and their endemic species (Muraleedharan, 2011; Pramod & Pradeep, 2021).

The laterite plateaus of the Malabar Plains are facing mounting threats from human activities and invasive species. Effective conservation measures are essential to safeguard these fragile ecosystems and ensure the survival of their endemic plant species.

1.3. Endemism in Plants

Endemism in plants refers to species that are geographically restricted and evolutionarily unique, often confined to specific regions due to historical, ecological, and environmental factors. These endemic species play a vital role in maintaining biodiversity and ensuring ecosystem stability but face significant conservation challenges. The primary drivers of plant endemism include climatic stability, geographic isolation, and environmental heterogeneity, which foster both neoendemism (recently evolved endemic species) and paleoendemism (ancient endemic species).

Endemic plant species are particularly vulnerable to environmental changes and human activities. Key threats include habitat loss and fragmentation due to land-use changes, land cover conversion, and the introduction of invasive species. Climate change escalates these challenges by causing severe weather events, altering habitat conditions, and threatening species' survival with narrow ecological niches (Hobohm & Barker, 2023).

Many endemic plants are characterized by small or declining population sizes, making them highly susceptible to extinction. Anthropogenic pressures, such as overexploitation, nutrient pollution, and urbanization, act independently or synergistically to drive these species toward rarity. Identifying and prioritizing regions with high endemism, such as biodiversity hotspots and specific biogeographic provinces, is essential for developing effective conservation strategies (Carmona *et al.*, 2024).

Understanding the underlying causes of endemism—whether they arise from geographic isolation, ecological specialization, or evolutionary history—is crucial for conserving endemic species. Protecting these unique plants requires targeted efforts, including habitat restoration, climate-resilient strategies, and stricter regulations to mitigate human impacts.

1.4. *Crinum malabaricum*

Crinum malabaricum Lekhak & S.R. Yadav, commonly known as the Malabar River lily (Amaryllidaceae), is an aquatic perennial plant endemic to the northern districts of Kerala. It is categorized as critically endangered under the IUCN Red List criteria (Lansdown, 2016). Initially, this species was discovered in a seasonal stream in Periye village, Kasaragod district, Kerala, with a population of fewer than 300 individuals (Lekhak & Yadav, 2012). Subsequent studies identified three additional populations in Embate and Aravanchal (Kannur district) and Cheemeni (Kasaragod district).

C. malabaricum is reported to contain several medicinally significant alkaloids with analgesic, antiviral, and antitumor properties. Among Indian *Crinum* species, it exhibits the highest concentration of galantamine, a selective and reversible competitive inhibitor of acetylcholine esterase. Galantamine is widely recognized for its therapeutic potential, particularly in treating Alzheimer's disease. Additionally, the species contains lycorine alkaloids, which exhibit anti-inflammatory, antimalarial, and antitumor properties (Ghane *et al.,* 2018).

The natural habitats of *C. malabaricum* are severely threatened by anthropogenic activities, leading to rapid population declines. Moreover, all known habitats of this species are located on non-protected lands, and are labelled as "wastelands." The pressures of urbanization and industrial development have further jeopardized the survival of this critically endangered species in its natural environment (Patil *et al.*, 2015).

1.5. Conservation of Threatened Species

Effective conservation planning necessitates a comprehensive approach that encompasses assessing, planning, and implementing actions to address species' conservation needs. The IUCN SSC Conservation Planning Specialist Group advocates for scientifically rigorous and well-resourced conservation plans to reverse species decline (Byers *et al.*, 2022). A key component of this approach is species prioritization, which involves ranking species based on their extinction risk, ecological significance, and socioeconomic value. For example, in the Indian Himalayan Region, conservation efforts have prioritized species that are at the highest risk of extinction and those playing pivotal ecological roles (Mehta *et al.*, 2020). This targeted prioritization ensures the optimal allocation of limited conservation resources to safeguard the most vulnerable and ecologically significant species.

The conservation of threatened aquatic plants is of particular significance due to their critical ecological functions and increasing vulnerability to anthropogenic pressures and climate change. Conservation strategies for these plants range from genetic diversity studies and *in vitro* regeneration techniques to habitat restoration and *ex situ* conservation. Much research on endangered aquatic species highlights the importance of integrating genetic analysis with species distribution modelling to guide seed banking, habitat restoration, and reintroduction efforts (Guo *et al.*, 2019; Makuch *et al.*, 2023). Furthermore, ecological assessments of aquatic plants have provided crucial insights into habitat requirements, guiding conservation actions tailored to specific environmental conditions (Azzella *et al.*, 2024).

The present study focuses on the conservation and sustainable use of *Crinum malabaricum*, a species characterized by high habitat specificity and endemism. This research addresses critical questions related to the species' conservation by predicting its potential geographical distribution under current and future climatic scenarios. It aims to understand the ecological and evolutionary factors driving its endemism, assess its phylogenetic relationships with closely related species, and investigate the evolutionary processes that influence its current status. The study further examines the conservation status of *C. malabaricum* to develop comprehensive strategies for both *in situ* and *ex situ* conservation. A primary objective is identifying the medicinally significant alkaloids present in the species, thereby highlighting its potential for pharmaceutical applications. This research integrates diverse methodologies, including species niche modelling, phylogenetic analysis, and phenological studies, to generate critical insights into the ecological and evolutionary dynamics of *C. malabaricum.* Ultimately, these findings will inform the development of effective conservation strategies and promote sustainable utilization of this endangered species.

Objectives

1. Ecological niche modelling to define, predict and reflect the adaptive landscape of *C. malabaricum.*
2. Phenology studies on *C. malabaricum* to understand the limiting factors in the survival of the population.
3. Molecular phylogenetic analysis of *Crinum* spp. of India to assess genetic variations and relationships within the genus.
4. Chemical profiling of *C. malabaricum* for potential pharmacognostic evaluation.
5. Development of a conservation strategy for *C. malabaricum*.

Chapter 2

Review of Literature

Chapter 2

Review of Literature

2.1. Conservation Perspectives of Aquatic Plants of India

India is a mega-diversity centre of aquatic flora, which has attracted the attention of botanists since the early twentieth century. Even though their conservation was not adequately addressed, many studies documented Indian aquatic biodiversity starting from Biswas and Calder (1984), who were the first to provide a comprehensive description of the aquatic flora of the Indian subcontinent. Subramanyam (1962) recorded the morphology and distribution of 117 taxa of aquatic vascular plants in India long before the Ramsar Convention acknowledged the importance of wetlands to the world. Deb (1975) described the distribution and condition of 144 aquatic and wetland species from several Indian states. The major remarkable work was Cook's *'Aquatic and Wetland plants of India'* (1996), significantly contributing to the field of study. Later, many studies were conducted based on the economic and medicinal aspects of aquatic plants. Lavania *et al.* (1990) developed a list of wetland plants of the Indian subcontinent, which comprised 470 species from aquatic and semi-aquatic habitats. The present-day comprehensive study from Kerala in this field was *'Aquatic and Wetland Flora of Kerala: Flowering Plants'* by Ansari *et al.* (2016).

Aquatic plant diversity of India is integral to the country's biodiversity, contributing significantly to ecosystem stability, phytoremediation, and aquatic food webs. The country is home to many aquatic plant species, many of which are endemic and essential for sustaining freshwater ecosystems. Freshwater ecosystems, including rivers, lakes, and wetlands, serve as crucial habitats supporting diverse aquatic flora (Sengupta & Dayanandan, 2022).

Despite India's rich aquatic plant diversity, these ecosystems face significant threats. The pace of growth of the Indian economy and rates of industrial and urban development are noted in tune with the conservation needs of its freshwater ecosystems and remarkably high species diversity. The consideration of this biodiversity during economic growth is mainly due to the lack of adequate information on freshwater species' diversity distribution and status (Molur *et al.*, 2011). The threats to aquatic plants range from habitat destruction, eutrophication, pollution, invasive species, and climate change. Rapid urbanization, agricultural expansion, and industrialization have led to habitat degradation and declining water quality (Sengupta & Dayanandan, 2022). The uncontrolled spread of invasive aquatic species, such as *Eichhornia crassipes* and *Salvinia molesta*, disrupts native plant communities by outcompeting indigenous species and altering aquatic habitats (Kawade *et al.,* 2023; Sedyaaw *et al.,* 2024). Climate change-induced shifts in precipitation patterns and rising temperatures further modify species distributions and increase ecosystem vulnerability (Arya, 2021). Additionally, crucial habitats such as estuaries are experiencing rapid degradation due to pollution and encroachment, necessitating immediate conservation measures (Freeman *et al.,* 2019). The current IUCN Red List criteria may not comprehensively assess the extinction risks faced by smaller aquatic plant species, highlighting the need for refined evaluation methodologies to ensure effective conservation planning (Manawaduge *et al.,* 2019).

Addressing these challenges requires an integrated conservation strategy that combines *in situ* and *ex situ* approaches. Habitat protection through continuous monitoring and restoration efforts is essential to safeguarding aquatic ecosystems (Chemeris *et al.,* 2019). *Ex situ* conservation initiatives, such as establishing artificial wetland ecosystems, botanical gardens, and seed banks, are crucial in preserving genetic diversity and supporting research on threatened aquatic plant species. Community participation in conservation programs has proven vital for sustainable ecosystem management. Technological advancements, including GIS-based mapping and satellite monitoring, facilitate biodiversity assessments and enable early detection of environmental changes. Strengthening legislative frameworks, such as enforcing the Biological Diversity Act of 2002 and implementing targeted conservation policies, is critical for ensuring the long-term protection of aquatic plant diversity. Additionally, the management of invasive species through biological control methods has shown promise in mitigating their ecological impact (Kapoor & Usha, 2020; Sengupta & Dayanandan, 2022; Kawade *et al.,* 2023; Saravanan & Panda, 2024; Sedyaaw *et al.,* 2024). Advances in ecological survey techniques, including stable isotope analysis and ecological modelling, further enhance our understanding of aquatic plant ecosystems and inform evidence-based conservation strategies (Cherry & Pec, 2022).

The need for the conservation of plants and their related diversity is of utmost importance, not just due to the unprecedented loss of biological diversity but also due to the benefits rendered by them, in terms of economic, social, and spiritual benefits. Aquatic plants are crucial in various domains, including medicine, economy, culture, and environmental sustainability. They are rich in bioactive compounds such as alkaloids, flavonoids, and saponins, which contribute to their antimicrobial, antioxidant, and antidiabetic properties (Aasim *et al.,* 2019; Arya *et al.,* 2021; Singh, 2024). Beyond their medicinal applications, aquatic plants also hold economic significance, particularly in aquaculture, where they serve as natural immunostimulants, promoting the health of aquatic organisms and reducing reliance on antibiotics (Liao *et al.,* 2024). Despite their commercial potential in pharmaceuticals and as food sources, their market remains underdeveloped due to limited awareness and research (Aasim *et al.,* 2019). Additionally, these plants are deeply embedded in cultural and spiritual traditions, influencing medicinal practices based on socio-economic and familial knowledge systems (Aasim *et al.,* 2019). Their ecological importance is equally notable, as they contribute to biomonitoring and phytoremediation, helping to filter pollutants and sustain aquatic ecosystems (Ansari *et al.,* 2020). By regulating nutrient cycles and supporting biodiversity, aquatic plants are essential for maintaining ecological balance (Aasim *et al.,* 2019). While their multifaceted benefits are well-documented, further research and conservation efforts are needed to fully harness their potential and ensure their sustainable utilization.

India's aquatic biodiversity is shaped by a wide array of unique habitats, including estuaries, mangroves, floodplains, river basins, reservoirs, ponds, and high-altitude wetlands. Additionally, ephemeral water bodies such as seasonal pools on laterite plateaus and monsoon-fed rock pools support specialized aquatic plant communities adapted to fluctuating water availability. These diverse ecosystems contribute to India's rich aquatic flora, each playing a crucial role in sustaining regional biodiversity.

2.2. Laterite Plateaus of Malabar Plains

Laterite is a soil and rock type rich in iron and aluminium, formed in hot and wet tropical areas through intense and prolonged weathering of the underlying parent rock. In India, laterite formations are predominantly found along the Malabar Plains of the

Western Ghats, extending from Dapoli in Ratnagiri, Maharashtra, to Malappuram District in Kerala (Figure 1) (Balakrishnan *et al.,* 2010). In Kerala, these laterite deposits are primarily located at elevations below 600 meters, forming low, flat-topped ridges and hills between the foothills of the Western Ghats and the Arabian Sea, especially from Malappuram to Kasaragod (Varghese & Byju, 1993).

These laterite plateaus are often misclassified as 'wastelands' in official records (Anonymous, 2019). However, they are ecologically significant, characterized by harsh environmental conditions such as high temperatures and low moisture during the summer months. These extreme conditions have led to the evolution of specialized vegetation, with many species exhibiting unique adaptations to survive in this environment. The severe climate influences the development of seasonal flora, with most plant species completing their life cycles during the monsoon period.

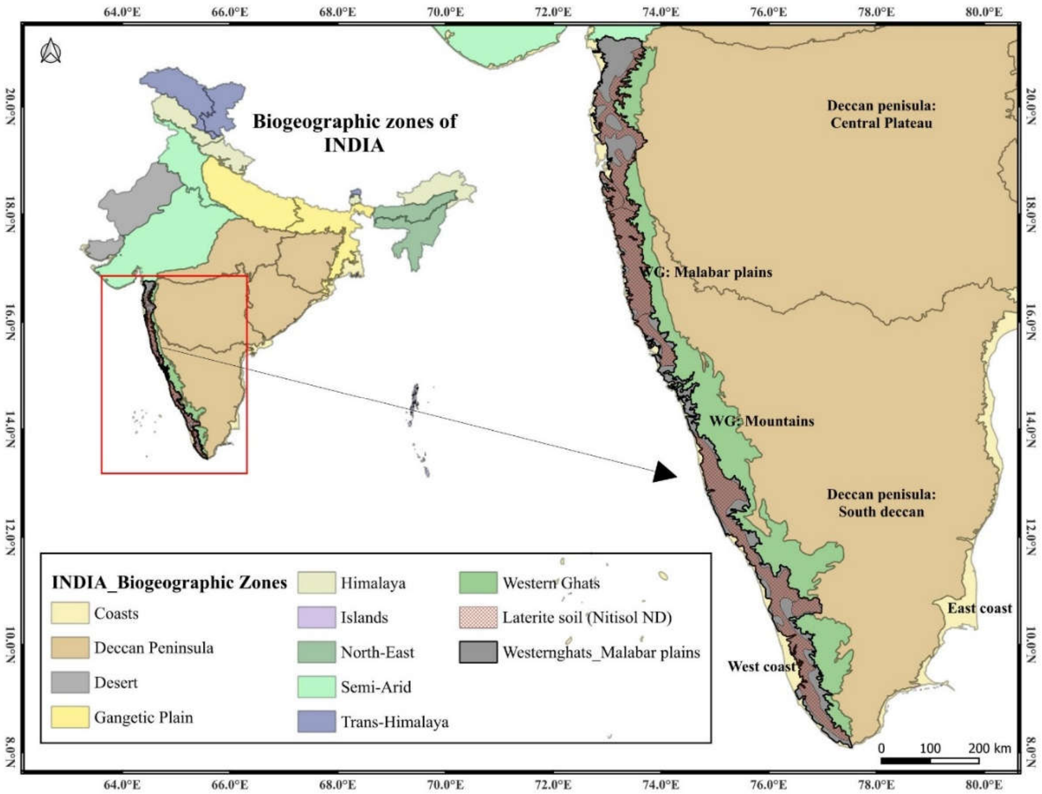


Figure 1. Biogeographic zones of India highlighting the distribution of laterite soil in the Malabar Plains of the Western Ghats

During the rainy season, the impermeable nature of the laterite rock surfaces leads to waterlogging, resulting in the formation of ephemeral pools, larger ponds and seasonal streams from the plateaus. These temporary aquatic habitats support a diverse array of hydrophytic (water-loving) plants, including several endemic species that are rare and specialized (Pramod & Pradeep, 2021). Despite their ecological importance, these plateaus are under threat due to anthropogenic activities. The lack of woody vegetation or forest cover often leads to their misinterpretation as barren lands in remote sensing images, reinforcing their designation as 'wastelands.' This misconception has led to overexploitation, including mining, the conversion of land to monoculture plantations, industrial development, and infrastructure expansion. Such activities alter drainage patterns and microhabitats, posing significant threats to the unique biodiversity of these areas (Bhattarai *et al.,* 2012; Sreejith *et al.,* 2017).

Botanical studies have reported numerous narrow-niched endemic and habitat-specialist angiosperms from these laterite plateaus throughout the Western Ghats. These regions continue to reveal new botanical discoveries, with several angiosperm species described in recent years (Biju *et al.,* 2018; Kolte *et al.,* 2018; Chandore, 2019; Anilkumar *et al.,* 2020; Borude *et al.,* 2020; Yadav, 2020; Chandore *et al.,* 2021; Gosavi *et al.,* 2022; Chappan,2024). Unfortunately, the 'wasteland' label has facilitated their degradation, as these habitats are increasingly subjected to mining, monoculture plantations, industrial activities, and infrastructure development.

Currently, only a small portion of these lateritic hills remains undisturbed. The pressures from human activities are often mismanaged due to a lack of understanding of the unique ecological features of these habitats (Watve, 2013). The lateritic hillocks in northern Kerala are particularly neglected from a conservation standpoint, as they appear barren for at least seven months (November to May). Moreover, the flora is predominantly composed of herbaceous annuals (90%), which are often undervalued compared to woody vegetation in forest ecosystems (Sreejith *et al.,* 2016).

The laterite plateaus of the Malabar Plains are unique ecosystems characterized by high biodiversity and ecological significance, particularly for specialized and endemic aquatic plants. Despite their 'wasteland' designation, these habitats support a rich array of life forms adapted to their extreme environmental conditions. *Crinum malabaricum* exemplifies the region's unique flora, exhibiting microhabitat specificity. Given the ongoing anthropogenic threats to these fragile ecosystems, urgent conservation efforts and increased awareness are essential to ensure their protection.

2.3. *Crinum malabaricum* Lekhak & S.R.Yadav

Kingdom : Plantae
Phylum : Streptophyta
Class : Equisetopsida
Subclass : Magnoliidae
Order : Asparagales
Family : Amaryllidaceae
Genus : Crinum
Species : *Crinum malabaricum*

C. malabaricum is a fully immersed herb with tunicate bulbs rooted in the watercourse bed. The bulbs are whitish, occurring in clusters of 20–30, globose, measuring 3.5–10 × 2.5–8 cm in diameter, and gradually narrowing into a neck. The neck is 3–7 × 2–3.5 cm, white to purple. Leaves are 8–19 per bulb, measuring 200–400 × 2–6 cm, whitish at the base and dark green above, spirally arranged, cartilaginous, flat, strap-shaped, many-nerved, and floating on the water surface. They gradually narrow from the base to the apex, which is acute, with margins minutely spinulate; spinules are pointed forward.

Peduncles arise laterally from the bulb, measuring 50–120 × 1.3–3.6 cm, dorsiventrally compressed, green to dark green with a purple tinge above, and whitish at the base. Involucral bracts are two, measuring 3–9 × 0.3–2.5 cm, many-nerved, initially hyaline, turning reddish-tinged at anthesis, with inflexed margins. Umbels bear 6–13 flowers. Flowers are white and sweet-scented. The pedicel is up to 1.5 cm long at anthesis.

Floral bracts measure 6.5–9 × 0.3–0.5 cm, 3–5-nerved, drooping, hyaline, sometimes reddish-tinged, broader at the base, and gradually narrowing at the apex, which is thickened. The perianth tube is 7–12.5 cm long, pale green to reddish, with shining white perianth segments measuring 5–10 × 0.7–1.3 cm, drooping at anthesis, and thickened and hooded at the apex.

Stamens six, arcuate-erect; filaments 5–7 cm long, dark red in the distal half; anthers black, measuring 1–1.5 cm long. The ovary measures 1.5–2 × 0.3–1 cm, is three-loculed, and contains ovules (Lekhak & S. R. Yadav, 2012).

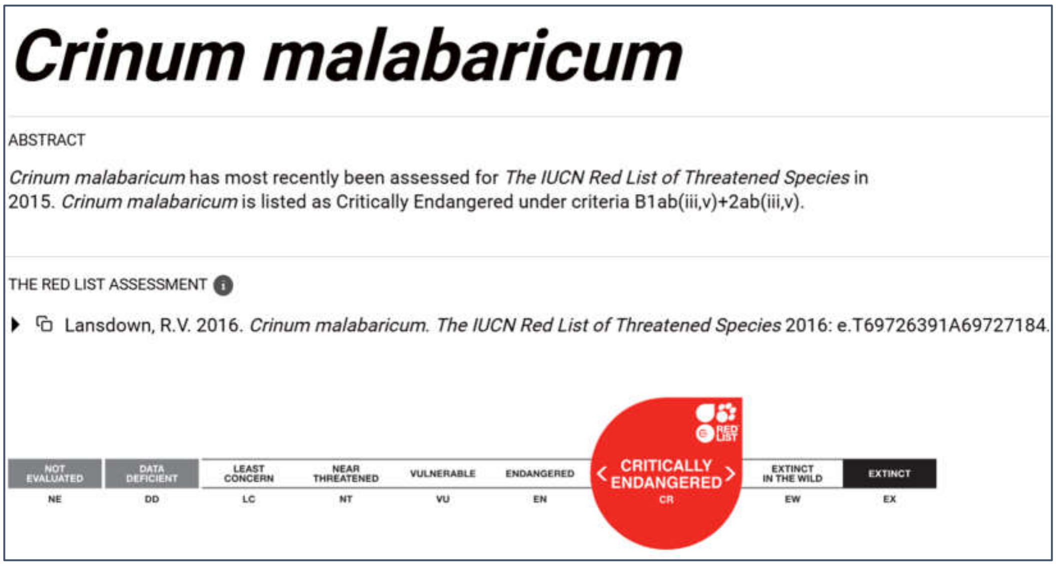
C. malabaricum morphologically resembles *C. thaianum* J. Schulze, a species endemic to the coastal plains of Ranong and Phang Nga Provinces in Thailand, but it differs in karyotype. *C. malabaricum* exhibits polyploidy with $n = 44$, which is unique among Indian *Crinum* species (Lekhak & S. R. Yadav, 2012). In India, *C. malabaricum, C. reddyii, C. solapurense* and *C. viviparum* are the species that inhabit aquatic environments, whereas the rest of the species are terrestrial. *C. viviparum* prefers marshy habitats and is an emergent species, while *C. malabaricum* is completely immersed in the watercourse. Apart from *C. thaianum* and *C. malabaricum*, other *Crinum* species exhibiting similar aquatic characteristics include *C. natans* Baker and *C. calamistratum* Bogner & Heine, which are endemic to West and West-Central Tropical Africa and Cameroon, respectively.

2.3.1. Conservation Status of *C. malabaricum*

The species was categorized as Critically Endangered (CR) under the IUCN criteria B1ab(iii,v) + 2ab(iii,v) because, at the time of assessment in 2015, it was only known from its type locality in Periye, Kasaragod. The known extent of occurrence (EOO) was limited to 1 km^2, with an estimated population of approximately 1,000 individuals (Lansdown, 2016) (Figure 2). Subsequent explorations in 2017 identified three additional habitats within a 100 km range from the type locality, specifically in Cheemeni (Kasaragod District) and Aravanchal and Embate (Kannur District, Kerala). Based on these findings, the study recommended reclassifying *C.*

malabaricum as Vulnerable, as its EOO exceeded the threshold for the Critically Endangered category (Lansdown & Molur, 2017).

However, this assessment did not fully account for the long-term threats to the species, including population decline and habitat degradation. Currently, *C. malabaricum* is found in only four natural habitats within a 50 km range, with each population consisting of approximately 100 to 500 individuals. None of these habitats are under formal protection, and two are designated as ‘waste lands.’ The species' dependence on specific habitat conditions, coupled with its limited dispersal ability, has led to a significant decline in population. Additionally, rapid urbanization and expanding industrial activities further threaten its survival in the wild (Patil *et al.,* 2015). Given these ongoing anthropogenic and natural threats, immediate conservation measures are essential to ensure the species' persistence.



Crinum malabaricum

ABSTRACT

Crinum malabaricum has most recently been assessed for *The IUCN Red List of Threatened Species* in 2015. *Crinum malabaricum* is listed as Critically Endangered under criteria B1ab(iii,v)+2ab(iii,v).

THE RED LIST ASSESSMENT

Lansdown, R.V. 2016. *Crinum malabaricum. The IUCN Red List of Threatened Species* 2016: e.T69726391A69727184.

Figure 2. Conservation status of *Crinum malabaricum* in IUCN Red List

2.3.2. Conservation Efforts for *C. malabaricum*

Attempts have been made to conserve *C. malabaricum*, particularly through *in vitro* techniques. Studies have explored *in vitro* regeneration using twin scales, meta-topolin-mediated germination, and the production of synthetic seeds, all of which have successfully facilitated the propagation of *C. malabaricum* under controlled *in vitro* conditions. These approaches not only aids in conservation but also supports the sustainable use of the plant for its medicinal properties. (Priyadharshini *et al.,* 2020a, 2020b; Chahal *et al.,* 2022). However, the primary threat to the species is not its propagation. *C. malabaricum* naturally exhibits high vegetative propagation, producing up to 8 to 16 bulbs per mother plant and a high seed-setting rate during the growing period (Lekhak& S. R Yadav, 2012; Lansdown & Molur, 2017). The significant factors contributing to its endemism and vulnerability are its restricted distribution, habitat specificity, and habitat destruction. Understanding its current status, phenology, ecological interactions, and the threats it faces is essential for developing effective conservation strategies.

2.4. Application of IUCN Red List Criteria in Plant Conservation.

Applying IUCN Red List criteria is essential for assessing extinction risks and guiding conservation efforts. Over the past 40 years, the Red List has developed into a key conservation tool, applied at both local and global scales. Its strength lies in its data-driven methodology, which ensures consistent species assessments while compiling extensive supporting information (Figure 3) (Rodrigues *et al.,* 2006).

The IUCN framework provides a systematic approach for evaluating species based on multiple criteria, which can be adapted to local contexts as demonstrated in several studies. The Israeli Red List successfully applied IUCN criteria to assess plant species, revealing a strong correlation between habitat vulnerability and extinction risk (Vine *et al.,* 2024). In Nigeria, IUCN assessments identified 97% of evaluated tree species as threatened, underscoring the urgent need for targeted conservation strategies (Nodza *et al.,* 2024). Adaptations of the criteria have also been made for specific contexts, such as isolated plant populations and endemic species in regions like the Kashmir Himalaya and Gabon, improving extinction risk assessments and conservation prioritization (Abeli *et al.,* 2009; Tali *et al.,* 2015; Texier *et al.,* 2021). Notably, geographic range size within the IUCN framework has proven useful in identifying species at extremely high risk of extinction due to ongoing population declines (Breton *et al.,* 2019).

Although the Red List criteria offer significant advantages, their application presents several challenges. These include data limitations, biased size distributions, varying measurement scales, missing data, difficulties in assessing rare species, and the necessity for localized conservation strategies. Addressing these issues is critical for improving conservation outcomes for endemic plants. Sensitivity analyses suggest that modifying the criteria, such as excluding very small locations and refining measurement scales, can help reduce biases (Keith *et al.,* 2000). Additionally, tools like the ConR R package facilitate large-scale preliminary assessments by automating the calculation of key geographic range parameters, expediting the Red List assessment process (Dauby *et al.,* 2017). This approach is particularly beneficial for regions with many unassessed species.

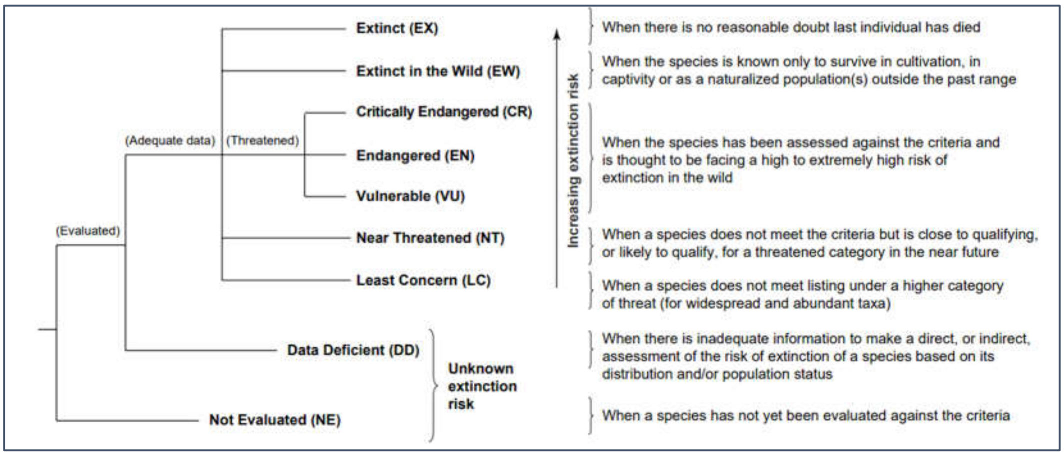


Figure 3. IUCN Red List categories (IUCN, S. S. C., 2001; Rodrigues *et al.,* 2006)

2.5. Phenology Studies in Plant Conservation

Phenology, the study of periodic plant life cycle events and their relationship with environmental factors, plays a crucial role in conservation biology. By understanding plant phenology, conservationists can develop effective strategies to preserve plant species, maintain ecological interactions, and mitigate climate change impacts (Morellato *et al.,* 2016). Various phenological data sources and ecological scales are employed to achieve conservation goals, ranging from species-level studies to ecosystem-wide assessments. The following sections explore how phenology contributes to conservation efforts, highlighting practical applications and case studies that illustrate its significance.

1. **Establishment of Seed Collection Calendars:** Developing a phenological calendar aids in collecting seeds and other plant resources for both *in situ* and *ex situ* conservation. Direct ground observations of plant phenophases, such as leafing, flowering, and fruiting, allow for the identification of optimal collection periods. The Kew Millennium Seed Bank Project exemplifies this approach, utilizing information on fruiting, seed germination, dormancy, and storage to implement conservation techniques for over 27,000 plant species (Ali & Trivedi, 2011). This ensures that seeds are harvested at the right time for successful conservation and restoration projects.

2. **Understanding Flower and Fruit Production in Threatened Species:** Monitoring flowering and fruiting patterns provides valuable insights into species survival and reproductive success. Assessing these patterns in threatened plant species supports conservation strategies by identifying critical pollination and seed dispersal periods. For instance, studies on the rare *Horsfieldia kingii* revealed limited fruit availability for its primary seed disperser, emphasizing the need for targeted conservation efforts (Datta & Rane, 2013). These observations help design habitat restoration and pollinator support strategies to improve reproductive outcomes.

3. **Enhancing Genetic Diversity and Population Resilience**: Phenological studies help maintain intra-population genetic diversity by reducing mismatches between flowering plants and their pollinators. Research on *Centaurea scabiosa* demonstrated that topographical variations minimized the risk of pollination failures, ensuring genetic diversity within populations (Hindle *et al.,* 2015). By identifying environmental factors influencing phenology, conservationists can take measures to enhance plant-pollinator interactions and support long-term population stability.

4. **Preserving Pollination Vectors and Ecosystem Services:** Flowering and fruiting phenology at different ecological scales ensures resource availability for pollinators. Managing natural and agricultural landscapes to support continuous resource availability sustains pollination services and ecosystem stability (Schellhorn *et al.,* 2015). Conservation strategies incorporating phenological data can enhance pollinator-friendly landscapes, benefiting wild and cultivated plant species.

5. **Controlling Herbivory and Its Impact on Plant Populations:** Synchronization between plant leafing and herbivore emergence influences population dynamics. Studies have shown high synchrony between leaf-feeding Lepidoptera and host plant phenology can lead to herbivore outbreaks. In contrast, climate change-induced disruptions in this synchrony may threaten plant and herbivore survival (van Asch & Visser, 2007). Understanding these interactions allows conservationists to predict and manage herbivore pressures on vulnerable plant populations.

6. **Sustainable Harvesting of Non-Timber Forest Products (NTFPs):** Phenological data on flowering and fruiting times are essential for sustainable harvesting of economically valuable plant species. Surveys on the Brazilian golden-grass (*Syngonanthus nitens*) demonstrated the importance of integrating phenological monitoring with ethno-ecological knowledge to manage harvesting sustainably (Schmidt *et al.,* 2007). This approach helps balance conservation with local economic needs.

7. **Conservation of Frugivore-Dependent Ecosystems:** Many vertebrate frugivores rely on seasonal fruit resources. Studies from Barro Colorado Island, Panama, revealed that abnormal declines in fruit production due to El Niño events led to frugivore famines, highlighting the importance of maintaining fruit availability for animal populations (Wright *et al.,* 1999). Conservationists can use phenological data to ensure habitat management supports plant and animal communities.

8. **Identifying Keystone Plant Species:** Long-term phenological monitoring allows for detecting keystone species that provide crucial resources during periods of scarcity. An eight-year study in Cocha Cashu, Peru, identified seven keystone species that significantly contributed to ecosystem stability (Diaz-Martin *et al.,* 2014). Prioritizing the conservation of these species can enhance ecosystem resilience.

9. **Assessing Climate Change Impacts on Phenology:** Herbarium collections and historical records provide long-term phenological datasets to evaluate climate change effects. In the Indian Himalayan Region, phenological patterns of high-value medicinal herbs were reconstructed to understand climate-induced shifts in flowering and fruiting (Gaira *et al.,* 2011). This information is crucial for adapting conservation strategies to climate-driven changes.

10. **Carbon Stock Estimates and Growth Model Development:** Tree growth phenology, derived from dendrochronological studies, provides baseline data for conservation and carbon sequestration strategies. Long-term observations have demonstrated how cambial activity responds to environmental changes, aiding forest management (Behera *et al.,* 2022). Incorporating such data helps refine forest conservation policies and carbon offset programs.

11. **Forecasting Vulnerability and Resilience to Climate Change:** Integrating phenological data with phylogenetic analysis helps identify plant groups more susceptible to climate change. Studies on Neotropical Myrtaceae revealed that species with conservative phenological patterns are at higher risk, aiding conservation prioritization (Staggemeier *et al.,* 2015). This approach strengthens species-specific conservation planning.

12. **Facilitating Ecological Succession:** Certain pioneer plant species provide trophic resources supporting frugivores and aiding in ecosystem recovery. Neotropical arborescent palms, which exhibit prolonged fruiting seasons, facilitate the establishment of late-successional species by maintaining food

resources year-round (Peres, 1994). Understanding succession dynamics enables better restoration planning.

13. **Monitoring Ecosystem Disturbances and Recovery:** Plant phenology can serve as an indicator of ecosystem health following disturbances such as wildfires and the proliferation of invasive species. Research on Madagascar's Tapia woodlands demonstrated that high fire frequency reduced flowering and fruiting, impairing natural regeneration (Alvarado *et al.,* 2014). Tracking phenological shifts helps evaluate ecosystem recovery efforts.

14. **Leveraging Citizen Science for Large-Scale Phenological Monitoring:** Citizen science initiatives, such as Canada's Plant Watch program, offer cost-effective means to collect phenological data, allowing conservation managers to track climate change effects on plant communities (Gonsamo *et al.,* 2013). Engaging the public in data collection expands monitoring capabilities and awareness.

15. **Remote Sensing Applications in Phenology and Conservation:** Remote sensing techniques provide spatially explicit data on plant phenology across landscapes. The US Geological Survey (USGS) integrates satellite imagery with field phenological datasets from the USA National Phenology Network (USA-NPN) to monitor climate-induced changes in vegetation dynamics (Crimmins, 2021). This enhances large-scale conservation planning.

Phenological studies provide essential insights into plant life cycle events and their ecological interactions, guiding conservation efforts at multiple scales. By leveraging field observations, long-term datasets, and advanced technologies such as remote sensing and citizen science, conservationists can develop strategies to protect plant biodiversity, mitigate climate change effects, and maintain ecosystem services. Phenology thus remains a cornerstone in conservation planning, bridging species preservation with broader ecological stability.

2.6. Phytochemistry of *C. malabaricum*

The Amaryllidaceae members has a long and rich history in traditional medicinal practices (Brenan & Breyer-Brandwijk, 1962; Bastida *et al.,* 2006; 2011; Nair & Van Staden, 2013; Nair *et al.,* 2017). Among the Amaryllidaceae members, the genus *Crinum* is most studied. Indian *Crinum* species exhibit diverse medicinal properties such as antioxidant, antimicrobial, antimalarial, antiviral, anti-inflammatory, antitumor, anti-diabetic, and immuno-stimulating effects due to the presence of pharmacologically active alkaloids (Ghosal *et al.,* 1985; Uddin *et al.* 2012; Refat *et al.* 2013; Ghane *et al.* 2018).

Most of the *Crinum* members are used in Asian folk and traditional medicine as rubefacient, tonic and for the treatment of allergic disorders and tumour diseases. (Ghosal *et al.,* 1985). These activities are attributed to the presence of Amaryllidaceae alkaloids. These are specific type of isoquinoline alkaloids exclusive to the family. They are structurally diverse, biogenetically related have wide range of biological activities (Bastida *et al.,* 2011). Phytochemical investigations have been done on Indian *Crinum* species for their alkaloid contents (Ghosal *et al.,* 1985a; Refat *et al.* 2013; Jagtap *et al.* 2014; Ghane *et al.* 2018). Study of Fennell and Staden (2001) on traditional and modern medicinal uses of *Crinum*, ethnomedical study on *C. asiaticum* by Haque *et al.* (2014), Study on medicinal use of *C. jagus* (Alawode, 2024a, 2024b) are remarkable. Chahal *et al.,* (2022) studied the medicinal potential of 11 Indian *Crinum* spp. Jagtap *et al.* (2014) studied the medicinal aspect and presence of alkaloids in five *Crinum* spp. Ghane *et al.* (2018) studied antioxidant, anti-diabetic, acetylcholinesterase inhibitory potential and alkaloid presence of Indian *Crinum*.

Crinum malabaricum contains several medicinally important alkaloids with analgesics, antiviral, and antitumor characteristics. Among the Indian *Crinum* species, *C. malabaricum* contains the highest concentration of galantamine (GAL), a selective, reversible competitive inhibitor of acetylcholine esterase (Jagtap *et al.,* 2014; Ghane *et al.,* 2018), and it is now the most promising treatment option for Alzheimer's disease under the trade names Reminyl® and Nivalin® (Heinrich, 2010). *C. malabaricum* also is a source for lycorine (LY), a toxic crystalline alkaloid with a chemical structure of $C_{16}H_{17}NO_4$ (Figure 4). It is a potential plant growth-inhibitor (Iqbal *et al.,* 2006) and exhibit cytotoxic (Giordani *et al.,* 2011), anti-inflammatory (Kang *et al.,* 2012), antitumor (Li *et al.,* 2007), anti-viral, anti-bacterial, anti-malarial properties (Cao *et al.,* 2013).

Studies are carried out on the root and leaf extract of *C. malabaricum* for their pharmacogenetic properties. The roots of *C. malabaricum* are a promising source of bioactive compounds with potential medicinal applications. It exhibited significant antioxidant potential, with high scavenging activity recorded in methanolic extracts. And it has a significant inhibitory effect on α-amylase, α-glucosidase, due to high flavonoid content, indicating potential anti-diabetic properties. Also, aqueous extract shows metal chelation potential. Also, the leaf extract of the species exhibits various medicinal properties, including anti-diabetic, anti-tumour, immune-stimulating, analgesic, cytotoxic, antiviral, anti-inflammatory, antioxidant, anti-malarial, and antimicrobial effects (Ghane *et al.,* 2018; Lekhak *et al.,* 2022).

2.6.1. Galantamine and Acetylcholine Esterase Inhibition

Galantamine is an acetylcholinesterase (AChE) inhibitor used in the management of Alzheimer's disease, where it elevates acetylcholine levels in the brain, thereby improving cognitive function and memory. Its chemical structure is $C_{17}H_{21}NO_3$, and it is identified as CID 9651 in the PubChem database (Figure 4).

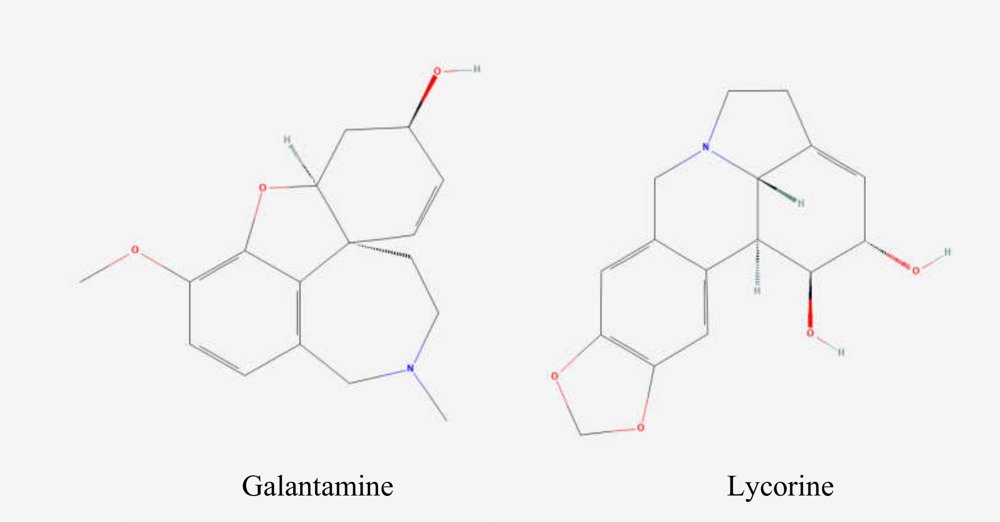


Figure 4. Chemical structures of Galantamine and Lycorine, retrieved from PubChem (National Center for Biotechnology Information, 2024)

Acetylcholine, a neurotransmitter primarily produced by cholinergic neurons, plays a crucial role in memory formation and learning. In Alzheimer's disease, the progressive degeneration of cholinergic neurons leads to a significant decline in acetylcholine levels, contributing to cognitive impairment. Additionally, AChE, the enzyme responsible for breaking down acetylcholine in the synaptic cleft, remains active despite the cholinergic loss, further exacerbating the neurotransmitter deficiency. The severity of dementia in Alzheimer's disease is strongly associated with cholinergic depletion, along with the accumulation of neurofibrillary tangles and amyloid-β plaques, which are key neuropathological markers of the disease (Razay & Wilcock, 2008; Kalola *et al.,* 2025).

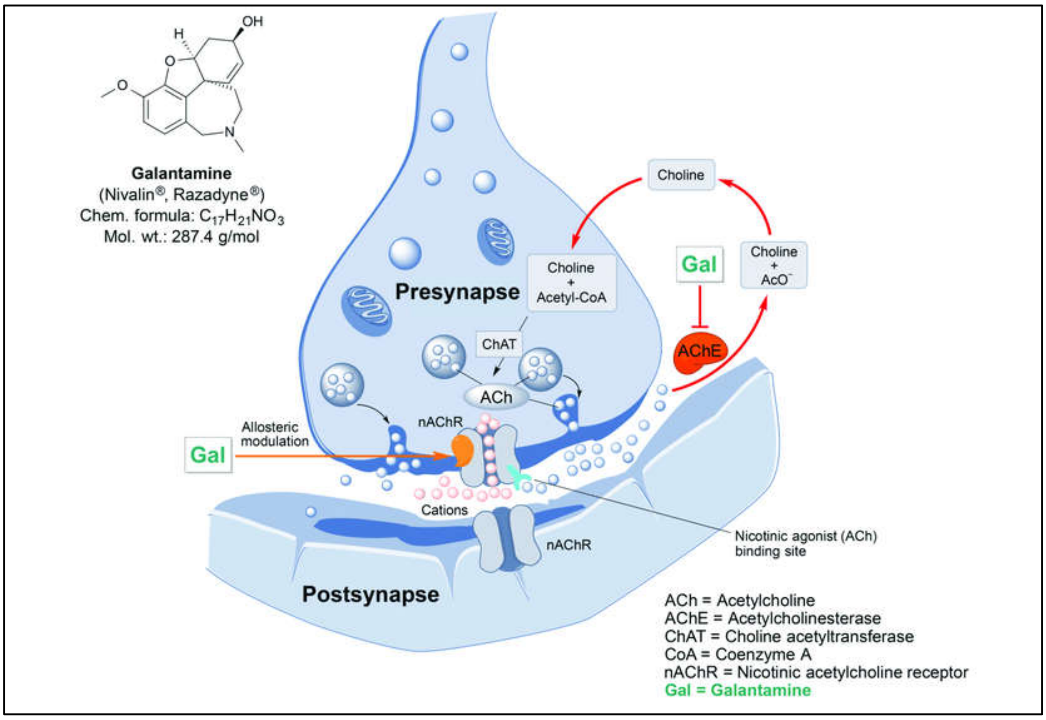


Figure 5. Graphical representation of Acetylcholine esterase inhibition activity of Galantamine (Tewari *et al.,*2018)

Galantamine inhibits acetylcholinesterase (AChE) through a dual mechanism. Primarily, it acts as a reversible, competitive inhibitor of AChE, binding to the enzyme's active site to prevent the breakdown of acetylcholine. This inhibition leads to increased acetylcholine levels in the synaptic cleft, enhancing cholinergic transmission and improving cognitive functions. Additionally, galantamine serves as an allosteric potentiator of nicotinic acetylcholine receptors (nAChRs), binding to sites distinct from acetylcholine and inducing conformational changes that increase receptor sensitivity (Figure 5). This modulation further amplifies cholinergic signalling, contributing to its therapeutic effects in conditions like Alzheimer's disease (Tewari *et al.,*2018).

Studies have confirmed the presence of bioactive compounds, including alkaloids and phenolics, in *C. malabaricum*, highlighting its medicinal potential. However, research on this species remains limited, necessitating further investigation to elucidate its complete chemical profile. Advanced analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) should be employed to identify and characterize its bioactive constituents. Additionally, studies focusing on the quantification, extraction efficiency, and pharmacological evaluation of these compounds are essential to fully exploring the therapeutic potential of *C. malabaricum.*

2.7. Genus *Crinum* in India

2.7.1. Taxonomy of Genus *Crinum*

The classification of lilioid monocots has long been a taxonomic debate (Chase *et al.,* 2009). Historically, all lilioid monocots, including the genus *Crinum*, were placed within a broad concept of the family Liliaceae (Cronquist, 1981). However, with advancements in phylogenetic studies, the Angiosperm Phylogeny Group reevaluated its classification, ultimately placing *Crinum* within the family Amaryllidaceae (APG IV, 2016).

The genus *Crinum* was first described by Linnaeus in 1737, initially recognizing four species: *C. asiaticum*, *C. africanum*, *C. americanum* and *C. latifolium* (Linnaeus, 1753; Nordal, 1977). Baker (1881) divided the genus into three subgenera based on floral morphology: Stenaster, Platyaster, and Codono Crinum. Species with actinomorphic flowers and linear petals were placed in Stenaster, those with actinomorphic flowers and lanceolate petals in Platyaster, and species with zygomorphic, funnel-shaped flowers in Codono Crinum. Later, Baker (1888) merged Platyaster into Stenaster, renaming it as subgenus *Crinum* since it contained the type species *C. americanum* (Meerow *et al.,* 2003). Over time, extensive taxonomic revisions and phylogenetic studies have expanded the genus, which is now known to comprise approximately 115 species distributed across Asia, Africa, the Americas, and Australia (POWO, 2024).

In India, *Crinum* is represented by 15 species: *C. asiaticum* L., *C. amoenum* Roxb.ex Ker Gawl, *C. brachynema* Herb., *C. humile* Herb., *C. lorifolium* Roxb. *C. latifolium*, *C. malabaricum* Lekhak & S.R.Yadav, *C. reddyi* M. Patel & H. Patel, *C. pusillum* Herb., *C. solapurense* S.P.Gaikwad, K.U.Garad & R.D.Gore, *C. stenophyllum* Baker, *C. stracheyi* Baker, *C. viviparum* R. Ansari & V.J. Nair, *C. woodrowii* Baker and *C. wattii* Baker (Lekhak *et al.,* 2015; Patel & Patel, 2019). These species are distributed across diverse ecological niches, ranging from coastal regions to inland plateaus. Among Indian *Crinum* species, *C. brachynema*, *C. woodrowii*, and *C. latifolium* belong to the subgenus Codono Crinum, while the remaining species, including *C. malabaricum*, are classified under Stenaster. These taxonomic insights highlight the evolutionary and morphological diversity of the genus in India.

2.7.2. Distribution and Endemism

The genus *Crinum* exhibits its greatest diversity in Africa, particularly in sub-Saharan regions. Biogeographical analyses suggest its origin is in southern Africa (Meerow *et al.,* 2003; Kwembeya *et al.,* 2007). *Crinum* is native to tropical and subtropical regions and is widely distributed across Asia, Africa, the Americas, and Australia (Meerow *et al.,* 2003).

In India, *Crinum* species are broadly distributed across the country, though some exhibit restricted ranges. Species such as *C. asiaticum* and *C. latifolium* are widely distributed in India, and they are valued for their elegant, fragrant flowers, which range from pure white to pink-tinged, and are often cultivated for ornamental purposes. Additionally, exotic species, such as *C. jagus* (J.Thomps.) Dandy, along with hybrids like *Crinum* × *amabile* Donn ex Ker Gawl., are commonly found in Indian gardens.

Among the endemic species, *C. malabaricum* is confined to the lateritic plateaus of the Malabar Plains, while *C. brachynema* and *C. woodrowii* are restricted to the Northern Western Ghats. *C. brachynema* only known from a single locality in Mahabaleshwar, Maharashtra. (Lekhak & Yadav, 2012; Lekhak & Yadav, 2012a; Lansdown, 2016).

C. lorifolium has a distribution range extending from India to Myanmar and is also reported from Tropical Africa. In India, the species has been reported from Maharashtra, Karnataka, and parts of Madhya Pradesh. *C. amoenum* is distributed from the Central Himalayas to Myanmar and primarily occurs in the northeast states and West Bengal in India. *C. wattii* is distributed in the Indo-Burma region and has been reported from Manipur in India. Some Indian species are known only from their protologue, with no further observations reported. *C. pusillum* is known only from the Nicobar Islands, originally reported by Herbert in 1837. Similarly, *C. humile* was described based on a bulb flowered at Spofforth. Its type specimen was not mentioned in the protologue, and only an illustration remains as the lectotype (Lekhak *et al.*, 2015).

Some species of the genus are described as Indian species, even though they are not reported from the present-day political boundaries of India. *C. stenophyllum* was reported by Baker (1881) based on a specimen from Wallich's Herbarium, which was collected from Tavoy, southeastern Myanmar. *C. reddyii* and *C. solapurense* are recently described aquatic plants, reported only from the banks of the Tapi River in Gujarat and the Bhima River in Solapur, Maharashtra, respectively (Table 1).

2.8. Phylogenetic Studies for Conservation

Molecular phylogenetic studies have become indispensable tools in conserving endangered plant species, providing critical insights into their evolutionary history, genetic diversity, and adaptive potential. By elucidating phylogenetic relationships, these studies enable conservationists to prioritize species and populations based on their evolutionary significance and genetic health, ensuring that conservation efforts are practical and scientifically informed (Avise, 1989; Smith & Wayne, 1996).

Sl. No.	Species	Geographical distribution (Global/ regional)	Protologue
1.	*C. amoenum* Roxb. ex Ker Gawl.,	Central Himalaya to Myanmar; West Bengal and North-east India	J. Sci. Arts (London) 3: 106 (1817)
2.	*C. asiaticum* L.	Tropical & Subtropical Asia to SW. Pacific. And Indian Ocean Islands; All over India	Sp. Pl. 1: 292 (1753)
3.	*C. brachynema* Herb.	Mahabaleshwar, Satara district Maharashtra, India	Edwards's Bot. Reg. 28(Misc.): 36 (1842).
4.	*C. humile* Herb.,	India	Bot. Mag. 53: t. 2636 (1826)
5.	*C. latifolium* L.	India to S. China and Indo-China; All over India	Sp. Pl. 1: 291 (1753)
6.	*C. lorifolium* Roxb.	India to Myanmar & Tropical Africa; Maharashtra and Karnataka, India	J. Sci. Arts (London) 3(5): 110 (-111) (1817).
7.	*C. malabaricum* Lekhak & S.R.Yadav	Kannur & Kasaragod, Kerala, India	Kew Bull. 67(3): 521 (2012).
8.	*C. pusillum* Herb.	Nicobar Islands, India	Amaryllidaceae: 255 (1837)
9.	*C. reddyi* M.Patel & H.Patel	Ghuntvel, Tapi District, Gujarat, India	Nordic J. Bot. 37(4)-e02172: 2 (2019
10.	*C. solapurense* S.P.Gaikwad, Garad & Gore	Machnur, Solapur district, Maharashtra, India	Kew Bull. 69(2)-9505: 1 (2014).
11.	*C. stenophyllum* Baker	India, Bangladesh and Myanmar	Gard. Chron. 1: 786 (1881)
12.	*C. stracheyi* Baker	Uttarakhand, India	Gard. Chron., n.s., 16: 72 (1881)
13.	*C. viviparum* R.Ansari & V.J.Nair	India to the Indo-Burma region. All over India	J. Econ. Taxon. Bot. 11(1): 205 (1988).
14.	*C.woodrowii* Baker ex W.Watson	Maharashtra, India	Forest 10(no. 495): 324 (1897).
15.	*C. wattii* Baker	Indo – Bruma; Myang Khong Valley, Manipur, india	Handb. Amaryll.: 76 (1888)
16.	*C.* × *amabile* Donn ex Ker Gawl.	Hybrid of *C. asiaticum* × *C. zeylanicum*.; Ornamental	Bot. Mag. 39: t. 1605 (1814)
17.	*C. jagus* (J.Thomps.) Dandy	Tropical Africa; Ornamental in India	J. Bot. 77: 64 (1939)

Table 1: Distribution of Genus *Crinum* in India

One of the primary contributions of molecular phylogenetics is its role in resolving taxonomic uncertainties. Many endangered species are misclassified due to morphological similarities, leading to inadequate or misplaced conservation efforts. Molecular analysis help correct these errors by delineating true evolutionary lineages, ensuring that conservation resources are allocated to distinct and evolutionarily significant units (Meerow *et al.,* 2003; Kwembeya *et al.,* 2007). For example, phylogenetic studies on *Zieria* species have clarified genetic relationships, confirming taxonomic validity and guiding conservation strategies (Bayly, 2023). Similarly, identifying cryptic diversity within threatened taxa can prevent overlooking genetically distinct populations that may require independent conservation measures (Teixeira & Nazareno, 2021).

Beyond taxonomic classification, molecular phylogenetics provides crucial information about genetic diversity within and between populations. Genetic variation is fundamental for species' resilience against environmental changes, and phylogenetic studies help assess whether populations harbour sufficient genetic diversity for long-term survival (Kim *et al.,* 2021; Teixeira & Nazareno, 2021).

The genomic analysis of *Pellacalyx yunnanensis* revealed low genetic diversity and adaptive constraints, emphasizing the need for targeted conservation actions to enhance population viability (Shao *et al.,* 2024). Similarly, studies on *Malania oleifera* have demonstrated how genomic data can predict species responses to climate change and habitat loss, informing proactive conservation planning (Shen *et al.,* 2024).

Phylogenetic analyses also contribute to identifying species of high conservation value based on their evolutionary distinctiveness. Some species represent ancient or unique evolutionary lineages, and their loss would result in the disappearance of significant evolutionary history (Faith, 1992; Hopper, 2000). The Fair Proportion index, for instance, prioritizes species based on their evolutionary isolation, highlighting taxa that contribute disproportionately to phylogenetic diversity (Wicke *et al.,* 2023). By incorporating such metrics, conservation strategies can move beyond species richness alone and focus on preserving evolutionary potential and functional biodiversity (Pio *et al.,* 2011; Rapacciuolo *et al.,* 2018).

Moreover, molecular phylogenetics enhances extinction risk assessments by integrating evolutionary relationships into conservation models. Species with low genetic diversity and long branches in the phylogenetic tree may be more vulnerable to environmental changes due to their limited adaptive capacity (Yessoufou *et al.,* 2012; Matsuba *et al.,* 2024). Understanding these relationships allows conservationists to implement measures that mitigate extinction risks, such as prioritising habitat protection for evolutionarily unique species or maintaining genetic reservoirs through *ex situ* conservation (Serrano *et al.,* 2021; Chung *et al.,* 2023).

Conservation planning also benefits from phylogenetic insights at the population level. As habitat fragmentation increases, phylogenetic networks help identify genetically distinct populations crucial for maintaining overall species diversity (Volkmann *et al.,* 2014). This information is particularly valuable for species with highly restricted distributions, such as *Crinum malabaricum*, where conservation efforts must account for microhabitat specificity and genetic uniqueness. In some cases, molecular data reveal that specific populations harbour unique alleles that may not be represented in *ex situ* collections, necessitating broader sampling strategies to ensure comprehensive genetic conservation (Bayly, 2023).

Despite their immense value, molecular phylogenetic studies also face challenges. A significant limitation is the need for extensive and representative genetic data across diverse taxa. In some cases, conflicting phylogenetic trees can complicate prioritization efforts, requiring integrative approaches that combine morphological, ecological, and genetic data (Wicke *et al.,* 2023). Nevertheless, advances in conservation genomics continue to refine these methodologies, allowing for more precise assessments of evolutionary and ecological significance of the species (Ma *et al.,* 2023).

Molecular phylogenetics provides a robust framework for conservation of endangered species by identifying taxonomically and evolutionarily significant units, assessing genetic diversity, predicting extinction risks, and guiding conservation strategies. By integrating phylogenetic diversity into conservation planning, efforts can be directed toward preserving species numbers, their evolutionary potential, and ecological functions (Davies *et al.,* 2008; Donoghue, 2008). This approach ensures that conservation priorities are both scientifically grounded and forward-thinking, enhancing the resilience of biodiversity in the face of ongoing environmental change.

2.9. Molecular Markers in Phylogenetic Studies

Molecular markers are genetic sequences that represent variations between individual organisms or species, which are located in close proximity to genes and act as ‘signs’ for variations, but do not affect the phenotype of the trait of interest. Molecular markers are specific DNA sequences positioned in the genome of an organism at known locations that reveal sites of variation in DNA. Molecular markers show polymorphism due to mutations such as point mutations, insertions, deletions, or replication errors in DNA (Hartl & Clark, 1997). These markers are typically located in non-coding regions, making them selectively neutral and unaffected by environmental factors or plant growth stages (Winter & Kahl, 1995).

Molecular markers play a crucial role in plant phylogenetic analysis, enabling to elucidate evolutionary relationships with high precision. Among these, chloroplast DNA (cpDNA) is widely favored due to its high mutation rate and maternal inheritance, making it particularly useful for studying angiosperm phylogeny (Yang *et al.,* 2001; Fineschi *et al.,* 2002). The rbcL gene, which encodes a subunit of RuBisCO, is one of the most commonly used markers in plant systematics. Due to its high level of conservation and slow mutation rate, it is particularly useful for resolving deeper evolutionary relationships in plants (Zitouna *et al.,* 2014). Another frequently utilized chloroplast marker is the MatK gene, which exhibits high sequence variation, making it valuable for clarifying relationships among seed plants (Jankowiak *et al.,* 2004).

In addition to chloroplast markers, nuclear DNA markers such as Random Amplified Polymorphic DNA (RAPD) and various types of repetitive sequences, including short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs), are also employed for phylogenetic studies (Grechko, 2002). Similarly, tandem repeats, such as microsatellites and minisatellites, are useful in assessing genetic variation and evolutionary divergence. The Internal Transcribed Spacer (ITS) region is another widely used molecular marker in plant phylogenetics, particularly for resolving relationships among closely related species. The ITS region, a non-coding segment of nuclear ribosomal DNA, exhibits high variability, making it well-suited for species-level identification and phylogenetic reconstruction (Figure 6) (Blattner, 1999).

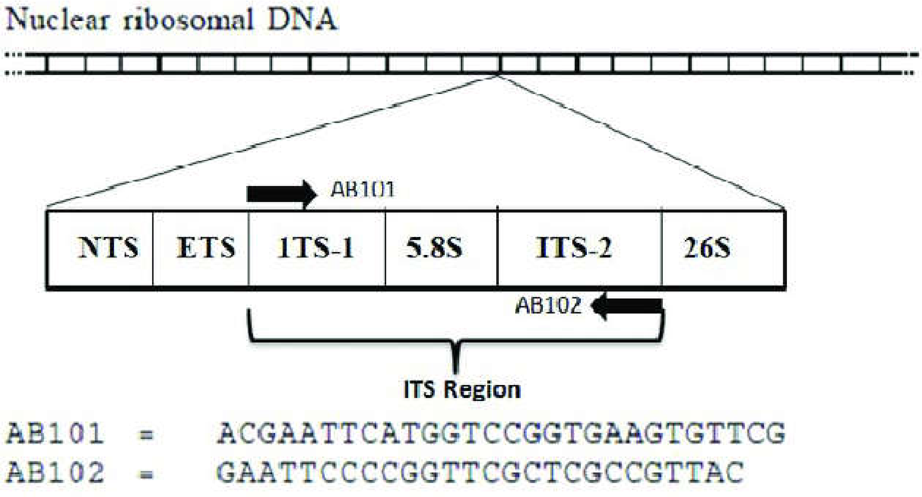


Figure 6. Structure of ribosomal ITS molecular marker region

Most phylogenetic studies on the genus *Crinum* have relied on ribosomal DNA ITS1/5.8S/ITS2 and the chloroplast spacer region *trnL-F* as molecular markers. The ITS region is commonly amplified using primers AB101 and AB102, which have proven effective for distinguishing *Crinum* species. For the *trnL-F* region, the d and e primers suggested by Taberlet *et al.* (1991) are used to amplify the trnL exon and the *trnL-F* spacer region (Figure 7) (Douzery, 1999; Meerow *et al.,* 1999).

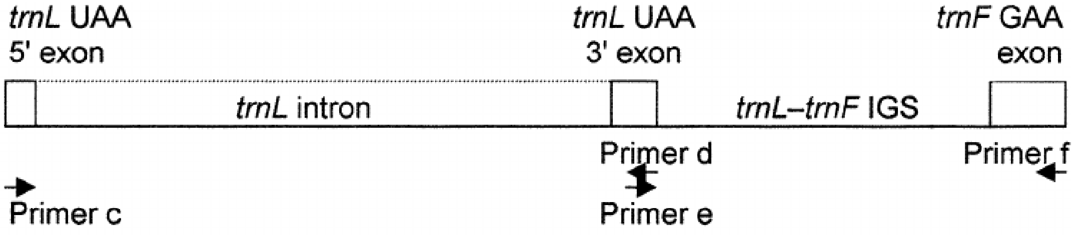


Figure 7. Structure of chloroplast *trnL-trnF* molecular marker region

Molecular markers provide a robust toolkit for reconstructing plant phylogenies and understanding evolutionary dynamics within plant lineages. Their application in *Crinum* phylogenetics has significantly contributed to clarifying species relationships and evolutionary history.

2.10. Phylogenetics of Genus Crinum

The genus *Crinum* is of significant evolutionary importance within the family Amaryllidaceae, and molecular phylogenetic studies provide critical insights into its biogeographical history and evolutionary relationships. Understanding these relationships is essential for resolving taxonomic uncertainties and tracing dispersal patterns across continents.

Molecular phylogenetic analyses using nuclear ITS and plastid *trnL-F* sequences have identified three distinct clades within *Crinum*. These include a monophyletic American group comprising tropical and North African species, a clade containing southern African and Australian species, and another grouping that includes species from Madagascar, Australasia, southern Africa, and the Sino-Himalayan region (Meerow *et al.,* 2003; Kwembeya *et al.,* 2007).

The evolutionary history of *Crinum* suggests that the genus likely originated in southern Africa, followed by radiation events leading to its present distribution. This includes two independent entries into Australia and distinct dispersal events to the Sino-Himalayan and Australasian regions (Meerow *et al.,* 2003). The phylogeny of *Crinum* exhibits strong geographical and ecological trends, with species distributed across Africa, Madagascar, Australasia, and the Americas (Kwembeya *et al.,* 2007). Certain species, such as *C. binghamii* and *C. purpurascens*, form a sister group to the American clade, providing crucial insights into biogeographical connections between southern Africa and tropical West African species. This relationship supports the hypothesis of an ancient dispersal event from Africa to the Americas.

A particularly intriguing phylogenetic placement is that of *C. flaccidum*, an Australian species nested within a clade primarily composed of southern African species. This suggests that the genus underwent long-distance dispersal events, challenging earlier assumptions about its biogeographical history.

Within the broader tree of life, *Crinum* is closely related to the genus *Ammocharis* (POWO, 2024). Phylogenetic analyses further reveal that certain species, such as *C. baumii,* exhibit closer relationships with *Ammocharis* and *Cybistetes* than with *Crinum* sensu stricto. These findings indicate a complex evolutionary relationship within the subtribe Crininae (Meerow *et al.,* 2003; Kwembeya *et al.,* 2007).

Floral evolution in *Crinum* demonstrates significant morphological transformations, particularly in perianth structure. The transition from zygomorphic to salverform, actinomorphic perianths has occurred multiple times independently, suggesting that subgeneric classifications based solely on floral morphology may not be phylogenetically valid (Meerow *et al.,* 2003; Kwembeya *et al.,* 2007). This underscores the necessity of integrating morphological data with molecular evidence for a more accurate assessment of evolutionary relationships within the genus.

The synthesis of molecular and morphological data highlights the intricate evolutionary pathways of *Crinum*, emphasising its complex phylogenetic history characterised by multiple radiations and dispersal events. The genus exhibits significant morphological diversity, with independent evolutionary changes in floral structure occurring across different lineages. These findings reaffirm the importance of molecular phylogenetics in elucidating the evolutionary and biogeographical dynamics of the genus *Crinum* and provide a foundation for further studies to refine its classification and conservation strategies.

However, these studies have primarily focused on African and American species, with limited representation from India, including only *C. asiaticum* and *C. latifolium* (Meerow *et al.,* 2003). This leaves a significant gap in understanding the evolutionary relationships of Indian *Crinum* species, many of which are endemic or geographically restricted. Given India's unique biogeographical history and diverse climatic zones, phylogenetic studies can provide insights into whether these species share ancestry with African lineages, dispersed via the Indo-Malayan route, or represent ancient Gondwanan relics.

Understanding the genetic relationships of Indian species is also critical for conservation planning, as many inhabit ecologically sensitive habitats threatened by habitat loss and climate change. Expanding phylogenetic studies to include a broader representation of Indian *Crinum* species, utilizing high-resolution molecular data, will enhance our understanding of their evolutionary history, systematic placement, and conservation priorities.

2.11. Ecological Niche Modelling in Species Conservation

2.11.1. Ecological Niche Modelling (ENM)

Ecological niche modelling (ENM), or habitat suitability modelling, is a computational approach that integrates species occurrence data with environmental variables to predict potential species distributions. Ecological niche of a species is the range of environmental conditions necessary for its survival and reproduction (Hutchinson, 1957). ENM is vital in conservation biogeography, helping researchers understand species-environment relationships and predict habitat suitability under different environmental scenarios (Franklin, 2010; Dolci & Peruzzi, 2022).

By analyzing the environmental parameters that define the niche of a species, ENM can project current distributions and predict potential future shifts due to environmental changes, including climate change (Drake *et al.,* 2006; Rinawati *et al.,* 2013). Despite inherent challenges, ENM has been widely adopted for biodiversity conservation, particularly in identifying and prioritizing areas for protection, species translocation, and restoration efforts (Franklin, 2013).

2.11.1.1. Uses of Ecological Niche Modelling

ENM provides significant advantages in conservation planning, including its predictive power in forecasting species distributions under climate change scenarios, cost-effectiveness by reducing the need for extensive field surveys, and broad applicability across taxa, ecosystems, and geographic scales

2.11.1.1.1. Predicting Potential Habitats

ENM is extensively used to identify geographic regions that provide suitable environmental conditions for species, particularly rare, endangered, or poorly studied. By analyzing the relationship between species occurrence data and environmental variables, ENM can predict potential habitats, even in areas where the species has not been previously recorded.

For example, the Maxent model has been employed to predict the distribution of 25 rare and endangered plant species in Northwest Yunnan, China. This study identified key areas for conservation, enabling targeted efforts to protect these species in their natural habitats (Ye *et al.,* 2020). Similarly, ENM has been used to locate suitable habitats for *Vanda thwaitesii,* a rare orchid species in the Western Ghats of India. The model's predictions guided field surveys and conservation actions, ensuring the protection of this threatened species (Decruse, 2023).

ENM is particularly valuable for species with limited occurrence data, as it can extrapolate potential distributions based on environmental similarities. This capability is crucial for conserving cryptic or poorly studied species and for identifying potential habitats for reintroduction programs.

2.11.1.1.2. Conservation Planning

ENM plays a critical role in conservation planning by assessing habitat suitability and identifying areas of high conservation value. It helps prioritize regions for protection, especially in landscapes where human activities, such as urbanization, agriculture, or infrastructure development, threaten biodiversity.

A notable example is the use of ENM to assess the impact of hydroelectric projects on the potential habitats of *Lagerstroemia minuticarpa*, a rare tree species endemic to the Indian Eastern Himalayas. The study revealed that proposed hydroelectric projects could significantly reduce the species' suitable habitat, prompting conservationists to advocate for alternative development plans (Adhikari *et al.,* 2019). ENM also supports the design of protected area networks by identifying biodiversity hotspots and corridors that facilitate species movement. By integrating ENM with spatial planning tools, conservationists can optimize resource allocation and ensure the long-term survival of threatened species.

2.11.1.1.3. Climate Change Impact Assessment

ENMs evaluate how climate change affects species distributions, identifying areas for conservation efforts. One of the most critical applications of ENM is assessing the impacts of climate change on species distributions. By projecting future habitat suitability under different climate scenarios, ENM helps identify species and regions most vulnerable to climate change.

For instance, a study on *Malania oleifera*, a rare tree species in China, used ENM to predict significant habitat loss under future climate scenarios. The findings underscored the urgent need for conservation interventions, such as assisted migration or *ex situ* conservation, to safeguard the species (Shen *et al.,* 2024). Similarly, ENM has been used to assess the impacts of climate change on alpine and Arctic species, which are particularly sensitive to warming temperatures (Kolanowska *et al.,* 2024). These projections enable policymakers and conservationists to develop adaptive management strategies, such as creating climate-resilient protected areas or restoring degraded habitats, to mitigate the impacts of climate change on biodiversity.

2.11.1.1.4. Invasive Species Forecasting

ENM is a valuable tool for predicting the potential spread of invasive species, which pose significant threats to native ecosystems and biodiversity. By modelling the environmental conditions suitable for invasive species, ENM can identify areas at risk of invasion and inform early detection and management efforts.

For example, ENM has been used to predict the spread of invasive plants, such as *Lantana camara* and *Chromolaena odorata*, in tropical and subtropical regions. These predictions help land managers prioritize areas for monitoring and control, reducing the ecological and economic impacts of the invasive species (Kishore *et al.,* 2024). Additionally, ENM can assess the potential effects of climate change on the distribution of invasive species, providing insights into how their ranges may shift in the future. This information is crucial for developing long-term management strategies to prevent the establishment and spread of invasive species.

2.11.1.1.5. Discovering New Populations

ENM is instrumental in guiding field surveys to discover new rare or endangered species populations. By predicting areas with suitable environmental conditions, ENM helps researchers focus on regions most likely to harbour undiscovered populations.

A notable example is the application of ENM to locate additional populations of *Gymnocladus assamicus*, a critically endangered tree species in northeastern India. The model's predictions led to the discovery of new populations, significantly enhancing *in situ* conservation efforts for this species (Menon *et al.,* 2010). This application is particularly valuable for species with limited known occurrences, as it reduces the time and resources required for field surveys. By identifying potential habitats, ENM increases the likelihood of discovering new populations, critical for conserving rare and endangered species.

2.11.1.1.6. Assessing Genetic Connectivity

Integrating ENM with genetic analyses provides valuable insights into population connectivity and evolutionary dynamics. By combining species distribution data with genetic information, researchers can identify barriers to gene flow, assess the impacts of habitat fragmentation, and design conservation strategies that maintain genetic diversity.

For example, a study on *Emmenopterys henryi*, a rare tree species in China, used ENM to analyze fine-scale niche dynamics and assess population connectivity. The findings revealed significant genetic differentiation among populations, highlighting the need for targeted conservation actions to enhance gene flow and ensure the species' long-term survival (Feng *et al.,* 2024). This integrated approach is beneficial for species with fragmented distributions, as it helps identify critical corridors and stepping-stone habitats that facilitate gene flow. By maintaining genetic connectivity, conservationists can enhance the resilience of populations to environmental changes and other threats.

2.11.2. Challenges and limitations of ENM

Ecological niche modelling (ENM) is a powerful tool for predicting species distributions and informing conservation strategies, but it faces several challenges and limitations. These include dependence on high-quality occurrence data, sensitivity to environmental variability, and the complexity of modelling algorithms, which necessitate rigorous validation (Phillips *et al.,* 2006; Peterson *et al.,* 2008; Ray & Ramachandra, 2017). While ENM is valuable for conserving endangered species, its effectiveness depends on addressing data limitations, environmental variability, and model complexity.

2.11.2.1 Data Limitations

The availability of occurrence records heavily influences the reliability of ENM. Limited data, particularly for rare species, can reduce model accuracy. For instance, a study on *Dianthus pseudocrinitus* demonstrated that multiple modelling iterations were required to improve predictive performance, highlighting the challenges posed by data scarcity (Behroozian *et al.,* 2022).

2.11.2.2 Environmental Variability

ENM outcomes are susceptible to species-specific ecological preferences and environmental conditions. Research on relic shrubs in western Ordos revealed that different species responded uniquely to environmental constraints, emphasising the need for tailored

2.11.2.3. Climate Change Projections

Predicting future habitat suitability under climate change scenarios adds complexity to ENM. For example, a study on *Malania oleifera* projected significant habitat loss under climate change, underscoring the importance of integrating genomic data with ENM to enhance predictive accuracy (Shen *et al.,* 2024).

2.11.2.4. Model Complexity and Accuracy

Inappropriate modelling methods, ignoring spatial autocorrelation, and not accounting for variable correlations can reduce model accuracy (Sillero & Barbosa, 2020). The choice of modelling algorithms and handling pseudo-absence data significantly impacts model reliability. Studies on *Syzygium travancoricum* highlighted the need to use multiple algorithms to refine predictions, particularly for data-deficient taxa (Ray & Ramachandra, 2017). Additionally, the misuse of metrics like the Akaike information criterion (AIC) can lead to inaccurate assessments of model performance, as low AIC values do not always correlate with high geographical prediction accuracy (Velasco & González-Salazar, 2019).

2.11.3. Ecological Niche Modelling (ENM) Algorithms

Ecological Niche Modelling (ENM) employs various statistical and machine learning algorithms to predict species distributions based on environmental conditions and occurrence records. The algorithms used in ENM differ in their approach, data requirements, and complexity, allowing researchers to choose the most suitable model for their study. ENM algorithms can be broadly classified into three categories:

1. **Statistical Methods:** These methods rely on statistical relationships between species occurrence and environmental variables. e.g.: Generalized Linear Models, Generalized Additive Models.

2. **Clustering-Based Approaches:** These methods group similar environmental conditions based on species occurrences. e.g.: Kernel Density Estimation (KDE), Marble (MA), Bioclim.

3. **Machine Learning Methods:** These approaches use computational techniques to identify complex patterns in species distributions. e.g.: Maximum Entropy Model (Maxent), Genetic Algorithm for Rule-set Prediction (GARP), Random Forest (RF), Boosted Regression Trees (BRT), Support Vector Machines (SVM).

The table 2 provides a comparative analysis of algorithms. Each algorithm has trade-offs, and the choice depends on study objectives, data availability, and computational resources. A combination of models is often used to improve prediction accuracy and reliability.

2.11.4. Factors influencing ENM

Several factors shape the accuracy of ecological niche models, including the choice of environmental predictors, species traits, collinearity, grain size, genetic information, model complexity, and sample size. Addressing these factors is critical for enhancing the reliability and applicability of ENM in conservation planning.

Environmental Predictors: The choice and combination of environmental predictors, such as climate, land-use/cover, and ecosystem functional attributes, significantly influence model performance and transferability. Integrating multiple types of predictors generally enhances model accuracy (Regos *et al.,* 2019).

Species Traits: Species-specific traits can affect model accuracy, but their impact on temporal transferability is inconsistent. While traits may enhance predictive accuracy, they do not always improve the model's ability to project distributions across different time periods (Regos *et al.,* 2019).

Collinearity: While collinearity among predictors does not significantly affect model performance during training, shifts in collinearity and environmental novelty can reduce model transferability to new regions or time periods (Feng *et al.,* 2019).

Grain Size and Niche Breadth: The spatial resolution of environmental predictors (grain size) and the niche breadth of species influence model accuracy. Models tend to perform better for habitat specialists and when the grain size aligns with the species' ecological scale (Connor *et al.,* 2018). **Sample Size:** Adequate sample size is crucial for model accuracy. While machine-learning methods can perform well with smaller datasets, predictive accuracy improves with larger sample sizes (Stockwell & Peterson, 2002).

Algorithm	Approach	Data Type	Advantages	Disadvantages	Reference
Generalized Linear Models (GLM)	Extends linear regression by incorporating non-normal response distributions and link functions.	Presence-absence	Well understood, interpretable, and flexible.	Assuming a predefined relationship between predictors and response, complex interactions may not be captured well.	Guisan & Zimmermann (2000)
Generalized Additive Models (GAM)	Extends GLMs by using smoothing functions to model nonlinear relationships.	Presence-absence	Captures nonlinear ecological relationships more effectively than GLMs.	Computationally intensive and challenging to interpret.	Guisan *et al.* (2002)
KDE	Calculates density based on the spatial distribution of presence points, with higher densities indicating areas with favourable environmental conditions.	Presence-only	Does not assume a specific distribution for the data. Works well with sparse occurrence data.	Sensitive to kernel bandwidth selection, which affects the resolution of the output.	Silverman (1986)
MA	Setting predefined rules or thresholds for environmental conditions and classifying areas based on how well they match known occurrence conditions.	Presence-only	Rules are explicitly defined. Can integrate expert knowledge into modelling.	Subjective rule selection can lead to biased results. Less flexible than machine learning models.	Qiao *et al.* (2015)
Bioclim (Bioclimatic Envelope Model)	Defines species' niche based on min-max values of climate variables from known occurrence points.	Presence-only	Simple, fast, easy to interpret	Assumes environmental tolerances are uniform and does not account for interactions between variables.	Busby (1991)

Maxent (Maximum Entropy Model)	Estimates probability distribution with maximum entropy	Presence-only	Handles small sample sizes, generates probabilistic outputs	Sensitive to sampling bias, risk of overfitting	Phillips *et al.* (2006)
GARP (Genetic Algorithm for Rule-set Prediction)	Generate rules that best separate occurrence points from randomly selected pseudo-absences.	Presence-only	Handles complex relationships	Model selection can be inconsistent due to its stochastic nature.	Stockwell & Peters (1999)
Random Forest (RF)	An ensemble machine learning algorithm that builds multiple decision trees and aggregates predictions for classification or regression.	Presence-absence	Handles large datasets, complex interactions, and nonlinear relationships.	Computationally expensive and may require tuning to avoid overfitting.	Cutler *et al.* (2007)
Boosted Regression Trees (BRT)	Uses gradient boosting to combine multiple regression trees iteratively, improving predictive accuracy by reducing bias and variance.	Presence-absence	Handles complex interactions and missing data, and provides high predictive performance	Computationally intensive and requires tuning.	Elith *et al.* (2008)
Support Vector Machines (SVM)	Finds a hyperplane to separate classes based on presence/absence data.	Presence-absence	Good for high-dimensional data and nonlinear relationships.	Computationally demanding and requires parameter tuning.	Drake *et al.* (2006)

Table 2: Comparison of major algorithms used for Ecological niche modelling based on their approach, data requirements, advantages, and disadvantages.

2.11.5. Common Pitfalls in doing ENM

- **Misuse of Presence-Background Methods:** A common mistake is treating presence-background methods (e.g., Maxent) as pseudo-absence models, leading to incorrect interpretations. Clear differentiation between the two is necessary.

- **Spatial Autocorrelation and Clustering:** Improper filtering of occurrence data due to confusion between spatial autocorrelation and clustering can distort model predictions.

- **Mismatched Spatial Resolution:** Using environmental variables at a finer scale than species records can introduce errors. Ensuring consistency in resolution is crucial.

- **Ignoring Variable Correlations:** Failing to account for correlations between environmental variables biases model predictions. Prior evaluation of variable redundancy improves accuracy.

- **Lack of Model Replication:** Not replicating machine learning models reduces reliability. Multiple iterations are necessary for robust validation.

- **Using Unprojected Coordinate Systems:** Topographical variables derived from unprojected coordinate systems can introduce inaccuracies. Using projected coordinate systems ensures precision.

2.12. Species Translocation in Conservation

Species translocation refers to the deliberate movement of organisms from one location to another, primarily as a conservation strategy to prevent extinction and enhance biodiversity (Seddon, 2010). This practice is particularly vital for endangered plant species, as it offers a proactive approach to preserving biodiversity in the face of habitat loss, climate change, and other anthropogenic pressures. Translocation encompasses several types, each tailored to specific ecological and conservation goals, including reintroductions, augmentations, within-range introductions, and conservation introductions (Serota, 2024).

2.12.1. Types of Species Translocations

1. **Reintroductions:** This involves restoring species to areas within their historical range where they have become locally extinct. Reintroductions aim to restore ecological balance and historical populations (Serota, 2024).

2. **Augmentation:** Also known as reinforcement, this strategy involves adding individuals to existing populations to enhance their viability. Augmentation is beneficial for addressing demographic threats such as low genetic diversity or environmental stochasticity (Zimmer *et al.,* 2019).

3. **Within-Range Introductions:** This approach establishes populations in areas within the species' natural range but where they have no historical record. It is often employed to enhance biodiversity or restore ecosystem functions (Serota, 2024).

4. **Conservation Introductions:** This involves relocating species beyond their known range, often termed assisted colonization. It is used when species face imminent threats in their native habitats and require new environments for survival (Seddon, 2010).

2.12.2. Key Roles and Benefits of Species Translocation

Species translocation plays a pivotal role in conservation by addressing multiple ecological and genetic challenges:

Species translocation plays a crucial role in conservation by addressing ecological and genetic challenges associated with threatened plant populations. One of its primary benefits is reducing extinction risk by establishing or augmenting populations, thereby enhancing species persistence in the face of environmental pressures (Feng *et al.,* 2019; Zimmer *et al.,* 2019). Translocation is particularly relevant in response to large-scale habitat destruction caused by infrastructure development, often leading to severe biodiversity loss. By relocating species to suitable habitats, translocation mitigates the adverse effects of anthropogenic disturbances and provides a means for species survival that would otherwise be impossible (Sullivan *et al.,* 2015; Tojibaev *et al.,* 2019).

The success of translocation efforts depends on rigorous site selection, often guided by experimental trials and long-term monitoring. These assessments ensure that the recipient site meets the ecological requirements of the translocated species and provides adequate conditions for population establishment and growth (Volis & Blecher, 2021; Andres *et al.,* 2022).

Environmental conditions play a significant role in determining translocation success. Climate suitability is a primary factor, as populations introduced to environments with similar climatic conditions to their native habitats exhibit higher persistence rates (Bellis *et al.,* 2025). Additionally, species with broader ecological niches demonstrate greater recruitment and maturity success, particularly regarding precipitation tolerance. Research indicates that species from biotically limiting environments - where interactions with other organisms constrain growth and survival - often show higher establishment rates following translocation (Bellis *et al.,* 2025).

Proximity to existing populations is another key determinant of success, as it facilitates ecological interactions such as pollination, seed dispersal, and genetic exchange. Species translocated near extant populations benefit from established ecological networks, contributing to improved adaptation and long-term survival (Jonas *et al.,* 2023).

Genetic diversity is critical in translocation success, influencing both short-term survival and long-term population stability. High genetic diversity enhances population resilience by providing adaptive potential against environmental changes and disease pressures (Schäfer *et al.,* 2020). To maximize the effectiveness of translocation, incorporating genomic data into planning efforts ensures that genetic variation is adequately represented in new populations. This approach helps maintain evolutionary potential and promotes long-term viability (Rossetto *et al.,* 2023).

Post-translocation monitoring is essential to assess genetic diversity, population dynamics, and adaptation to new environments. Continuous tracking allows for adaptive management strategies, enabling conservationists to make data-driven decisions that improve translocation outcomes. By evaluating genetic and ecological parameters over time, researchers can refine translocation methodologies and enhance species conservation efforts (Rossetto *et al.,* 2023).

2.12.3. Challenges in Species Translocation

Despite being a critical conservation strategy, species translocation faces numerous challenges that can hinder its success. These challenges arise from ecological, genetic, and monitoring complexities, which must be carefully addressed to achieve desired conservation outcomes.

2.12.3.1. Defining Success Criteria

One of the fundamental challenges in species translocation is the lack of well-defined success criteria. Without clear benchmarks, conservation efforts may be misinterpreted, leading to misleading conclusions about the effectiveness of translocation initiatives. Success criteria must align with specific objectives and be measurable within realistic timeframes to provide meaningful assessments of translocation outcomes (Cowen *et al.,* 2024). Additionally, inadequate monitoring strategies can further complicate evaluations, as species' behaviors and interactions with their environment may not be effectively captured, making it difficult to determine whether populations have truly established themselves (Cowen *et al.,* 2025).

2.12.3.2. Ecological and Genetic Considerations

The ecological consequences of translocating species outside their natural range present significant risks. Introduced species may compete with native flora and fauna, alter existing ecological interactions, or disrupt local ecosystems, necessitating rigorous risk assessments and regulatory frameworks to mitigate adverse impacts (Liu *et al.,* 2015; Tarkan *et al.,* 2024).

Genetic diversity is another crucial factor in translocation success. Establishing genetically robust populations is often challenging due to limitations in available genetic material, which can reduce adaptability and resilience in translocated populations. Insufficient genetic variation may lead to inbreeding depression, lowering long-term survival rates and reducing the ability of populations to respond to environmental changes (Bragg *et al.,* 2020).

2.12.3.3. Context-Dependent Factors

Demographic characteristics, genetic factors, habitat quality, and species-specific behaviours influence translocation success. For instance, a Scandinavian arctic fox translocation study found that only a small proportion of released individuals successfully established themselves, highlighting the importance of species behaviour and environmental compatibility in translocation efforts (Wallén *et al.,* 2022). Cost is another major constraint, as translocation has traditionally been considered an expensive and high-risk conservation strategy. However, studies suggest that the financial burden of translocation is increasingly comparable to other conservation interventions, reinforcing the need for well-planned and efficiently executed projects to maximize success (Zimmer *et al.,* 2019).

2.12.3.4. The Need for Guidelines and Coordination

Successful translocation requires structured guidelines and coordinated national efforts. Countries like China and Canada have made notable progress in developing frameworks that guide translocation initiatives, ensuring that best practices are followed and conservation goals are met effectively (Swan *et al.,* 2018). Establishing comprehensive policies can help streamline translocation efforts, improve post-release monitoring, and facilitate adaptive management strategies.

Species translocation remains a vital conservation tool for mitigating extinction risks and enhancing biodiversity, particularly for endangered plant species. While it offers significant benefits, its success depends on careful consideration of ecological, genetic, and geographic factors and rigorous planning and monitoring. Addressing the challenges associated with translocation and leveraging scientific advancements can enhance its effectiveness, ensuring that it remains a viable strategy for biodiversity conservation in an era of rapid environmental change.

2.13. Cryopreservation as a Method of *Ex situ* Conservation

Cryopreservation has emerged as a reliable and innovative approach for safeguarding the genetic material of plant species, ensuring year-round availability for hybridization, research, and conservation efforts (Rajasekharan & Rohini, 2023). Pollen cryopreservation involves preserving pollen grains at ultra-low temperatures, typically using liquid nitrogen at -196°C. This technique is particularly crucial for the long-term conservation of plant genetic resources, especially for species that are difficult to propagate through traditional methods (Pence *et al.,* 2020). Cryopreservation plays a vital role in conserving plant biodiversity and addressing the challenges posed by species decline in the wild by securing germplasm for future restoration.

The concept of cryopreservation has evolved significantly over the years. Initially, the focus was primarily on seeds and vegetative tissues. However, as understanding of plant biology advanced, researchers began to explore the potential of cryopreserving pollen to enhance genetic diversity and facilitate breeding programs. Early methods of pollen cryopreservation were rudimentary, often involving simple freezing techniques. Over time, more sophisticated methods have been developed, including using cryoprotectants to prevent ice crystal formation within pollen grains. This advancement is critical, as ice formation can damage cellular structures, leading to reduced viability upon thawing (Engelmann, 2004).

Pollen cryopreservation has become an invaluable tool in plant breeding and conservation. It allows for the storage of pollen from elite or rare plant varieties, enabling breeders to access genetic material that may not be available during the growing season. This is particularly beneficial for species with limited flowering periods or endangered ones. Moreover, cryopreserved pollen conserves nuclear genetic variability, which can be utilized for improved seed set, species recovery, and eco-rehabilitation programs (Rajasekharan *et al.,* 2013).

Recent studies have demonstrated that cryopreserved pollen can retain its viability and fertilization potential after thawing, making it a reliable method for long-term storage. The development of species-specific protocols has further expanded the applicability of this technique across a broader range of plants, enhancing its utility in both conservation and agricultural contexts (Pence *et al.,* 2020).

Pollen cryopreservation offers significant benefits for the conservation of endangered species by providing a secure, long-term method to preserve genetic diversity. This technique is particularly valuable for species that cannot be stored in seed banks or are threatened by habitat loss and climate change. By maintaining genetic variability, cryopreservation supports species' resilience and adaptability, ensuring their survival in changing environments (Rajasekharan & Rohini, 2023).

The future of pollen cryopreservation is promising, with ongoing research focused on refining techniques and understanding the underlying biological mechanisms. As the demand for plant genetic conservation increases, pollen cryopreservation is expected to play a critical role in preserving biodiversity and supporting agricultural sustainability. Advances in cryoprotectant formulations, storage protocols, and thawing techniques will further enhance the viability and applicability of this method (Engelmann, 2004).

2.13.1. Key Benefits of Pollen Cryopreservation

Genetic Diversity Preservation: Cryopreservation maintains endangered species' nuclear genetic diversity (NGD), which is crucial for future conservation and restoration efforts. This is particularly important for species with limited breeding information, as it allows for conserving genetic variation expressed through pollen (Rajasekharan & Ganeshan, 2019; Rajasekharan *et al.,* 2013).

Long-term Storage: Pollen cryopreservation allows for the long-term storage of genetic material at ultra-low temperatures, ensuring that the genetic resources remain viable for decades. This method is effective for species that are desiccation-tolerant and can be stored in liquid nitrogen without losing viability (Rajasekharan *et al.,* 2013; Pence *et al.,* 2020; Kaviani & Kulus, 2022).

Support for Restoration Programs: The preserved pollen can be used in controlled pollination and breeding programs to enhance seed set and support species recovery and eco-restoration efforts. This is particularly beneficial for rare, endangered, and threatened species that require immediate conservation attention (Rajasekharan *et al.,* 2013; Edesi *et al.,* 2019).

Cost-Effectiveness and Efficiency: Cryopreservation reduces the need for space, lowers the risk of contamination, and minimizes operator errors compared to other conservation methods. It is a low-cost and efficient strategy for maintaining plant biodiversity (Kaviani & Kulus, 2022; Merzougui *et al.,* 2023).

Flexibility Across Species: The technique applies to a wide range of plant species, including those with recalcitrant seeds or those that are vegetatively propagated. This flexibility makes it a versatile tool in conserving both tropical and subtropical species (Żabicki *et al.,* 2021; Kaviani & Kulus, 2022).

Pollen cryopreservation is a powerful tool for the conservation of endangered species. It offers a reliable method to preserve genetic diversity and support restoration efforts. Its ability to maintain genetic resources over the long term makes it an essential component of modern conservation strategies.

2.13.2. Limitations of Pollen Cryopreservation

While pollen cryopreservation is a promising technique for plant genetic conservation, it faces several limitations, including viability issues, cryoprotectant toxicity, species-specific protocols, storage challenges, limited genetic diversity, and the requirement for technical expertise. Addressing these limitations is essential for enhancing the effectiveness of pollen cryopreservation in conservation and breeding programs.Here are some key limitations of pollen cryopreservation:

Viability Issues: One primary challenge is ensuring pollen's viability after thawing. Not all pollen types respond equally well to cryopreservation, and some may exhibit reduced germination rates or fertilization potential post-thaw. This variability can limit the effectiveness of the technique across different species. (Pence *et al.,* 2020).

Cryoprotectant Toxicity: Cryoprotectants help prevent ice crystal formation, but they can be toxic to pollen grains at specific concentrations. Finding the right balance between effective cryoprotection and toxicity is crucial, as it can vary significantly among plant species (Rajasekharan *et al.,* 2013).

Species-Specific Protocols: There is no one-size-fits-all protocol for pollen cryopreservation. Each species may require specific conditions for successful freezing and thawing, which can complicate the process. This necessitates extensive research and optimization for each target species, making it time-consuming and resource-intensive (Pence *et al.,* 2020).

Storage Duration and Conditions: The long-term storage of cryopreserved pollen can also pose challenges. While many studies indicate that pollen can remain viable for extended periods, the exact duration can vary widely. Additionally, the conditions under which pollen is stored (e.g., temperature fluctuations) can impact its viability (Engelmann, 2004).

Limited Genetic Diversity: Pollen cryopreservation may not capture the full genetic diversity of a species, especially if only a limited number of individuals are sampled for preservation. This can be a significant concern for conservation efforts to maintain genetic variability within populations (Rajasekharan & Rohini, 2023).

Technical Expertise Required: Successful pollen cryopreservation requires specialized knowledge and technical skills. This can be a barrier for many institutions, particularly in regions with limited resources and expertise.

Chapter 3

The Species- Threats and Phenology

Chapter 3

The Species- Threats and Phenology

3.1. Introduction

3.1.1. Habitat Exploration and Threat Assessment

Habitat exploration is integral to designing effective conservation strategies for endangered species, as it provides critical insights into their ecological needs, behaviours, and vulnerabilities. Understanding the dynamics of species habitats enables conservationists to identify specific requirements for survival, assess the suitability of current and potential habitats, and develop targeted measures to address threats. Additionally, habitat exploration informs the identification of climate-resilient areas that can support the recovery and persistence of endangered species, enhancing their long-term survival prospects (Leopold *et al.*, 2024).

A key tool for habitat assessment and threat evaluation is the application of spatial metrics like the Extent of Occurrence (EOO) and Area of Occupancy (AOO), as outlined by the International Union for Conservation of Nature (IUCN) under Criterion B. EOO measures the geographic spread of a species within the shortest continuous boundary that encompasses all known or inferred occurrences, excluding irregular movements or vagrancy (IUCN Standards and Petitions Committee, 2024). In contrast, AOO quantifies the area occupied within the EOO, providing a finer-scale understanding of habitat utilization, particularly for species with fragmented or patchy distributions.

Combining EOO and AOO metrics is vital for identifying species at risk due to restricted ranges and fragmented habitats. These metrics facilitate the prioritization of conservation actions by highlighting critical areas requiring immediate attention. Furthermore, integrating these spatial assessments with ecological monitoring enables conservationists to identify threats such as habitat degradation, fragmentation, and land-use changes. Through these efforts, habitat exploration and threat assessment serve as foundational steps in mitigating risks, preserving biodiversity, and ensuring the resilience of ecosystems.

3.1.2. Relevance of Phenology in Conservation

Phenology is the study of recurring biological events in the life cycles of organisms, such as flowering, fruiting, and breeding, and their relationship with environmental factors, particularly climate. Climate is a critical regulator of phenological events, influencing species distributions and the timing of life cycle transitions. The impacts of global warming on phenology are well-documented, with shifts in leaf change, flowering times, and reproductive phases being observed across ecosystems (Chuine & Beaubien, 2001; Menzel *et al.*, 2006). Such changes have cascading effects on species interactions, community dynamics, and ecosystem processes, highlighting the need for integrating phenological insights into conservation strategies.

Understanding phenological patterns provides essential information for biodiversity conservation and ecosystem management. Phenological data helps predict species' responses to climate change, including distribution shifts and adaptations to changing ecological niches. For instance, species distribution models (SDMs) informed by phenological responses have demonstrated the potential to improve predictions of species loss and turnover under climate change scenarios (Peng *et al.,* 2024). Furthermore, phenology-guided conservation actions, such as timing seed collection and monitoring dispersal agents, are crucial for maintaining ecosystem functions and species persistence. By revealing vulnerabilities or resilience within species, phenological studies enable targeted interventions to mitigate mismatches in ecological interactions between pollinators and plants and ensure long-term ecosystem health and biodiversity (Rosemartin *et al.*, 2014; Morellato *et al.*, 2016).

This comprehensive understanding of phenology, encompassing its role in species distributions, ecological interactions, and climate change adaptation, underscores its importance as a foundational tool in developing conservation and sustainable ecosystem management strategies.

3.2. Materials and Methods

3.2.1. Habitat Exploration

Field exploration was conducted in the natural habitats of *Crinum malabaricum* across different seasons from June 2020 to June 2024. Live plants were collected from various populations, and geographic data such as altitude, latitude, and longitude were recorded using a handheld Global Positioning System (Montana 360, Garmin) (Table 3). The associated floral and odonata diversity were documented. Photographs capturing various phenological stages of the plants and their habitats were taken using a digital camera (EOS 700D, Canon).

Live plants were introduced into the aquatic plant's conservatory, 'Aquagene', at KSCSTE-MBGIPS, Kozhikode, and the collected samples were studied in the systematics laboratory for taxonomic confirmation. Herbarium specimens were prepared from the collected samples and deposited in the MBGH Herbarium for future reference.

Sl. No.	Habitat	Latitude	Longitude	Elevation	Length (km)
1.	Embate, Kannur	12°4'31.14"N	75°18'34.03"E	80 m a.s.l.	4.7
2.	Aravanchal, Kannur	12°12'34.36"N	75°17'02.36"E	100 m a.s.l	3.7
3.	Cheemeni, Kasaragod	12°13'31.25"N	75°14'06.71"E	95 m a.s.l.	0.59
4.	Periye, Kasaragod	12°24'0.52"N	75° 06'0.57"E	100 m a.s.l	5.5

Table 3: Geographic details of the natural habitat of *C. malabaricum*

3.2.2. Assessment of Conservation Status

The conservation status of *C. malabaricum* was assessed following the IUCN Criteria B guidelines. Criterion B is designed to identify populations with restricted distributions that are severely fragmented, limited to few locations, experiencing ongoing decline, and/or exhibiting extreme fluctuations (Table 4). The spatial extent of the habitats was mapped using QGIS, version 3.28.4 (QGIS Development Team, 2023). The Extent of Occurrence (EOO) was calculated using the alpha hull method in the conR R package (Dauby *et al.*, 2017) and the minimum convex polygon approach using GeoCAT (Bachman *et al.*, 2011). The Area of Occupancy (AOO) was estimated using two approaches: a fine-scale Grid Method using 1×1 km grid cells and a Linear Habitat Method (total habitat length × average width), suitable for species inhabiting linear environments (IUCN Standards and Petitions Committee, 2024).

Population assessments were conducted in 2020 and 2024 using 10-meter permanently marked sections along the streams, spaced at intervals of 100 meters. The number of mature individuals within each marked section was recorded. The total length of the population extent was estimated using QGIS (version 3.34.11), and the average width and density of the population were calculated based on the collected data. Comparative differences between 2020 and 2024 were analyzed to assess population changes. Habitat conditions were also documented, and potential threats to the species were identified.

Criteria B: Geographic range in the form of either B1 (extent of occurrence) AND/OR B2 (area of occupancy)			
	Critically Endangered	Endangered	Vulnerable
B1. Extent of occurrence (EOO)	< 100 Km^2	<5,000 Km^2	<20,000 Km^2
B2. Area of occupancy (AOO)	< 10 Km^2	< 500 Km^2	< 2,000 Km^2
AND at least 2 of the following 3 conditions:			
(a) Severely fragmented OR Number of locations	=1	≤ 5	≤10
(b) Continuing decline observed, estimated, inferred or projected in any of: **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** area, extent and/or quality of habitat; **(iv)** number of locations or subpopulations; **(v)** number of mature individuals			
(c) Extreme fluctuations in any of: **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** number of locations or subpopulations; **(iv)** number			

Table 4: Guidelines for IUCN criteria B used to evaluate if a taxon belongs in an IUCN Red List threatened category.

3.2.3. Phenology

3.2.3.1. Study Area

Phenological data were collected from four permanent plots established in the natural habitats of *Crinum malabaricum* in Periye (Kasaragod district) and Embate (Kannur district), Kerala, India. The plots were marked 50 meters long within the streams.

The geographic coordinates of the permanent plots are:

- **E1**: Embate (12° 4' 26.23959" N, 75° 18' 22.10774" E)
- **E2**: Embate (12° 4' 31.14717" N, 75° 18' 34.03781" E)
- **P1**: Periye (12° 24' 21.70984" N, 75° 6' 59.56668" E)
- **P2**: Periye (12° 24' 32.80958" N, 75° 6' 43.54148" E)

3.2.3.2. Phenological Analysis

Phenological analysis was conducted through continuous field observations over 42 months, from December 2020 to May 2024. The vegetative and reproductive phenophases were recorded and classified following the methodologies proposed by Newstrom *et al.* (1994), Dafni *et al.* (1992) and Madhuri *et al.* (2016). Observations were made every three consecutive days at 15-day intervals during the reproductive stage and monthly during the dormancy stage.

Field observations included the stages of leaf sprouting and seed setting. Floral longevity was observed by marking buds in the pre-anthesis stage and monitoring them until floral senescence. Anthesis and anther dehiscence were examined using a hand lens based on the method outlined by Reddi and Bai (1981) and Ramasubbu *et al.* (2009). The floral morphology was analysed using a hand lens and a stereomicroscope (SAPO, Leica, Germany).

The species' phenophases are divided into seven principal growth stages: Leaf sprouting, Vegetative growth, Flowering, Fruit development, Fruit maturity, Senescence, and Dormancy. Each principal stage is further divided into secondary growth stages. Numerical values were assigned to each stage according to the BBCH scale for accurate monitoring of phenophases (Meier, 1997).

3.2.3.3. BBCH Scale

The BBCH scale employs a two-digit numerical code:

- **First digit:** Represents the principal growth stage (0–9).
- **Second digit:** Represents the secondary growth stage (0–9).

Principal growth stages correspond to the seven main developmental stages of the plant, while secondary growth stages indicate either the percentage of growth completed or the duration of each principal stage (Table 5).

Code	Principal growth	Subcode	Secondary growth stage
0	Leaf sprouting	9	Initiation of sprouting
1	Vegetative growth	1	10% of maximum biomass reached
		5	50% of maximum biomass reached
		9	100% of maximum biomass reached
2	Flowering	0	Floral Buds appears
		2	Inflorescence emergence
		3	First flowers open (solitary)
		4	10% flowering
		5	50% of flowers open
		8	Flowering about to finish
		9	End of flowering
3	Fruit development	0	Fruit formation
		1	Fruit achieving 10% size
		5	Fruit achieving 50% size
		9	Fruits achieving 100% size
4	Fruit maturity	5	Ripening or maturity of fruit or fruit colouration
		9	Fruit fallen or drying of peduncle
5	Senescence	0	Pigmentation of leaves
		9	Drying and complete senescence of leaves
6	Dormancy period	9	Dormancy

Table 5: Principal and secondary growth stages with corresponding BBCH values.

3.2.3.4. Correlation with Rainfall

Rainfall is the major environmental parameter determining the life cycle of this ecosystem. Daily Gridded Rainfall Data for the study area was obtained from the IMD Pune database (http://www.imdpune.gov.in/cmpg/), featuring a spatial resolution of 0.25 x 0.25 degrees. Rainfall measurements were recorded in millimetres (mm) (Pai *et al.*, 2014). The rainfall data were statistically correlated with the BBCH scale of phenological events in the study area using Pearson's correlation coefficient. The strength and direction of the correlation were evaluated based on the coefficient of determination (r^2 value) and the associated significance (ρ-value).

3.2.4. Reproductive Biology

3.2.4.1. Pollen Morphology

The morphology of pollen was studied using Scanning Electron Microscopy (SEM). Pollen samples were dehydrated in a desiccator to reduce moisture content and then carefully mounted on adhesive carbon tape affixed to polished aluminium stubs. To minimise electrical charging during SEM imaging and ensure high-quality visuals, the samples were coated with a thin layer of gold-palladium (Au/Pd) using a Sputter Coater SC7620 (Quorum). The prepared stubs were examined under a scanning electron microscope (EVO 18, Carl Zeiss, Germany). Pollen images were captured at appropriate resolutions, and key morphological parameters, including polar length (μm) and equatorial width (μm), were measured using the integrated SmartSEM software (Carl Zeiss).

3.2.4.2. Pollen Viability

Estimated the pollen viability by *in vitro* pollen germination method and two staining methods; tetrazolium test and Alexanders staining test.

Tetrazolium Test

The TTC staining solution was prepared by mixing 100 g L^{-1} sucrose with 5 g L^{-1} 2,3,5-triphenyl tetrazolium chloride (TTC) and adjusting the pH to 7 using either 1N HCl or 1N NaOH solution. Pollen from three flowers was mixed and gently shaken onto a microscope slide. The TTC solution was then pipetted onto the pollen, covered with a coverslip, and incubated at room temperature under humid conditions in the dark for 30 minutes. The preparation was observed under a compound microscope, and red-stained pollen grains, indicating the formation of formazan, were considered viable.

Alexander Staining Method

Alexander stain was prepared by dissolving 100 mg acid fuchsin, 20 ml ethyl alcohol (95%), and 20 mg malachite green. Gradual addition of 50 ml distilled water was followed by 40 ml glycerol and 5 g phenol, with thorough stirring after each step until fully dissolved. Finally, 1–2 ml lactic acid was added. The stain was stored in coloured reagent bottles in a cool, dark place for 8–10 days to age (Alexander, 1980).

One to two drops of Alexander stain were placed on a glass slide, and pollen from three flowers was dusted onto it, mixed thoroughly, and covered with a coverslip. After allowing the slide to stand for 5 minutes, it was examined under compound microscope (Leica). Pollen-stained crimson red was considered viable, while green-stained pollen was classified as aborted.

In vitro Pollen Germination

In vitro pollen germination was performed using the hanging drop method (Shivanna & Rangaswamy, 2012). The pollen germination medium (PGM) was based on a modified Brewbaker and Kwack's formulation, with varying concentrations of Poly Ethylene Glycol (PEG) (1%, 5%, and 10%) and sucrose (1%, 2.5%, 5%, 10%, and 15%) (Brewbaker & Kwack, 1963).

Pollen from three flowers was pooled, mixed, and dusted onto a drop of pollen germination medium placed on a 22 mm square coverslip. The coverslip edges were sealed with petroleum jelly, inverted onto a cavity slide, and incubated at 25°C in Petri dishes lined with moist filter paper. After the incubation period, the hanging drop was observed under a compound microscope (DM750, Leica, Germany).

Pollen germination percentage was calculated using the formula:

$$\text{Percentage of pollen germination } = \frac{\text{No. of pollen grains germinated}}{\text{Total no. of pollen grains observed}} \text{x100}$$

The experiments were conducted in a Completely Randomized Design (CRD) with three replications per sample. For each replication, a minimum of 500–600 pollen grains were observed across at least ten microscopic fields.

3.2.4.3. Pollen Count

Using a haemocytometer, pollen production per flower was calculated following the method proposed by Cruden (1977) and Barrett (1985). Before anther dehiscence, ten mature floral buds were collected. One anther was removed from these buds and placed in separate 1 ml microcentrifuge tubes. Each anther was suspended in a solution of 0.5 ml distilled water and 0.5 ml glycerine to ensure uniform distribution of pollen grains in the sample. A known dilution of the pollen suspension was introduced into the haemocytometer grid, and the number of pollen grains was counted under a microscope.

The pollen production per flower was calculated using the following formula:

$$\text{N } = \frac{(\text{ax v x 10000})}{\text{n}}$$

Where:

- N = Number of pollen grains per flower
- a = Mean number of pollen grains counted per corner square of the grid
- v = Volume of the suspension prepared with the anther
- n = Number of anthers used to prepare the suspension

3.2.4.4. Pollen – Ovule Ratio

The number of ovules per flower was determined by dissecting the ovaries of pre-anthesis flowers under a dissection microscope. The ovules within each ovary were carefully counted to obtain the mean number of ovules per flower.

The pollen-ovule ratio was calculated following the method proposed by Shivanna and Rangaswamy (2012). This was achieved by dividing the mean number of pollen grains per flower by the mean number of ovules per flower, using the formula:

$$\text{Pollen} - \text{Ovule Ratio} = \frac{\text{Mean no. of pollen grains per flower}}{\text{Mean no. of ovules per flower}}$$

3.2.4.5 Stigma Receptivity

Stigma receptivity was assessed using two enzymatic activity tests: the hydrogen peroxide test and the α-naphthyl acetate test. These tests provide insights into the presence and activity of peroxidase enzymes on the stigmatic surface, which are indicators of stigma readiness for pollen reception.

Hydrogen Peroxide Test

The hydrogen peroxide test was employed to evaluate the peroxidase activity on the stigma, a marker of receptivity. Upon applying hydrogen peroxide to the stigma, the formation of oxygen bubbles indicates the presence of peroxidase enzymes. The methodology followed was as described by Joshirao and Saoji (1989) and Kearns and Inouye (1993).

Fresh, mature, and unpollinated flower buds were collected, and the pistils were carefully excised. The excised pistils were placed on a clean, dry microscope slide. A single drop of 3% hydrogen peroxide (H_2O_2) was applied directly onto the stigmatic surface. The number of oxygen bubbles produced was counted within 1 minute. The data were statistically analysed using the Kruskal-Wallis test for independent measures to determine variations in receptivity across samples (McKigh & Najab, 2010). Results were expressed as mean ± standard deviation.

3.2.4.6. Floral Visitors and Their Behaviour

The temporal activities of the floral visitors and the foraging mode of insects visited were regularly observed during the flowering season between morning 6:00 am to evening 6:00 pm.

The number of floral visitors, visiting time, foraging nature, foraging hour, time spent in flower, stigma touch by insects, frequency of visit, contact with the anthers and the stigma are the parameters recorded.

After each visit, the stigma was inspected using a hand lens for the confirmation of pollen transfer to the stigma. The insect visiting the flowers were caught and fixed in 70% alcohol for identification. Observed them under stereo microscope for pollen load on the body parts. The pollinators are identified with the help of entomologists at Zoological Survey of India (ZSI), Kozhikode.

3.2.4.7. Breeding Experiments

Conduct breeding practices like open pollination, autogamy, geitonogamy, xenogamy, and apomixes (Wani *et.al.*, 2006)

Open Pollination: Unemasculated flower heads were tagged and allowed to open pollinate

Autogamy: Unemasculated flower heads were bagged to avoid foreign pollen deposition and allowed to self-pollination.

Geitonogamy: In emasculated flowers, stigmas were hand-pollinated with pollen from the anthers of the same plant and bagged.

Xenogamy: In emasculated flowers, stigmas were hand-pollinated with pollen from the anthers of genetically different plants and bagged.

Apomixis: Emasculated flowers were bagged to check the apomictic development of seeds

Seed Set: The percentage of seed set was calculated by the equation proposed by Lubber and Christenson, 1986:

$$\text{Percentage of Seed set} = \frac{\text{Total no. of seeds produced per plant}}{\text{Total no. of ovules borne by the plant}} \text{x} 100$$

3.3. RESULTS

3.3.1. The Habitat

Crinum malabaricum is distributed across four seasonal streams located in the northern districts of Kerala, India. These habitats are situated within the Malabar Plains of the Western Ghats, adjacent to the western coastal plains (Figure 8). The streams originate from the laterite plateaus in the region and flow into larger water bodies before eventually joining major rivers. All the streams supporting the species are seasonal, beginning to flow with the arrival of southwest monsoon or pre-monsoon rainfall (May–June) and drying up entirely by the onset of summer (January–February).

The streams are located at altitudes below 100 meters above sea level and are separated by distances of 10 to 20 kilometres. All populations are situated within 20 kilometres of the coastline. The streams run through valleys formed between low-lying laterite hills and have widths ranging from 1.5 to 6 meters. Based on terrain, bed material, and flow speed, the streams are divided into three distinct stages: upper, middle, and lower courses.

The middle course is gradually sloping with bed material of gravel with fine grit and small or medium-sized laterite stones and moderately to fast flowing. Lower course is with shallow water body with high deposition rate of slit and very slow running or stagnant and enters to a large water body like paddy field. Both the upper course and lower course does not support the growth of *C. malabaricum.* Only middle course supporting the growth of the species.

The upper course is characterized by steep slopes with continues exposed laterite bedrock and large-sized laterite pebbles. This section experiences the highest flow speeds and does not support the growth of *C. malabaricum*. The middle course is the longest segment of the streams and supports the largest population of the species. It is defined by gradually sloping terrain, with bed material composed of gravel, fine grit, and small to medium-sized laterite stones. The flow in this section is moderately to fast, and the long leaves of *C. malabaricum* often cover the entire streambed, forming dense populations. However, gaps in plant distribution are observed due to specific habitat conditions. The lower course, in contrast, consists of shallow water bodies with significant silt deposition. The flow in this section is slow-moving or stagnant, and enters into paddy fields. These conditions are unsuitable for the growth of *C. malabaricum*.

In the middle course, where the species thrives, certain factors influence its distribution. The optimal habitat is observed in regions where the stream depth is less than 0.5 meters. In areas modified for hydrological management, such as the creation of deeper channels, the population becomes sparse. Similarly, discrete patches of large laterite bedrock exposed above water disrupt the stream's normal flow, creating deeper regions. In these areas, *C. malabaricum* is restricted to rock crevices where gravel deposited.

Figure 8. Habitats of *Crinum malabaricum*

High silt deposition, which is common in curves, blockages, and shallow regions, also limits the growth of *C. malabaricum.* Anthropogenic activities, such as channel modifications, bridge construction, and the installation of check dams, significantly impact the habitat of *C. malabaricum*. These interventions alter the natural flow and structure of the streams, leading to the absence of the species in modified regions.

3.3.2. Ecological Interactions

C. malabaricum is the dominant species in its habitat, serving as an ecosystem architect for the small seasonal streams it inhabits (Figure 9). The long leaves of *C. malabaricum* form dense cover over much of the streambeds, creating a safe breeding ground for fishes and amphibians. This dense vegetation also provides a suitable environment for a high diversity of odonates (Table 6, Figure 10), which thrive due to the abundance of insects associated with the ecosystem formed by the plant. These plants offer a secure ground for breeding and egg-laying activities within their vicinity.

Sl. No	Species	Common name	Family
1.	*Phylloneura westermanni*	Myristica bamboo tail	Platycnemididae
2.	*Euphaea fraseri*	Malabar torrent dart	Euphaeidae
3.	*Agriocnemis clauseni*	Wisps	Coenagrionidae
4.	*Vestalis gracilis*	Clear-winged forest glory	Calopterygidae
5.	*Trithemis aurora*	Crimson marsh glider	Libellulidae
6.	*Trithemis festiva*	Indigo drop wing	Libellulidae
7.	*Orthetrum luzonicum*	Tricolored marsh hawk	Libellulidae
8.	*Heliocypha bisignata*	Stream ruby	Chlorocyphidae
9.	*Libellago indica*	Southern heliodor	Chlorocyphidae

Table 6: Odonata diversity in the habitat of *C. malabaricum*

The extensive leaf cover of *C. malabaricum* contributes to nutrient cycling within the streambed. As the streams dry out during summer, the decaying plant material enriches the soil's nutrient levels, benefiting other species within the ecosystem.

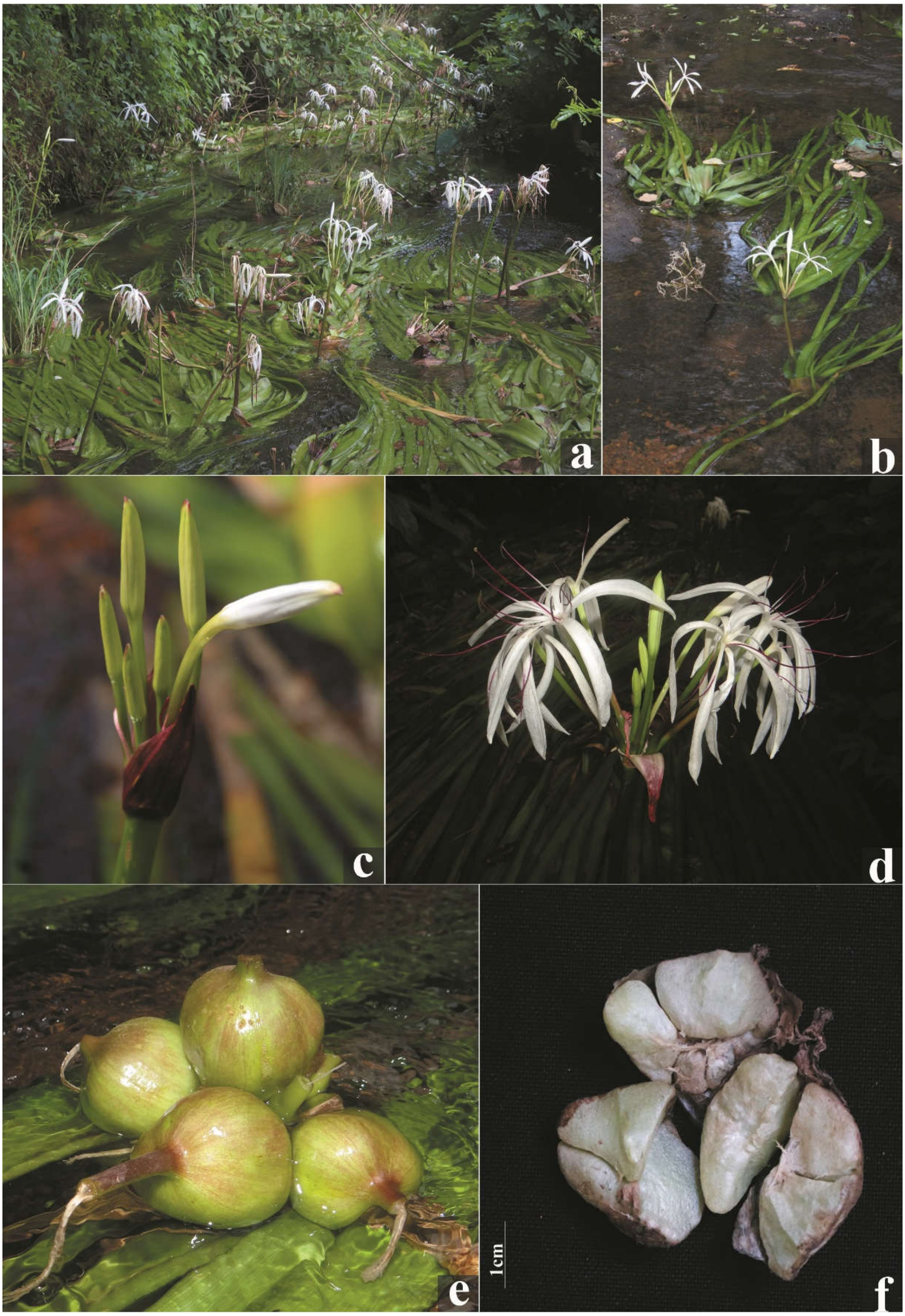


Figure 9. *Crinum malabaricum* Lekhak & S.R.Yadav; a & b. Habitat; c. Flower buds; d. Inflorescence; e. Fruits; f. Seeds

Figure 10: Odonata diversity in the habitat of *C. malabaricum*

Additionally, *C. malabaricum* is a host plant for the Indian lily moth caterpillar (*Polytela gloriosa*), which is particularly abundant during the drying period. The caterpillars aid in the rapid degradation of plant biomass, further enhancing the habitat's nutrient turnover.

Several plant species co-occur with *C. malabaricum* in these streams, often adapting to similar seasonal cycles. The major associated plants identified are *Eriocaulon* *heterolepis* and *E. kanarense,* which are abundant in all of their natural habitats and follow a similar life cycle to *C. malabaricum*, except that these are ephemeral plants. The floral diversity recorded from these streams is listed in the table 7.

Area of Habitat	Species
Along the running stream	*Eriocaulon heterolepis* *Eriocaulon kanarense* *Blyxa aubertii var. echinosperma* *Eriocaulon setaceum* *Cryptocoryne spiralis*
Stagnant regions	*Limnophila aquatica* *Limnophila repens* *Najas species* *Rotala kasargodensis*
Margin vegetation	*Isachne globosa* *Sasiola interrupta* *Oryza rufipogon* *Murdannia nudiflora*
Laterite outcrops	*Eriocaulon kanarense*

Table 7: Associated aquatic plant species of *C. malabaricum* in its habitats

3.3.3 Habitat Assessment

The habitat assessment of *C. malabaricum* reveals that the species occupies a total area of 11.42 km^2 across four streams in northern Kerala, India, with a population estimated at approximately 25,000 mature individuals as of 2020. However, due to habitat destruction, the population decreased significantly by 2024, resulting in a loss of 1,569 m^2 of habitat and a reduction in population size to approximately 22,000 mature individuals. Detailed observations for each habitat are presented below:

Embate: Embate holds the largest population of *C. malabaricum* with approximately 8,000 mature individuals inhabiting an area of 1.5 km in length and an average width of 2.5 m. Most of the streams running through human settlements and rubber estates. The population abundance ranged from 20 individuals per 10 m in narrower regions to 93 individuals per 10 m in broader sections of the channel, with an average of 55 individuals per 10 m.

The population density was calculated at 2.2 ± 0.005 individuals/m², covering a total area of 2,922 m² in 2020. However, the habitat assessment over four years shows that, 478 m² of the population was lost, leading to a population reduction to 6,721 ± 34 individuals by 2024 (Figure 11).

Figure 11. The extant of habitat of *C. malabaricum* in Embate showing population loss from 2020 to 2024 marked as red colour

Aravanchal: In Aravanchal, the streams mostly flow through the boundary of barren laterite plateaus and the Aravanchal sacred grove, with remaining sections passing through human settlements and plantations. The area supports approximately 7,200 mature individuals within a habitat measuring 1.1 km in length and an average width of 4 m.

Figure 12. The extent of habitat of *C. malabaricum* in Aravanchal showing population loss from 2020 to 2024 marked as red

The population abundance ranged from 45 individuals per 100 m in narrow sections to 75 individuals per 100 m in broader regions. The population density was 1.32 ±

0.005 individuals/m², covering an area of 4,400 m² in 2020. Habitat destruction resulted in a loss of 240 m², reducing the population size to 6,864 ± 21 individuals by 2024 (Figure 12).

Cheemeni: Cheemeni supports the smallest population of *C. malabaricum*, with an approximate population size of 2400 mature individuals recorded in 2020. The habitat spans 294 m in length, with an average width of 2 m. The habitat exhibited the highest density of 4.1 ± 0.005 individuals/m² among all locations, with an average of 82 plants per 10 m. The total area covered was 588 m² in 2020. Over four years, 122 m² of habitat was lost, reducing the population to 2,156 ± 9 individuals by 2024 (Figure 13).

Figure 13. The extant of habitat of *C. malabaricum* in Cheemeni showing population loss from 2020 to 2024 marked as red colour

Periye: Periye is the type locality of *C. malabaricum* and supports a population of 7,949 ± 24 individuals as of 2020. The population is 1.1 km long, with an average width of 3 m, running through areas with human habitation, rubber estates, and plantations. The population ranged from 40 individuals per 10 m in narrow sections to 120 individuals per 10 m in broader regions, with an average of 55 individuals per 10 m.

The density was calculated at 1.7 ± 0.005 individuals/m², covering a total area of 3,507 m² in 2020. However, this habitat experienced the most significant habitat loss, with 729 m² destroyed due to check dam co construction. Consequently, the population declined to 6,297 ±19 individuals by 2024 (Figure 14).

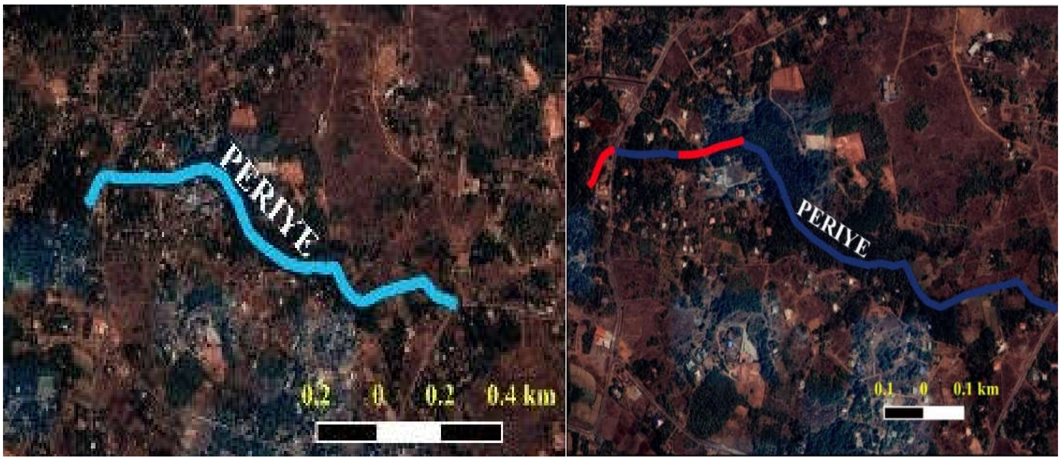


Figure 14. The extant of habitat of *C. malabaricum* in Periye showing population loss from 2020 to 2024 marked as red colour

Across all populations of *C. malabaricum*, significant variation in population size was observed over the four-year assessment period. The estimated overall population size declined from 25,655 ± 24 mature individuals in 2020 to 22,038 ± 20 mature individuals in 2024, indicating a notable loss. This represents a 14.1 ± 0.05% reduction in the species' overall population size (Table 8).

Population	Average width (m)	Density (Mature individual/m²)	Length of population (m) (2020)	Total area (m²) (2020)	Population size (2020)	Lost Area (m²) (2024)	Population size (2024)
Embate	2.5	2.2 ± 0.005	1461	2922	8035 ± 40	478	6721 ± 34
Aravanchal	4	1.32 ± 0.005	1100	4400	7260 ± 22	240	6864 ± 21
Cheemeni	2	4.1 ± 0.005	294	588	2410 ± 10	122	2156 ± 9
Periye	3	1.7 ± 0.005	1169	3507	7949 ± 24	729	6297 ± 19
Total					**25655 ± 24**	**1569**	**22038 ± 20**

Table 8: Population structure of *C. malabaricum* in the four natural habitats and the loss of area of habitat from 2020 to 2024

3.3.3.1. Extent of Occurrence (EOO)

Two approaches were implemented to plot the extant of occurrences. The species has an EOO of 160.5 sq.km. based on the minimum convex polygon method. In contrast, the Alpha hull method estimated the EOO at 235 sq. km. (Figure 15).

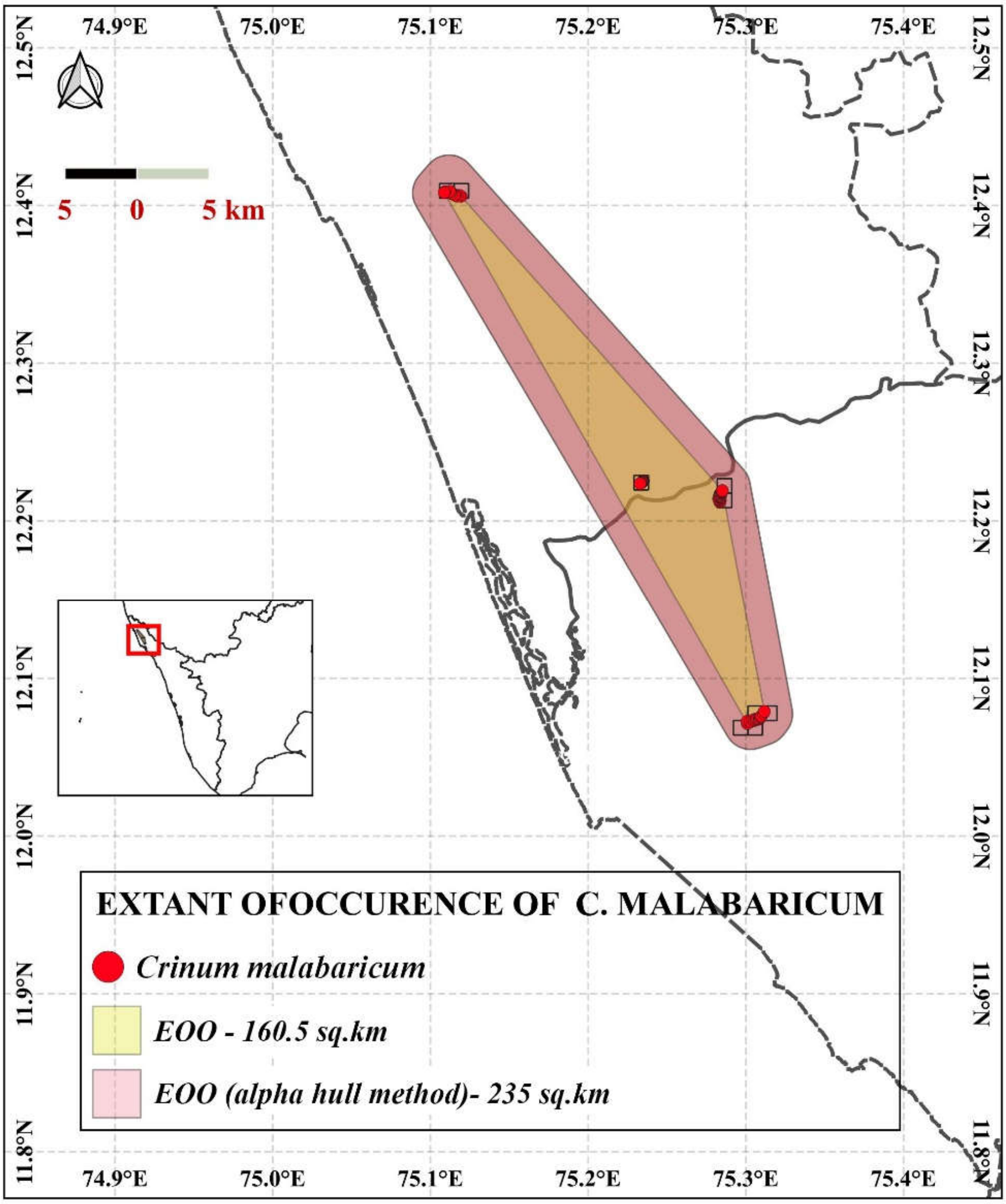


Figure 15. Extant of occurrences (EOO) of *C. malabaricum*

3.3.3.2. Area of Occupancy

The Area of occupancy (AOO) of the species is estimated as 9 sq. km based on 1x1 Km square grid method (Figure 16). Using the linear method, the AOO was recorded as 11.417 sq. km. in the 2020 assessment. However, rapid changes in the population affected the AOO, with the 2024 assessment recording an AOO of 9.98 sq. km. (Table 8).

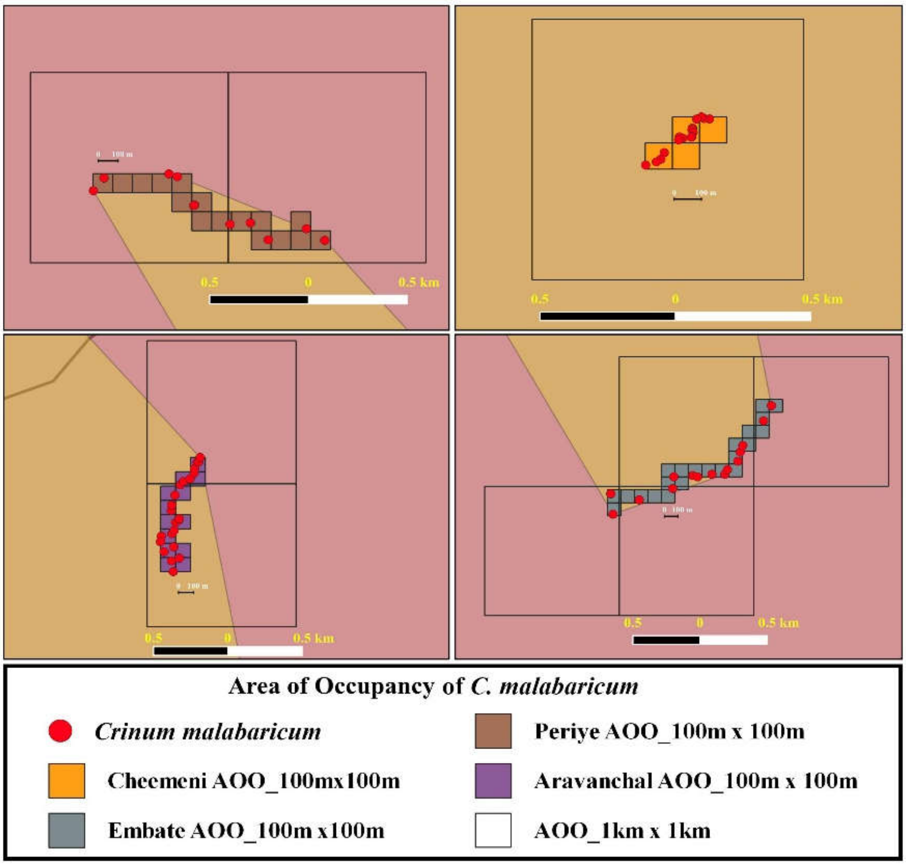


Figure 16. Area of occupancy (AOO) of *C. malabaricum*

3.3.3.3. IUCN Category

The initial assessment in 2016 categorized the species as Critically Endangered (CR) under the criteria CR B1ab(iii,v) + 2ab(iii,v) (Lansdown, 2016). A subsequent study in 2017 suggested reclassifying the species to the Vulnerable (VU) category (Lansdown & Molur, 2017) based on an increase in the number of known populations to four and the expansion of the Extent of Occurrence (EOO) to 235 sq. km.

However, the present study reveals a rapid decline in the species' Area of Occupancy (AOO), from 11.417 sq. km in 2020 to 9.98 sq. km in 2024, based on the linear method. The 1 × 1 km^2 grid-based AOO estimation also supports this trend, with the species occupying only nine grids (9 sq. km), which falls within the same critical threshold.

This decline qualifies the species once again for the Critically Endangered (CR) category under IUCN Criterion B2 (AOO less than 10 sq. km).

The population also meets the following sub-criteria: **(b)** Continuing decline observed, estimated, inferred, or projected: **(ii)** Area of occupancy, **(iii)** Area and quality of habitat, **(v)** Number of mature individuals. And **(c)** Extreme fluctuations in: **(ii)** Area of occupancy, **(iv)** Number of mature individuals.

Thus, this study strongly recommends including the population in the Critically Endangered (CR) category of the IUCN, based on the criteria B2b(ii,iii,v)c(ii,iv).

3.3.4. Threats to the Species

Through habitat exploration conducted over four years, noticeable threats and their significant impacts on *C. malabaricum* were identified. Threats to the species are classified into major and minor threats based on the scale of impact, range of influence, and difficulty of management or mitigation.

Major threats exert a widespread, long-term impact on the species and its habitat, often resulting in irreversible changes. These threats directly disrupt critical ecological processes like hydrology, temperature regulation, and habitat continuity of the species, making them more challenging to address. Conversely, minor threats are localized or temporary disruptions that, while detrimental, can be mitigated through targeted interventions. These threats are more manageable as they typically involve specific areas and actions. This classification helps to prioritize conservation efforts by focusing on the more pressing and complex challenges while ensuring that localized issues are not overlooked. The threats are listed below:

3.3.4.1. Major Threats

1. Uncontrolled Laterite Mining Alters the Water Cycle

Laterite mining directly impacts the species' habitat, as these streams depend on the laterite plateaus for their water source. Destruction of these laterite hills disrupts the water cycle of the streams, thereby affecting the species' lifecycle and ultimately destroying its habitat. This damage is often permanent and irreversible. Notably, four species' natural habitats are situated within areas potentially suitable for laterite mining.

During the study period, a laterite mine was initiated upstream at the origin of the population in Embate. The mining activity caused increased silt deposition, leading to a drastic population decline during the 2022 growing period. This followed the commencement of rapid laterite mining in December 2021. Satellite image analysis reveals the rapid changes in the laterite plateaus in this region (Google Earth, 2024) (Figure 17).

Figure 17. The satellite Images show a laterite mine (red arrow mark pointed) and the origin of the stream (yellow mark)

2. Change in Climatic Cycles Affects the Population

Life in the laterite streams is directly dependent on annual rainfall. Global climate changes disrupt rainfall patterns in this region, triggering abnormal events in these habitats and posing significant threats to the species.

For example, the species serves as a host for the Indian lily-moth (*Polytela gloriosae*). The caterpillars of this moth usually feed on senescent plant parts, facilitating natural degradation (Figure 18). Their activity typically increases during the natural drying period. However, with changes in temperature and rainfall cycles during the growing period (June to November), the streams may dry up prematurely. This elevates the caterpillar infestation, causing them to feed on live plants, bulbs, and seeds, leading to severe and massive damage to the population. Such unpredictable events can rapidly impact the population of the species.

3. Natural Barrier to the Spread of Populations

The species thrives in laterite soil, but natural geographical barriers restrict its distribution. The discontinuous distribution of laterite plateaus in the Malabar Plains prevents the species from spreading to suitable habitats when conditions in its current habitat become unfavourable. This lack of connectivity poses a significant challenge for the species' survival and expansion, leaving it highly vulnerable to habitat changes.

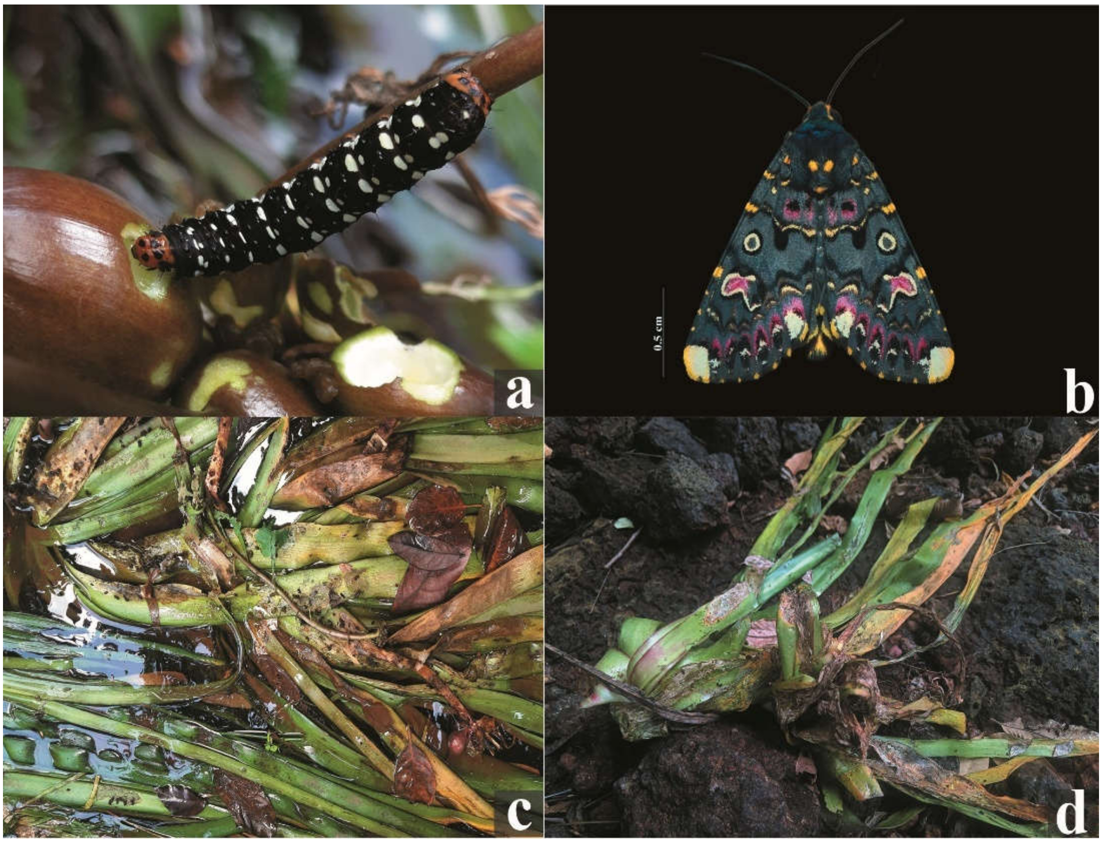


Figure 18. *Polytela gloriosae* Fabrici (Indian lilymoth): a. Caterpillar; b. Adult moth; c & d. Severe attack of the moth on *C. malabaricum*

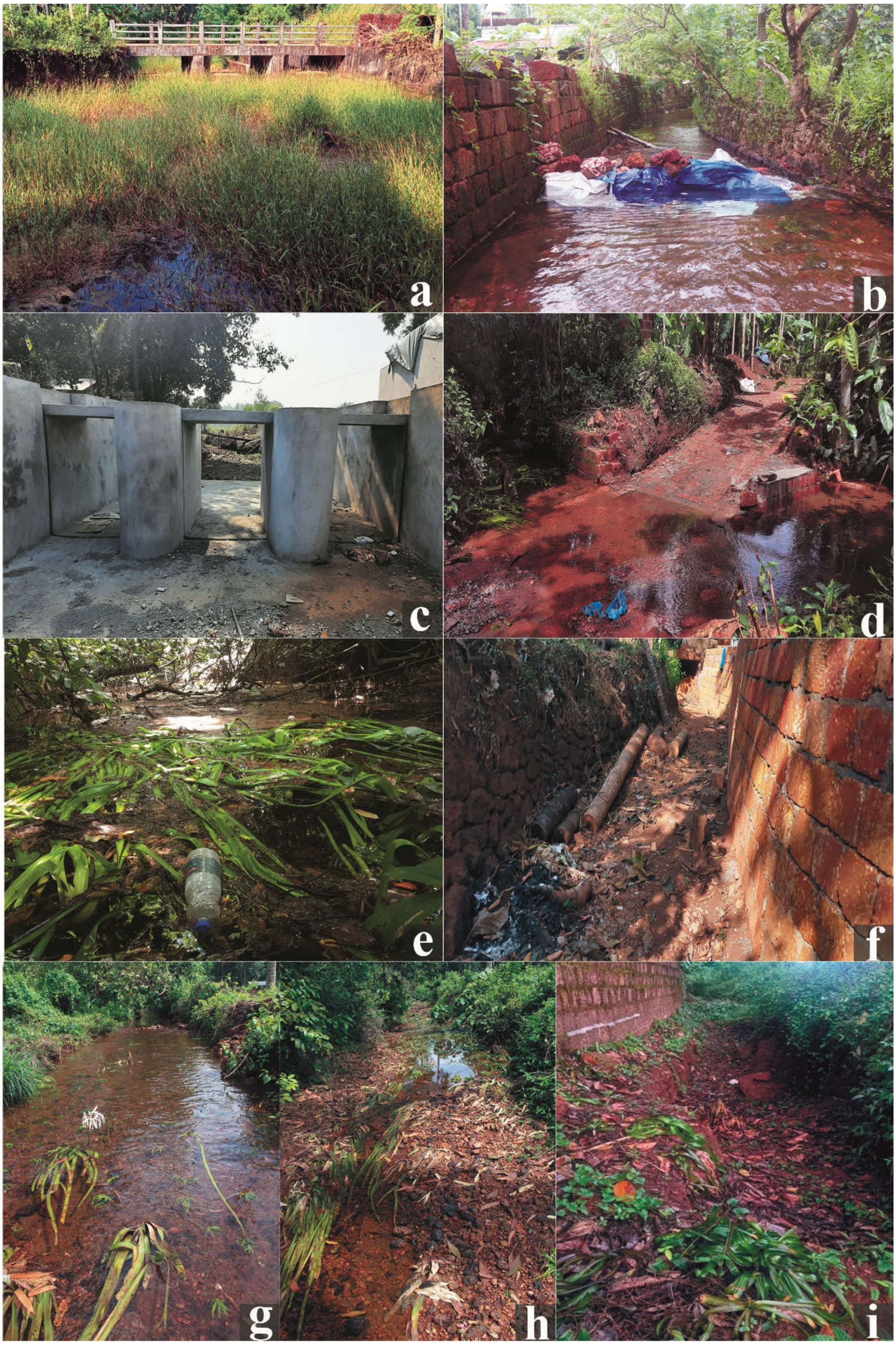


Figure 19. Threats in the habitats of *C. malabaricum*: a. Check dams; b. Clearing and blocking of the stream; c. Bridge construction; d. Constructions within the stream e. Waste dumping; f. Encroachment; g & h. Silt deposition; i. Soil removal

3.3.4.2. Minor Threats

1. Inappropriate Hydrological Management of the Streams

- **Construction of Check Dams**: Check dams Alter the natural flow of water, creating stagnant waterbodies, which prevents the growth of *C. malabaricum* in this area. The major check dam constructed in the stream of Periye created a large pond within the stream, simultaneously increasing slit deposition and destroying the population around 300 m. upstream and downstream (Figure 19.a).

- **Canal Construction along the Banks**: Diverts water and disrupts the habitat, affects the flow of the streams

2. Human Interventions in the Stream

- Impact of Bridges: The construction of bridges across streams destroys populations in the immediate vicinity. The stream beds under bridges are often levelled with concrete, permanently destroying the habitat and creating discontinuities in the population. Additionally, areas immediately upstream and downstream of bridges are significantly affected, resulting in habitat destruction and population decline (Figure 19.c).

- Waste Disposal: Dumping waste into streams covers the populations and adversely affects the species' survival. It also degrades water quality, further threatening the ecosystem (Figure 19.e).

- Silt deposition: Alternating stream flow, combined with the operation of laterite mines, increases silt deposition in the streams, which negatively affects the population (Figure 19.g&h).

- Clearing Stream Areas During Summer: Removing vegetation from streams during the dry season disrupts habitat stability and negatively impacts the population.

- Stream Clearing for Cloth Washing: Continuous removal of plants for washing clothes over an extended period leads to habitat degradation and population decline (Figure 19.b).

- Installation of Concrete Walls: Encasing stream banks with concrete walls severely alters the natural habitat, reducing population viability and disrupting ecological balance.
- Gravel Collection: Digging into stream beds to extract gravel disturbs the substrate and destroys the habitat, further endangering the population (Figure 19.i).
- Encroachment into Stream Areas: Private property encroachment reduces habitat space, alters natural water flow, and increases human-induced pressures on the ecosystem (Figure 19. d&f).
- Housing Development Near Streams: Housing construction near stream premises poses risks of direct habitat damage, increased sedimentation from runoff, and the introduction of untreated waste into the watercourse, all of which threaten the species' survival.

3.3.5. Phenology

3.3.5.1. Phenological Events

All events are summarized based on the phenophase that the majority of plants in the marked study area are undergoing. As bulbous perennial plants, some individuals may exhibit varied patterns of phenophases mentioned here, but we are considering only the phenophase observed in the majority of the plants (Figure 21). The summary of the phenology and floral biology of the species given in the table 13.

3.3.5.1.1. Leaf Sprouting and Development

The active growth stage of *C. malabaricum* begins with the sprouting of leaves from its underground bulbs, which have survived extreme temperatures during its dormancy period. This event typically commences with the first rains following the intense heat of summer, often during the "mango showers" (pre-monsoon rainfall). During this initial leaf-flushing period, the emerging leaves appear olive green, with a crimson red hue at the collar of the bulb.

With the onset of heavier rains, usually brought by the southwest monsoon or substantial pre-monsoon rainfall (May to June), the streams become active, and the leaves proliferate. The plant reaches a length of 1.5 to 2.5 meters. Mature leaves transition to a deep green colour as the plant enters an active growth phase. Along with the plant's growth, the surrounding ecosystem also rejuvenates, with streams supporting a variety of organisms, making the habitat vibrant once again.

3.3.5.1.2. Flowering Activity

The inflorescence primordia will appear three weeks after leaf sprouting is initiated. Flowering typically begins in late June, reaching its peak during August and September, gradually declining through October and November.

Across all studied populations, the inflorescence develops within 15 to 20 days. The plant produces an umbel inflorescence supported by a greenish peduncle measuring 50–120 cm in height. The involucral bracts are initially hyaline in colour, turning reddish-tinged at anthesis. After the first flower blooms, subsequent flowers open in a centripetal sequence.

Floral buds are greenish-white, becoming pure white as they develop. Anthesis occurs early in the morning, between 6:00 AM and 7:00 AM, with anthers dehiscing within the bud one day prior to anthesis. The flowers are strikingly showy, shiny white, and highly fragrant, with each flower lasting for three days. The flowering frequency is approximately 40 ± 5 days, and some bulbs may produce up to three flowers simultaneously.

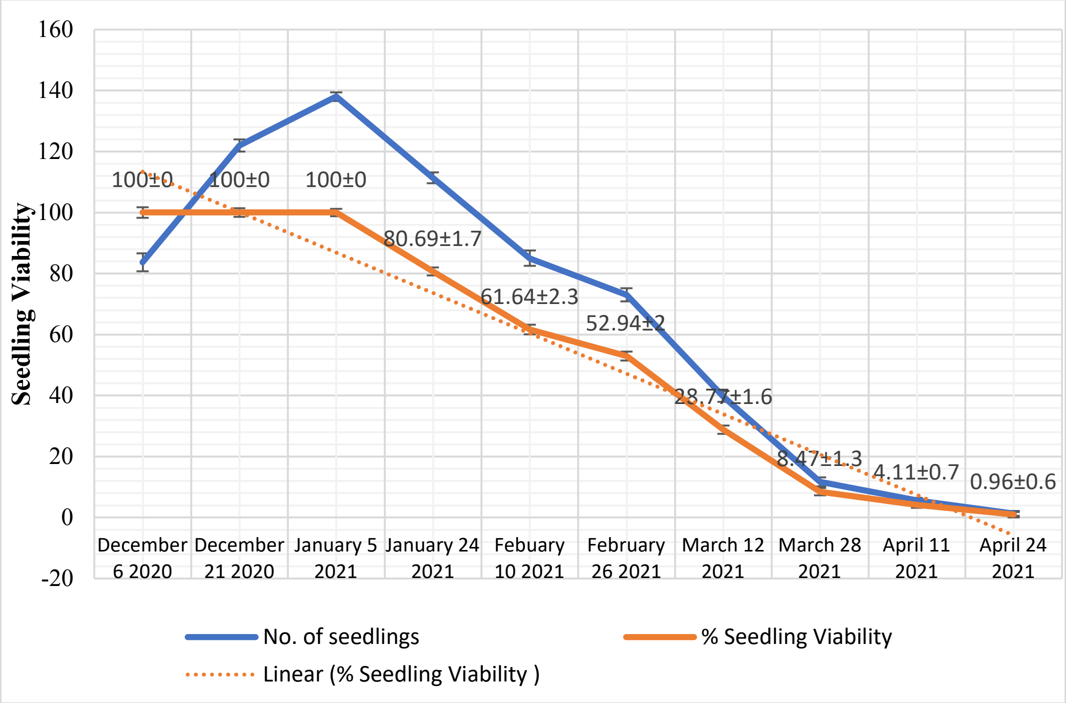
3.3.5.1.3. Fruiting and Seed Setting

Fruit development begins 8 - 14 days after anthesis and takes significant time to mature. Fruit formation occurs from mid-July to October, with peak fruiting observed in September. Fruit maturation and ripening take 45 - 50 days post-anthesis. Mature capsules turn crimson red, and the peduncle dries out, causing the fruit to fall onto the water body while remaining attached to the plant via the dried peduncle. The number of fruits per inflorescence varies from 5 to 13. Seeds exhibit extreme variation in number, size, and shape, ranging from 2 to 18 seeds per flower. Seeds are chlorophyllous and are released into the body of water as the capsule gradually decays.

3.3.5.1.4. Seed Dispersal and Seedling Viability

Dispersed seeds are transported by running water or deposited within the gravel of the streambed. It was observed that seeds cannot germinate underwater or survive submerged for more than 40 ± 3 days, after which they decay or are consumed by the Indian lily moth. Seeds that remain viable are those that survive until the streams dry, or those transported to stream margins with reduced water levels and flow.

The seed germination and seedling viability were observed using the quadrant method. Most of the seeds germinated with the drying of the streams (have a high germination rate of 89.6 ± 2%). Although with the increasing temperature and lack of water availability during the summer, most of the seedlings failed to thrive (survival rate = 0.87 ± 0.5%). The surviving seeds endure the summer by hiding under the plant's dried leaves, which act as a mulch for the seedlings. This will cause a lack of a successful dispersal mechanism for the species. Although the species is reproduced by vegetative propagation by bulbs, the dispersal of the population is hindered by the lack of proper seed dispersal.



Graph 1: Seedling viability of *C. malabaricum* with the drying of the streams. Values showing the percentage of seed viability with the standard deviation from the mean

Seed germination and seedling viability were assessed using the quadrant method. Most of the seeds germinated with the drying of the streams, with a high germination rate of 89.6 ± 2%. However, with the increasing temperature and lack of water availability during the summer season, most of the seedlings failed to thrive (survival rate = 0.96 ± 0.6%) (Graph 1).

Surviving seedlings endure the summer by sheltering beneath dried leaves of the parent plant, which act as natural mulch. This lack of a successful seed dispersal mechanism significantly limits the species' ability to spread. While *C. malabaricum* also reproduces vegetatively through bulbs, the absence of effective seed dispersal creates a significant barrier to population dispersal and expansion.

3.3.5.1.5. Phenological Events in BBCH Scale

The phenological events were converted to a two-digit BBCH scale for numerical comparison and visualisation. This approach simplifies the analysis of periodic behavioural changes in the plant's life cycle and their correlation with environmental parameters (Figure 20).

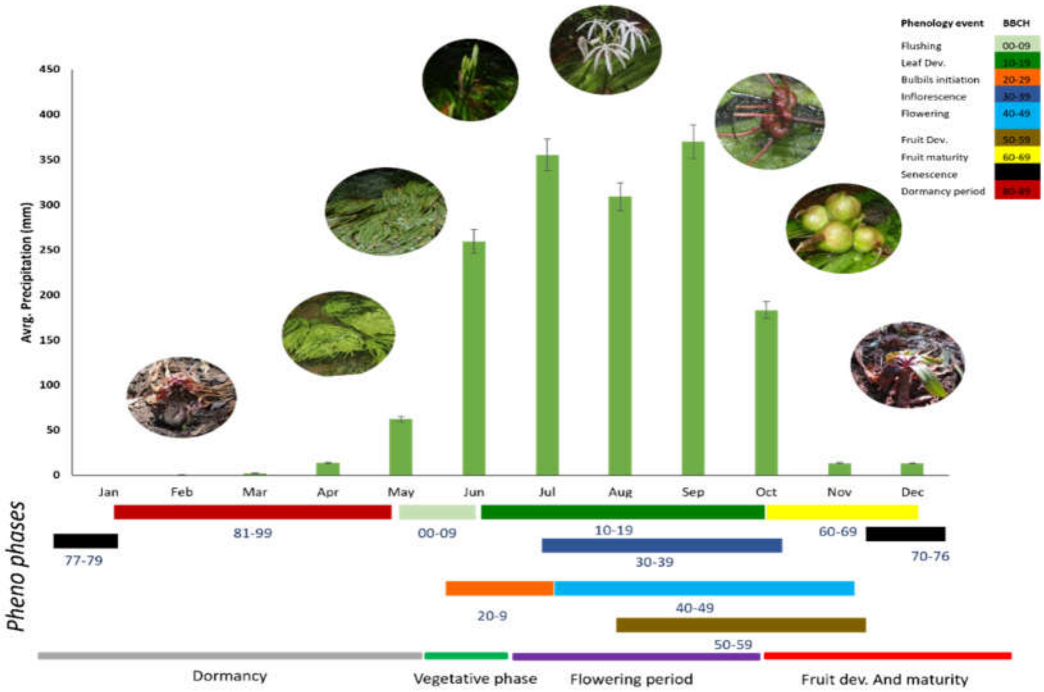
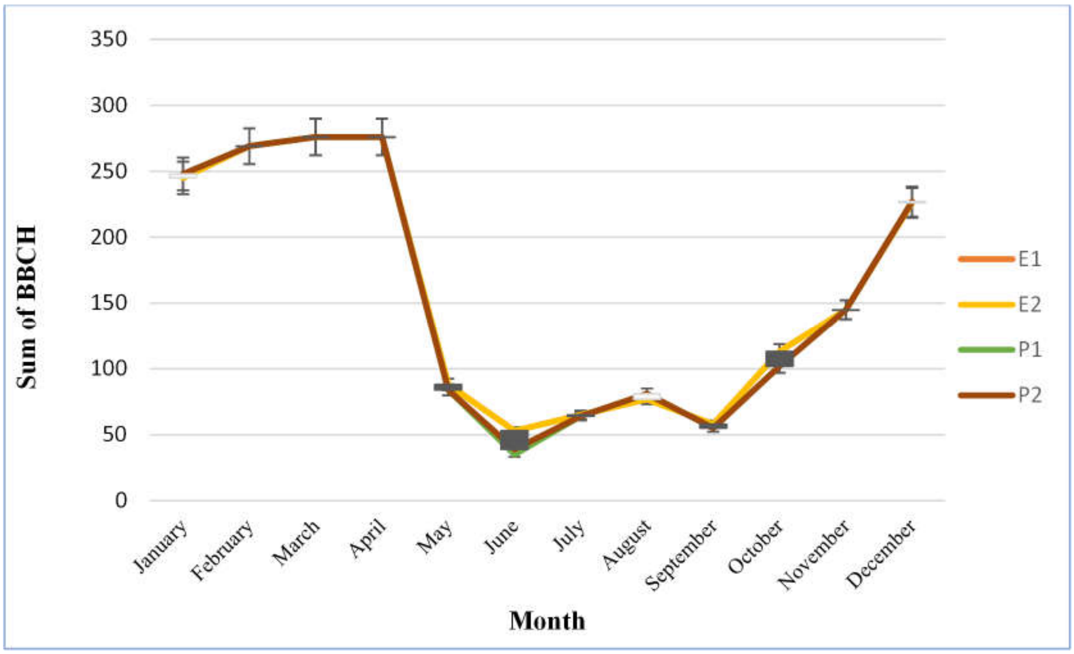


Figure 20. Graphical representation pf periodic events in the life cycle of *Crinum malabaricum* with the annual rainfall

The phenological events in both populations exhibited variations, as represented in the graph using the BBCH scale. Notable differences were observed in the initiation of the growth phase and the onset of senescence and dormancy (Table 9).

In both the study site at Embate, the growth phase begins between May and June, depending on the availability of pre-monsoon rainfall. In Periye, slight variations were observed between the study sites. In the upstream section (P2), the growth phase commenced earlier, from May to June, whereas in the downstream section (P1), it began in June (Graph 2). This variation can be attributed to physical barriers within the stream, such as laterite bedrock and check dams, which obstruct the flow of water from upstream to downstream, affecting water availability in different sections of the population. The variation was also observed during other phenophases, particularly during dormancy. Plants in the downstream section of Periye entered dormancy later than those in the upstream section. However, in Embate, this phenomenon was not observed, as the stream in this habitat has a continuous flow without noticeable barriers.



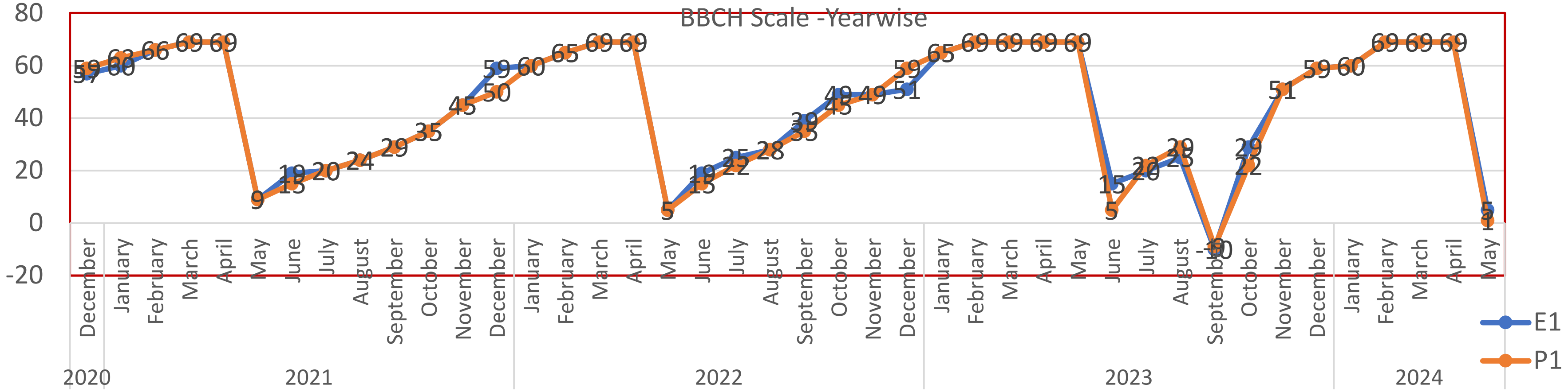
Graph 2: Sum of BBCH Scales representing the variations in the Phenophases in different plots

Phenophases	E1	E2	P1	P2
Leaf sprouting	May to June	May to June	June	May to June
Vegetative growth	June to July	June to July	June to July	June to July
Flowering	June to October	June to October	July to November	July to October
Fruit setting	July to November	July to November	August to November	August to November
Maturity of fruits	September to November	September to November	September to December	September to December
Senescence	November	November	December	December
Dormancy period	December to May	December to May	January to May	January to May

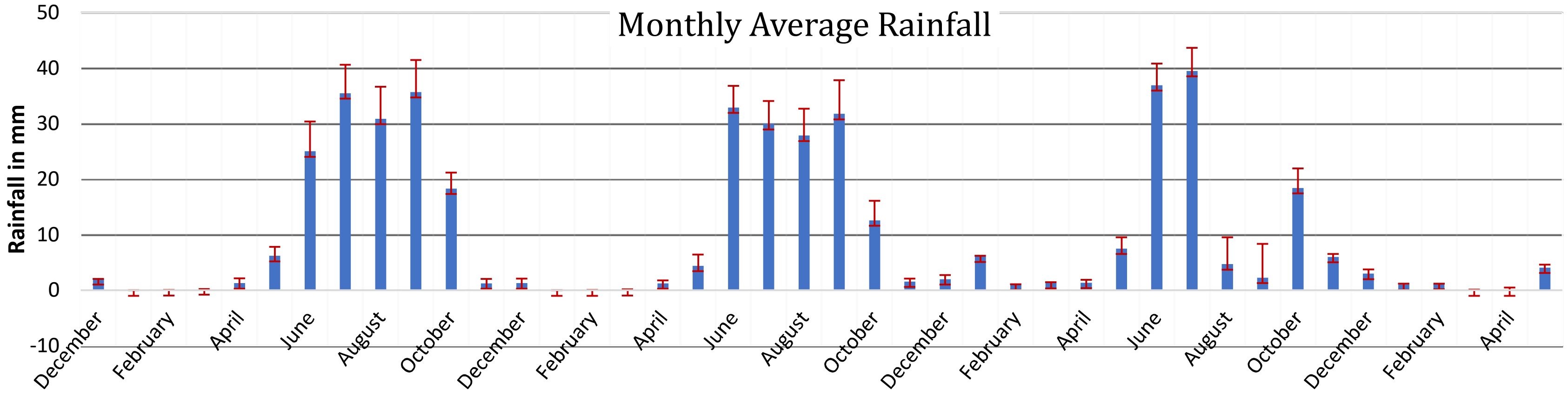
Table 9: Flowering phenology in the natural habitat of *Crinum malabaricum*

In *C. malabaricum* the phenological events are highly depend on rainfall. Rainfall influences the beginning of the growing period and the dormancy period. The phenological variation within the studied populations are due to the influence of rainfall. Also, the variation in the rainfall directly impacts the flowering cycle of the plant. The rainfall and the phenophases are highly correlated. The coefficient values for the rainfall and phenophases in Embate are, $r= 0.762$; $\rho < 0.05$ for 2021, $r = 0.746$; $\rho < 0.05$ for 2022 and $r= 0.785$; $\rho < 0.05$ for 2023. The correlation coefficients in Periye are $r= 0.745$; $\rho < 0.05$ for 2021, $r = 0.813$; $\rho < 0.05$ for 2022 and $r = 0.795$; $\rho < 0.05$ for 2023.

Graphs 3 and 4 represent the phenological events of *C. malabaricum* in the BBCH scale and the annual rainfall average in the study sites respectively. During August 2023, rainfall drastically decreased due to the reduction in the South West monsoon in all the study sites, which caused a drastic shift in the phenophases from the flowering stage to senescence. The return of northeast rain brings the plants back to active stages. However, this event dramatically affected the entire study site population.



Graph3: Comparison of phenological changes in Embate (E1) and Periye (P1) in BBCH scale



Graph 4: Average monthly rainfall in Periye and Embate from December, 2020 to May, 2024 (values are mean ± standard error)

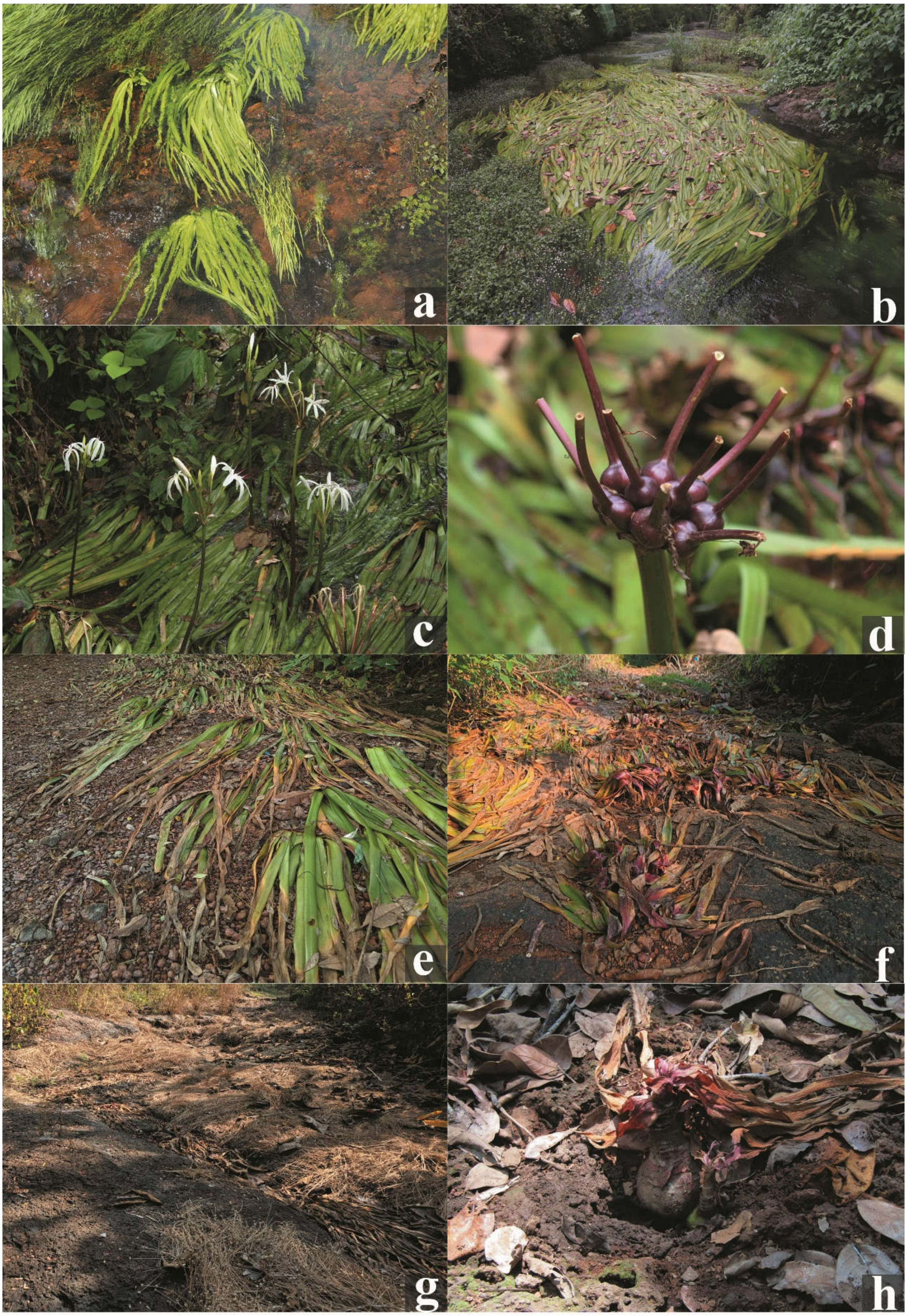


Figure 21. Phenophases of *C. malabaricum*; a. Leaf sprouting; b. Vegetative growth; c. Flowering; d. Fruit development; e & f. Senescence; g & h; dormancy stage

3.3.6. Reproductive Biology

3.3.6.1. Pollen Morphology

The pollen grains of *Crinum malabaricum* are mono-aperturate, ovoid, and perprolate, with an equatorial width of 45.27 ± 2.1 µm and a polar length of 105.47 ± 0.12 µm (Figure 22).

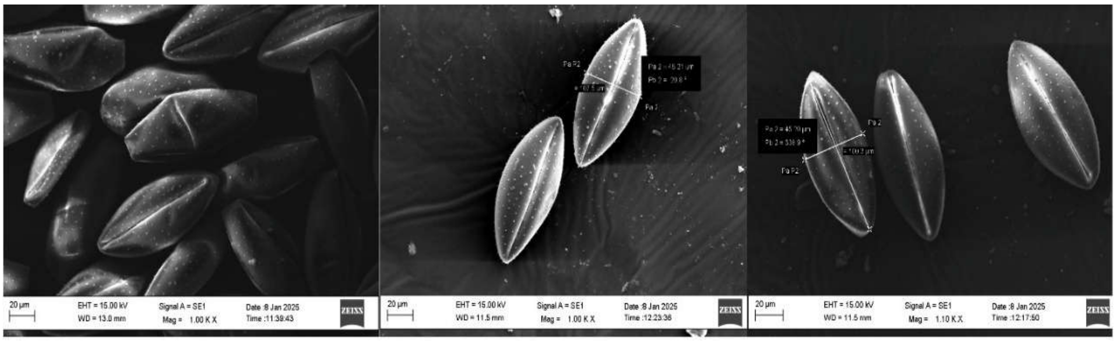


Figure 22. SEM images of *Crinum malabaricum* pollen grains show the Equatorial length and Polar width

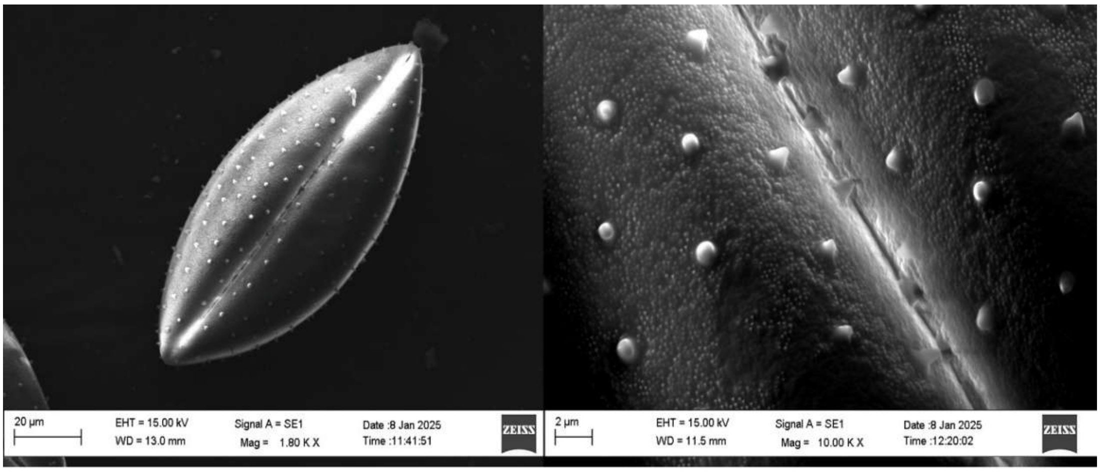


Figure 23. SEM images of pollen grains of *Crinum malabaricum*, showing individual pollen grain and the ornamentation

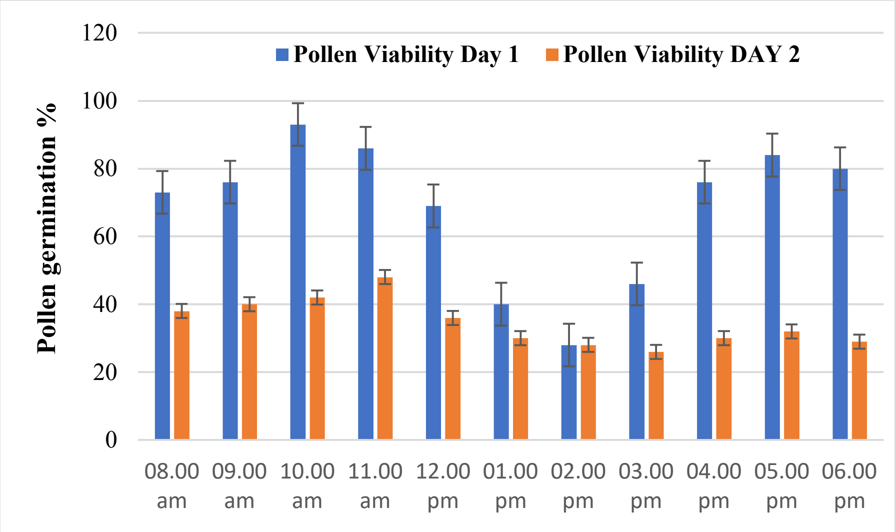
According to Walker and Doyle (1975), pollen grains measuring 100–199 µm fall into the very large category, placing *C. malabaricum* within this classification based on its polar length. Using the NPC system of pollen classification (Erdtman & Straka, 1961), which considers the number of apertures and morphological features, *C. malabaricum* pollen is characterized as tricolpate, having three equidistant furrows. The exine is micro-verrucate, exhibiting a warty surface with bulbous outgrowths (Figure 23).

3.3.6.2. Pollen Viability

Pollen viability of *Crinum malabaricum* was evaluated using three methods: two staining techniques (Alexander staining and the Tetrazolium test) and the *in vitro* pollen germination method. The pollen germination medium for the *in vitro* method was standardized with 10% polyethylene glycol, 2.5% sucrose, and B & K salts (Brewbaker & Kwack, 1964).

The *in vitro* pollen germination method recorded the highest germination rate of 91.6 ± 0.5% between 10:00 and 11:00 a.m. This rate decreased to 65.3 ± 0.7% by 2:00 p.m., followed by an increase to 86.75 ± 0.3% between 4:00 and 6:00 p.m. On the second day, the germination rate gradually dropped to 41.76 ± 1.2% by 10:00 a.m. (Graph 5, Figure 24).

In the Tetrazolium test (TTC), 89.6 ± 0.5% of pollen grains were viable during post-anthesis between 10:00 and 11:00 a.m., gradually declining to 65.72± 1.2% by 2:00 p.m. Pollen collected from second-day flowers exhibited significantly lower viability at 40 ± 0.5%. Alexander staining showed a similar pattern, with first-day pollen having a viability of 93.26 ± 0.3% between 10:00 and 11:00 a.m., which decreased to 42 ± 0.7% after 24 hours (Figure 24). Across all methods, pollen viability was highest on the first day between 10:00 and 11:00 a.m., with a marked decline on successive days after anthesis.



Graph 5: *In vitro* pollen viability assessments of *C. malabaricum*

3.3.6.3. Pollen Ovule Ratio

In *C. malabaricum*, the pollen production per anther was 8264 ± 26.78, and the pollen production per flower was 49584±164. The ovule number per flower, determined from fresh pistils, was 22.5±2.5. The calculated pollen-ovule (P/O) ratio is 1:2203.73, with a standard deviation of 244.97.

3.3.6.4. Stigma Receptivity

The stigma of *C. malabaricum* becomes receptive only after anthesis and remains receptive for up to 72 hours following flower opening. Maximum receptivity was observed on the first day, between 4 and 5 hours after anthesis, followed by a secondary peak at 10 hours post-anthesis (Table 10, Figure 25). Successful fertilization and subsequent seed set further confirmed stigma receptivity. Fertilized ovules developed into seeds with a high germination percentage, indicating efficient reproductive success.

Sl. No.	Time	Mean No. of Bubbles
1.	08.00 am	3.4 ± 1.13
2.	09.00 am	9.67 ± 1.003
3.	10.00 am	25.9 ± 1.23
4.	11.00 am	64.57 ± 1.26
5.	12.00 pm	88.67 ± 1.65
6.	01.00 pm	80.53 ± 1.2
7.	02.00 pm	71.3 ± 1.21
8.	03.00 pm	66.2 ± 1.365
9.	04.00 pm	82.93 ± 2.11
10.	05.00 pm	71.78 ± 1.73
11.	06.00 pm	62.22 ± 3.53

Table 10. The average number of bubbles produced during Hydrogen peroxide test for stigma receptivity. H= 8.8081, p=0.0453

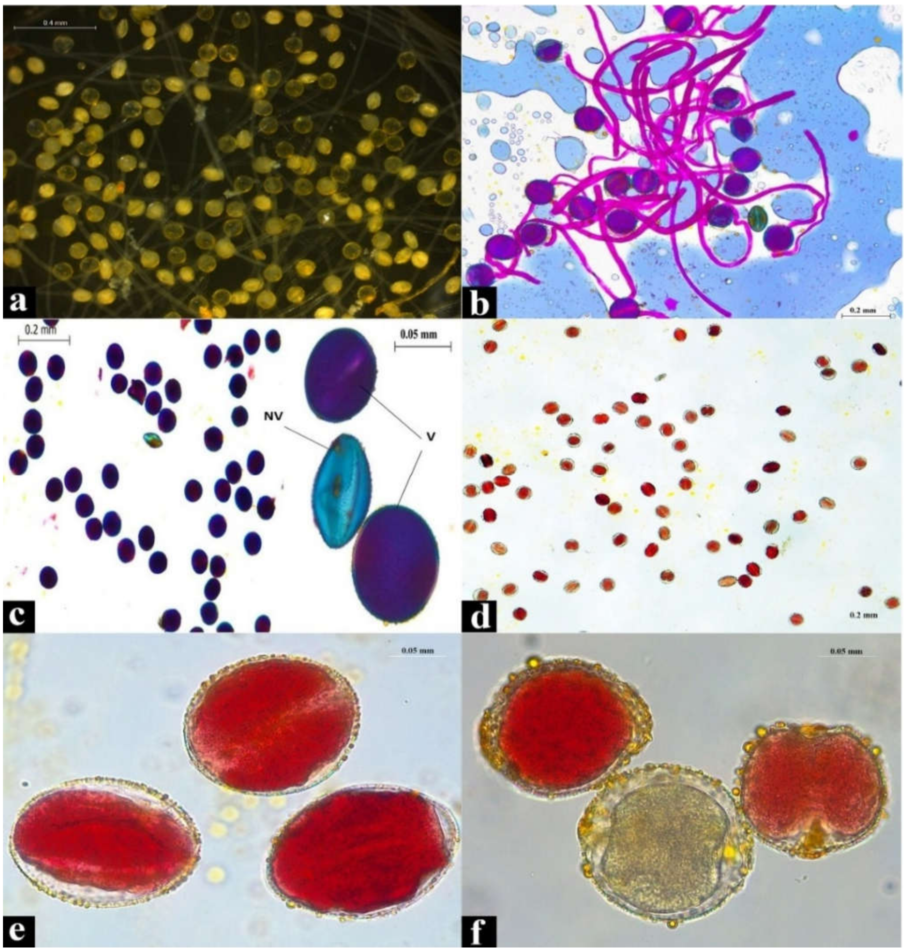


Figure 24. Pollen viability assessments: a. *In vitro* germination using the hanging drop method; b. stained to show germinated, non-germinated (densely stained), and sterile (green) pollen grains. c. Alexanders staining method d, e & f: Tetrazolium method

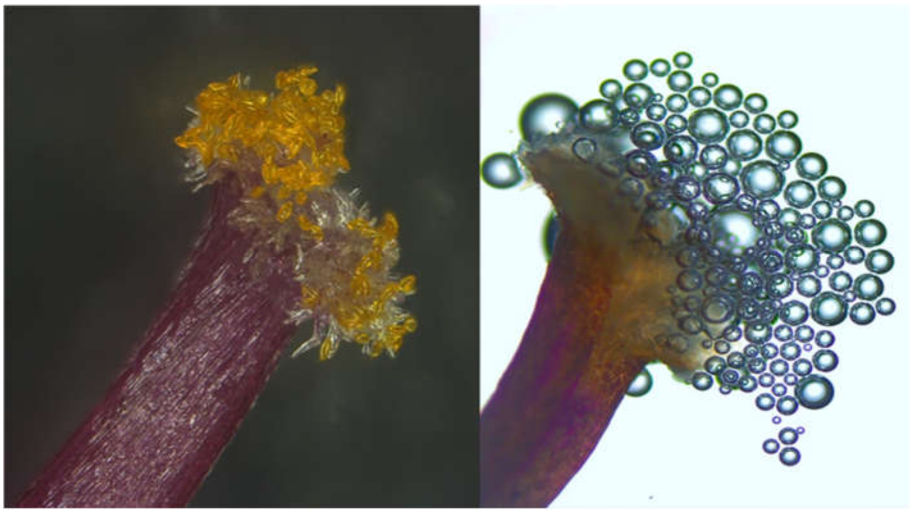


Figure 25. a. Stigma with pollen; b. Stigma bubbling with the application of hydrogen peroxide. The bubbling frequency corresponds to activity of the peroxidase enzyme

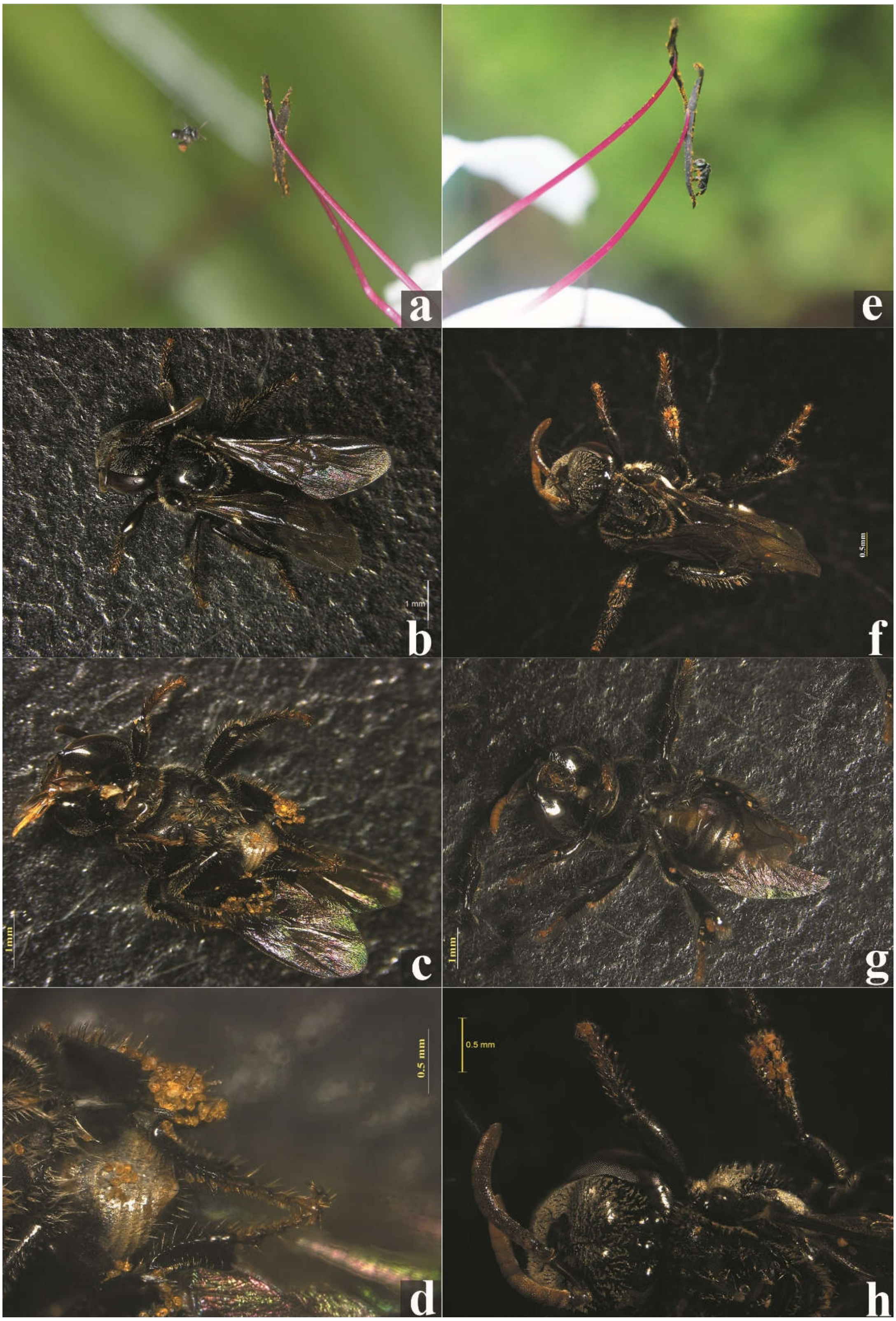


Figure 26. Pollinators of *C. malabaricum*: (a-d) T*etragonula perlucipinnae* & (e-h) *T. iridipennis.* Image represents different views: pollinator interacting with the anther, adaxial and abaxial perspectives and pollen grains carried by the pollinator

3.3.6.5. Floral Visitors and Foraging Mode

Insect are visit the flowers of *C. malabaricum* right after anthesis. Maximum visitation occurred between 9:am to 12:00 noon. Odonates, bees and flies are the most frequent visitors. *Tetragonula irridepennis* and *T. perlucipinnae* (Figure 96) are the identified pollinators among the visitors. Their pollination activity was confirmed by their frequent visit from stamens to stigma and the presence of viable pollen grains from the pollen sac of the insect was identified by microscopic observation. The foraging pattern of the pollinator are listed in the table. During the visiting time, the insects are rest on stamens and the pollen grains adhere to the insect body. They deposit pollens on the stigma of another flower while foraging for the next flower. The insects receive both pollen and nectar as a reward (Table 11).

Sl. No	**Insect**	**Visiting rate**	**Handling time**	**Peak foraging activity time**	**Frequency of visit**
1	*Tetragonula irridepennis*	4 ±2	15±5 sec	10:00 – 11:00 am	High
2	*T. perlucipinnae*	3± 2	1±5 sec	3:00 – 4:00 pm	High

Table 11: Foraging behaviour of the pollinators of C. *malabaricum*

3.3.6.6. Breeding Experiments

The breeding experiments on *C. malabaricum* revealed significant differences in fruit set percentages across various pollination mechanisms. Among the tested methods, xenogamy (cross-pollination involving pollen transfer between flowers of different plants) resulted in the highest fruit set percentage of 86.25 ± 4.8%, indicating a strong dependence on genetic exchange for optimal reproductive success. This was closely followed by open pollination, achieving a fruit set percentage of 83.75 ± 4.8%. In contrast, self-pollination methods, including autogamy (self-pollination within the same flower) and geitonogamy (pollination between flowers on the same plant), showed comparatively lower fruit set rates of 28.75 ± 4.8% and 60 ± 4.1%, respectively. These results suggest that while the species can have limited self-reproduction, cross-pollination significantly enhances reproductive outcomes. Additionally, apomixis was not observed in this species, indicating a reliance on sexual reproduction for seed production (Table 12).

Pollination Mechanism	No. of Flowers Pollinated	No. of Flowers Fruit Set	Percentage of Fruit Set
Open pollination	20	16.75 ±0.95	83.75 ± 4.8
Autogamy	20	5.75 ±0.95	28.75 ± 4.8
Geitonogamy	20	12 ±0.81	60 ± 4.1
Xenogamy	20	17.25 ± 0.95	86.25 ± 4.8
Apomixis	20	0	0

Table 12: Breeding experiments in *C. malabaricum*

Table 13: Summary of Reproductive and Floral Biology of *Crinum malabaricum*

Floral characters	Observation
Flowering period	July – November
Inflorescence type	Umbel
No. of flowers /inflorescence	6 - 13
Flower longevity	Three days
Flower type	Regular bisexual
Flower colour	White
Odour	Present
Nectar	Present
Anthesis time	6:00-7:00am
Anther dehiscence time	One day prior to anthesis
Anther dehiscence mode	Longitudinal
Perianth	Tube pale green with 6 shining white segments
Androecium	6 anthers/flower
No. of pollen grains/anther	8264 ± 26.78,
No. of pollen grains/flower	49584±164
Pollen shape	Ovoid
Pollen type	Tricolpate
Pollen viability	91.6 ± 0.5 % (at the day of anthesis 10:00am)
Gynoecium	3 loculed ovary, 5- 10 ovules per locule
Sigma type	Three lobes, dry
Stigma receptivity	11:00 am -12:00noon (at the day of anthesis)
Mean no. of ovules/flower	22.5 ± 2.5
Pollen ovule ration	1:2203.73
Fruit type	Capsules, irregularly Globose, 5-13 per inflorescence
Seed	Irregular in shape and size & chlorophyllous
Seed type	Recalcitrant seed
No. of seeds per fruit	2 to 18

3.4. Discussion

The Western Ghats is one of the most ecologically diverse regions in the world, harbouring unique habitats that support an incredible variety of life forms, making it a biodiversity hotspot of global significance. Among these unique habitats, the laterite plateaus of the Malabar Plains stand out as a distinctive ecological niche near India's coastal belt. These plateaus experience extreme seasonal changes brought about by the monsoon and summer, forming seasonal pools and streams that serve as unique habitats for various aquatic plants. Despite their ecological importance, aquatic plants remain understudied and are often overlooked, even though they are known to possess significant medicinal value and serve as sources of bioactive compounds (Swapna *et al.*, 2011).

Crinum malabaricum, a critically endangered and endemic aquatic species, is a unique plant thriving in the seasonal aquatic habitats of the laterite plateaus. The present study focuses on understanding the threats this species faces and its habitat, while identifying the ecological requirements necessary for its successful conservation. The high level of endemism of aquatic species in the laterite plateaus associated with the Malabar Plains of the Western Ghats has been addressed by many studies (Watve & Thakur, 2006; Lekhak & Yadav, 2012; Rao *et al.,* 2012; Pramod *et al.,* 2014). This endemism is primarily attributed to the unique climatic conditions and monsoon-driven seasonality. Furthermore, the fragmentation of these plateaus within the Malabar Plains further restricts the distribution of these species to highly localized areas, compounding their vulnerability.

3.4.1. Habitat Exploration and Threat Assessment

The species *Crinum malabaricum* was first discovered by Lekhak and S. R. Yadav in 2012 at its type locality in Periye, Kerala. Their preliminary investigation revealed a limited population size of 1000 individuals restricted to a small area (0.5 km^2) within its original habitat. In 2016, the IUCN assessed the species, categorizing it as Critically Endangered based on its single known population (Lansdown, 2016). Subsequent studies by Lansdown and Molur (2017) identified three additional natural habitats in Kannur and Kasaragod districts, expanding the species' known range.

In the present study, habitat assessments of *C. malabaricum* in four seasonal streams revealed that the species occupies an area of 11.417 km² with an estimated population of approximately 25,000 individuals. The species is depth-specific and confined to the middle course of these streams. However, observations from 2020 to 2024 indicate a rapid population decline and a reduction in the extent of occurrence (EOO). While earlier studies suggested reclassifying the species to the Vulnerable category due to the discovery of additional habitats (Lansdown & Molur, 2017), the current findings support retaining its status as Critically Endangered based on the IUCN criteria, given the observed decline in area of occupancy (AOO) and population size.

The species faces numerous threats that require attention both locally and globally. One of the immediate challenges is human intervention in the streams, as local communities often lack awareness of the species' rarity, ecological importance, and potential uses. Instead, *C. malabaricum* is frequently dismissed as an aquatic weed, reflected in its local names "Polathaali" or "Poothaali." (Lansdown & Molur, 2017). This perception leads to careless removal of plants and degradation of the streams through activities such as encroachment, waste deposition, gravel removal, and the construction of temporary check dams. These actions significantly threaten the species' survival.

At the administrative level, mismanagement escalates the issue. Government land surveys have categorized the laterite plateaus associated with these streams as wastelands (Anonymous, 2019). This classification has permitted the overexploitation and conversion of these areas for industrial activities, laterite mining, monoculture plantations, and housing or infrastructure development. The rising demand for land due to urbanization in Kerala, along with the rapid urbanization of rural areas, further intensifies these challenges (Thekkeyil *et al.*, 2023). Such demands often result in the unsustainable utilization of these lands without consideration of the biodiversity that depends on these unique ecosystems. Additionally, the management of human-induced pressures is often misguided due to a poor understanding of the special ecological features of the habitat (Watve, 2013). Effective conservation strategies require increased awareness, sustainable land-use practices, and informed policies to mitigate these threats and ensure the survival of *C. malabaricum* and its habitats.

The ecological survey highlights that *C. malabaricum* is not merely a species under threat but serves as a critical ecological architect due to its substantial size and dominant presence within relatively small stream ecosystems. This species forms a vital structural component of the habitat, linking the surrounding faunal and floral diversity to the ecosystem. It plays an essential role in the breeding and feeding odonates, fishes, amphibians, and reptiles that are part of the habitat's ecological network (Lansdown & Molur, 2017; Pulparambil & Pradeep, 2023). A comprehensive study of the ecosystem is crucial to understanding these interactions and further exploring the biodiversity richness associated with this unique habitat. During the present investigation, nine species of odonates were observed in association with *C. malabaricum*, along with a floral diversity of 16 angiosperms.

These findings underscore the ecological significance of the species as an ecosystem architect. Therefore, conservation efforts should not only focus on protecting *C. malabaricum* but also account for its broader ecological role, ensuring the preservation of the intricate relationships and biodiversity within its habitat.

3.4.2. Phenological Studies on *Crinum malabaricum*

Phenological studies are vital for understanding a species' life cycle, ecological interactions, and responses to environmental changes, thereby contributing significantly to conservation efforts. They provide critical insights into species' responses to climate change, inform restoration actions, and improve understanding of ecological interactions and species distributions (Morellato *et al.*, 2016). Phenological information is crucial for guiding effective future restoration efforts by increasing the success of ecosystem function recovery and species interactions (Rother et al., 2021).

This study represents the first comprehensive documentation of the phenological events of *C. malabaricum*, focusing on its periodic behaviour and responses to environmental factors. The classification of phenophases into seven principal growth stages provided a quantitative framework for correlating phenological data with climatic variables (Meier *et al.*, 2009). The phenological data collected over a period of 42 months revealed a strong correlation between the life cycle of the species and monsoon seasonality. The initiation of the growing period and leaf sprouting in *C. malabaricum* depends on the onset of the South-west monsoon, while senescence and dormancy phases aligned with the retrieval of the North-east monsoon. These timings, however, varied across the species' natural habitats, suggesting that local environmental factors modulate phenological events.

The findings also underscore the vulnerability of *C. malabaricum* to climate change-induced alterations in rainfall patterns. Changes in the timing and intensity of rainfall during the active growing period could negatively impact the species' growth and reproductive success. Such variations in rainfall, driven by climate change, have been shown to disrupt phenological events across taxa, influencing species-specific reproductive strategies, species interactions, and ecosystem dynamics (Fitchett *et al.*, 2015; Lai *et al.*, 2024).

Observations on floral development show that anthesis occurs in the early morning between 6-7 am, and pollen viability peaks after two to four hours of anthesis and afternoon, similarly stigma receptivity peaks between 11 am and 12 pm and 4 to 6 pm. The pollinators were identified as *Tetragonula iridipennis* and *T. perlucipinnae* and their forging behaviour in the morning and evening are corresponds to the peaking of pollen viability and stigma receptivity. The forging behaviour of insect pollinators is closely linked to the timing of pollen viability and stigma receptivity, which are critical for successful plant reproduction. This synchronization ensures that pollinators visit flowers when they are most likely to facilitate fertilization, thereby enhancing reproductive success (Brunet *et al.*, 2019; He *et al.*, 2017).

The species primarily propagates vegetatively, with mother bulbs generating clusters of smaller bulbs. However, this method is ineffective for long-distance dispersal, contributing to the species' limited range. While the seeds of *C. malabaricum* are recalcitrant and water-dispersed, their role in population expansion has been understudied (Lekhak & Yadav, 2012). Observations from the present study reveal that survivability of the seedling decrease with rising temperatures. Seeds germinate as streams dry, but only a few survive extreme climates in the summer. This limits the widespread dispersal of the species to adjacent similar habitats.

In aquatic plants, inadequate dispersal mechanisms can hinder colonization of new habitats, reduce genetic diversity, and increase vulnerability to environmental changes (Cuena-Lombraña, 2024). For instance, water-dispersed plants in fragmented landscapes often face connectivity loss, limiting their distribution and diversity (Favre-Bac *et al.*, 2017). Innovative dispersal strategies, such as the involvement of animal vectors seen in other aquatic species (Hoffmann *et al.*, 2024), underline the need to further explore mechanisms that could enhance the resilience and spread of *C. malabaricum*.

This study provides critical insights into the habitat assessment, ecological significance, reproductive biology, and phenological patterns of *C. malabaricum*, a critically endangered aquatic plant endemic to the laterite plateaus of the Western Ghats. The species' restricted distribution, lack of effective dispersal mechanisms, and sensitivity to climate-driven variations in rainfall and temperature underline its vulnerability. Integrating habitat conservation efforts with a deeper understanding of its ecological interactions and life cycle is essential for safeguarding this unique species and the biodiversity it supports.

Chapter 4

Bio Chemical Profiling of *C. malabaricum*

Chapter 4

Bio Chemical Profiling of *C. malabaricum*

4.1. Introduction

Bioprospecting systematically explores biological resources to discover compounds with commercial and societal applications, including pharmaceuticals, agriculture, and biotechnology (Beattie *et al.,* 2011). This process involves identifying bioactive molecules, genetic materials, and other valuable biochemical compounds that can be utilized for medical, industrial, or environmental purposes. Given the increasing demand for natural products in drug discovery, agriculture, and bio-based industries, bioprospecting has gained prominence as a tool for unlocking the untapped potential of biodiversity. However, many plant species, particularly endangered ones, remain underexplored; bioprospecting is a crucial approach to assess their biochemical potential. Demonstrating these species' practical and economic value encourages research, investment, and conservation initiatives.

Beyond its scientific and commercial significance, bioprospecting contributes directly to species conservation by creating economic incentives for habitat protection and sustainable resource management of endangered species (Barrett & Lybbert, 2000; Rausser & Small, 2000). Identifying valuable biochemical compounds in endangered plants highlights their ecological and economic importance, strengthening arguments for their conservation. This approach supports conservation goals with sustainable utilization, reducing overexploitation while promoting *in situ* and *ex situ* conservation strategies (Silva *et al.,* 2022). Additionally, international legal frameworks such as the Nagoya Protocol promote fair and equitable benefit-sharing derived from bioprospecting, encouraging local communities and stakeholders to participate in conservation efforts (Hamilton, 2004; Sava *et al.,* 2019). By integrating ecological sustainability with economic viability, bioprospecting supports long-term conservation strategies, safeguarding biodiversity while fostering scientific advancements.

Medicinal plants have played a vital role in drug development, drawing from their rich biochemical diversity and traditional uses. For thousands of years, they have been a key source of human medicine, with references found in ancient texts. The traditional use of medicinal plants has been the foundation of modern pharmacology, with many contemporary drugs derived from plant compounds. The pharmaceutical industry relies on these natural leads, as plant-based products remain crucial for drug discovery (Chaachouay & Zidane, 2024). The medicinal properties of the plants are largely attributed to their abundant secondary metabolites. Understanding the chemical composition of these metabolites is essential for discovering new therapeutic compounds and identifying novel sources of valuable phytochemicals. These compounds can contribute to drug development either directly, as bioactive molecules, or indirectly, by providing structural templates for the synthesis of complex and targeted drug systems. Recent advancements in biotechnology have enhanced the extraction and identification of bioactive compounds, leading to innovative therapeutic solutions. Techniques such as genetic engineering and tissue culture allow for the controlled production of bioactive compounds, improving yield and sustainability (Bangash *et al.,* 2024).

Mass spectrometry combined with chromatography, including GC-MS (Gas Chromatography-Mass Spectrometry) and HPLC (High-Performance Liquid Chromatography), identify and quantifies phytochemicals in plant extracts. GC-MS detects and identifies unknown phytochemicals, particularly volatile and semi-volatile compounds. Gas chromatography separates the components, while mass spectrometry provides structural information, enabling identification based on mass spectral libraries. HPLC is suitable for non-volatile and thermally unstable compounds. Various detectors, such as UV-Vis, PDA, or MS, aid in identifying unknown phytochemicals by analyzing retention time, spectral data, and comparison with known standards. These methods are also widely used for the quantification of phytochemicals. HPLC enables precise quantification of bioactive compounds like flavonoids, phenolics, and alkaloids in complex mixtures by measuring peak areas and comparing them with known standards (Betz *et al.,*1997; He *et al.,*2000; Filatov *et al.,* 2023). GC-MS and HPLC provide a comprehensive phytochemical profile, offering a detailed understanding of both volatile and non-volatile constituents. This is crucial for medicinal plant research, drug discovery, and quality control.

The genus *Crinum*, belonging to the Amaryllidaceae family, is well known for its diverse alkaloid profile, significantly contributing to its medicinal applications. Alkaloids such as lycorine, galantamine, crinamine, and haemanthamine are prominent in various *Crinum* species, exhibiting a wide range of pharmacological activities, including anti-inflammatory, antitumor, antioxidant, antimicrobial, antimalarial, antiviral, antidiabetic, and immunostimulatory effects (Ghosal *et al.,* 1985; Uddin *et al.,* 2012; Ji *et al.,* 2013; Refat *et al.,* 2013; Ghane *et al.,* 2018). This biochemical diversity highlights the therapeutic potential of *Crinum* and underscores its significance in traditional medicine. *Crinum malabaricum*, in particular, contains several medicinally important alkaloids with analgesic, antiviral, and antitumor properties. It is a rich source of galantamine, an acetylcholinesterase (AChE) inhibitor used in treating Alzheimer's disease, and lycorine, a compound with notable anticancer properties. Understanding AChE inhibition by plant-derived alkaloids is crucial for developing therapeutic agents for neurodegenerative diseases, particularly Alzheimer's disease. Alkaloids, as secondary metabolites, have shown significant potential in inhibiting AChE, which plays a vital role in maintaining acetylcholine levels in the brain. This inhibition can enhance cognitive function and serve as a foundation for novel drug development.

Previous studies on *C. malabaricum* have primarily focused on the pharmacogenetic properties of its root and leaf extracts (Lekhak *et al.,* 2022; Ghane *et al.,* 2018). The root extract has demonstrated significant antioxidant activity, metal chelation potential, and enzyme inhibition, indicating anti-diabetic potential. Likewise, the leaf extract exhibits various medicinal properties, including anti-inflammatory, antimicrobial, and cytotoxic effects. However, the bulb, a crucial storage organ rich in secondary metabolites, remains largely unexplored. This study addresses this gap by analyzing the phytochemical composition and bioactivity of *C. malabaricum* bulb extracts, with a comparative assessment between the dormancy and growing periods. Since the bulb functions as a reservoir for bioactive compounds, variations in its chemical profile across physiological stages could provide valuable insights into its medicinal potential and optimal extraction periods for pharmaceutical applications.

The present study investigates the phytochemical diversity of the critically endangered *C. malabaricum* using chromatographic and mass spectrometric techniques. Additionally, it assesses this species' antioxidant potential and acetylcholinesterase inhibitory activity. The study also quantifies major phytochemical groups such as flavonoids, phenols, and alkaloids, specifically focusing on the economically significant alkaloids galantamine (GAL) and lycorine (LYC) using HPLC. This bioprospecting research may pave the way for further in-depth studies to identify plant-based, cost-effective drugs while promoting the conservation and sustainable utilization of this valuable species.

4.2. Materials and Methods

4.2.1. Collection of Plant Materials

The bulbs of *Crinum* genotypes used in this study were collected from their natural habitats across various regions of India during their respective growing periods. *C. malabaricum* was collected from Periye, Kasaragod, Kerala, India, during two distinct phases: actively growing (August 2023) and dormancy (April 2023). This dual-phase collection was conducted to assess biochemical variations between these growth stages, providing insights into metabolic changes occurring in response to seasonal shifts. The collected specimens were carefully documented, and voucher specimens for each *Crinum* genotype were prepared and deposited in the MBGH herbarium (Table 14).

Sl. No.	Voucher No.	Species	Locality	Geographic coordinates	Collection Time
1.	18653	*C. malabaricum* (CMD)	Periye, Kasaragod,	12°24'21.5"N 75°06'59.2"E	April 2024
2.	18653b	*C. malabaricum* (CMG)	Periye, Kasaragod,	12°24'21.5"N 75°06'59.2"E	August 2024
3.	18601	*C. asiaticum* (CA)	Olavanna, Kozhikode	11°14'36.1"N 75°49'37.4"E	September 2024
4.	18643	*C. latifolium* (CLt)	SGNP, Thane, Maharashtra	19°14'55.8"N 72°56'39.3"E	July 2024
5.	18624	*C. lorifolium* (CLr)	Devgad, Maharashtra	16°22'47.7"N 73°24'54.1"E	July 2024
6.	18635	*C. reddyii* (CR)	Guntvel, Tapi, Gujrat	21°01'13.3"N 73°33'57.8"E	October 2023
7.	18615	*C. solapurense* (CS)	Machnur, Maharashtra	17°33'54.4"N 75°33'33.8"E	October 2023
8.	18602	*C. viviparum* (CV)	Olavanna, Kozhikode	11°14'09.8"N 75°49'46.3"E	September 2024

Table 14: Details of the voucher specimen of *Crinum* spp. used for biochemical profiling

4.2.2. Reagents and Standards

All chemicals and reagents used in this study were of high analytical grade to ensure the reliability and accuracy of experimental results. The details of these reagents are listed in the table. Solvents, including methanol, trifluoroacetic acid (TFA), and diethylamine (DEA), used in this study were of high-purity HPLC grade, ensuring optimal resolution and sensitivity in detecting biochemical compounds. All compounds used in the study had an average purity of 99%. Based on the SDS, proper storage conditions and handling protocols were followed to maintain reagent stability throughout the study.

Sl.No.	Regent/ Enzyme/ Standard	Brand
1.	2,2' azinobis (3-ethyl-benzothiozoline-6-sulfonic acid (ABTS)	Hi-media
2.	Acetyl thio-choline iodide (AChI)	Sigma-Aldrich (USA).
3.	Acetylcholinesterase (AChE) type VI-S	Sigma-Aldrich (USA).
4.	Ammonium molybdate	Hi-media
5.	Ascorbic acid	Hi-media
6.	Butylated hydroxytoluene (BHT)	Hi-media
7.	Bromocresol Green	Hi-media
8.	Chloroform	Hi-media
9.	Diethylamine (DEA)	Sigma-Aldrich (USA).
10.	Dimethyl sulfoxide (DMSO)	Hi-media
11.	1,1-diphenyl-2-picrylhydrazyl (DPPH)	Hi-media
12.	5,5' –di thio bis[2- nitro benzoic acid] (DTNB)	Hi-media
13.	Ferric chloride hexahydrate	Hi-media
14.	Ferrous sulphate	Hi-media
15.	Folin-Ciocalteu reagent	Hi-media
16.	Galantamine (GAL)	Sigma-Aldrich (USA).
17.	Gallic acid (GA)	Sigma-Aldrich (USA).
18.	Hydrochloric acid	Hi-media
19.	Lycorine (LY)	Sigma-Aldrich (USA).
20.	Methanol	Hi-media
21.	Petroleum ether	Hi-media
22.	Potassium persulphate	Hi-media
23.	Quercetin (CA)	Hi-media
24.	Sodium acetate	Hi-media
25.	Sodium hydroxide	Hi-media
26.	Sodium phosphate	Hi-media
27.	Sulphuric acid	Hi-media
28.	2,4,6- tripyridyl-2-triazine	Hi-media
29.	Trifluoroacetic acid	Sigma-Aldrich (USA).
30.	Tris [hydroxyl methyl] aminomethane (Tris buffer)	Sigma-Aldrich (USA).

Table 15: Reagents and standards used in this study

4.2.3. Sample Preparation

The bulbs of the six *Crinum* genotypes (Table 15) collected were washed thoroughly in running tap water 2-3 times to remove impurities like soil and debris, followed by rinsing with distilled water before drying. The samples were shade dried for nearly 30 days, and then the dried samples were ground into fine powder in a mixer grinder. All the samples were then subjected to methanolic extraction. 15 g of the samples were soaked in 50 ml 95% methanol and extracted in a magnetic stirrer at 64.7 °C for 4 hrs. The extracts were then filtered using Whatman filter paper grade 1 (Hi-media, India) and concentrated in a rotary evaporator at 65 °C. Weighed the extract and dissolved in 95% methanol to make an extract of 100 mg/mL concentration and used for further phytochemical analysis.

4.2.4. Phytochemical Analysis

4.2.4.1. Total Phenolic Content (TPC)

The extract's total phenolic content (TPC) was determined using the Folin-Ciocalteu method as described by Singleton and Rossi (1965), with slight modifications. The phenolic compounds reduce the Folin-Ciocalteu reagent, producing a blue complex that can be quantified spectrophotometrically at 765 nm. The intensity of the blue colour is directly proportional to the total phenolic content.

About 2 mL of plant extract (200 mg/mL) was mixed with 12 mL of 7.5% sodium carbonate and 12 mL of diluted Folin-Ciocalteu reagent. The mixture was vortexed and incubated in the dark at room temperature for 60 minutes. Absorbance was measured at 765 nm using spectrophotometer (Jasco, Japan) with a blank control of the reaction mixture without the extract. A standard calibration curve was prepared using gallic acid at different concentrations (20, 40, 60, 80, 100, 120 µg/mL). The percentage absorbance was plotted against concentration, and the linear regression equation was obtained as:

$$y = mx + c$$

Where, y = Absorbance at 765 nm

x = Gallic acid concentration (µg/mL)

m = Slope and c = Intercept

Using this equation, the gallic acid equivalent (GAE) concentration x for the sample was calculated, and the total phenolic content was expressed as mg gallic acid equivalent per gram of extract (mg GAE/g E) using the formula:

$$\text{mg GAE per g Extract} = \frac{x\,\mathrm{X}\,\mathrm{V}}{M}$$

Where, x = Equivalent concentration of Gallic acid (mg/mL)

V = Total extract volume used (mL)

M = Mass of extract used per mL (in g)

4.2.4.2. Total Flavonoid Content (TFC)

Aluminium chloride colorimetric method was used to determine TFC (Sakanaka *et al.*, 2005) with some modifications. In this assay, flavonoid compounds form a stable complex with aluminium chloride in acidic conditions, resulting in a yellow coloration. It can be quantified spectrophotometrically at 415 nm. The intensity of the colour is directly proportional to the total flavonoid content.

A reaction mixture was prepared by adding 2 mL of sample extract (200 mg/mL) to 2.5 mL of 5% sodium nitrite ($NaNO_2$) solution and 2.5 mL of distilled water. The mixture was incubated at room temperature for 6 minutes. Following incubation, 4.5 mL of 10% aluminium chloride ($AlCl_3$) solution and 15 mL of 1M sodium hydroxide (NaOH) solution were added. The reaction mixture was thoroughly mixed, and the absorbance was immediately measured at 415 nm using a spectrophotometer (Jasco, Japan). A reaction mixture without the sample extract was used as the blank control.

A standard calibration curve was prepared using quercetin at different concentrations (20, 40, 60, 80, 100, 120 µg/mL). The absorbance values were plotted against the concentration, and the linear regression equation was obtained as: $y = mx + c$

Where, y = Absorbance at 415 nm

x = Quercetin concentration (µg/mL)

m = Slope and c = Intercept

Using this equation, the quercetin equivalent (QE) concentration x for the sample was calculated, and the total phenolic content was expressed as mg quercetin equivalent per gram of extract (mg QE/g Extract) using the formula:

$$\text{mg QE per g Extract} = \frac{x \, \text{X} \, \text{V}}{M}$$

Where, x = Equivalent concentration of Quercetin (mg/mL)

V = Total extract volume used (mL)

M = Mass of extract used per mL (in g)

4.2.4.3. Total Alkaloid Content (TAC)

The total alkaloid content (TAC) of the extract was determined using the bromocresol green (BCG) method described by Ghane *et al.* (2018), with slight modifications. The BCG method is based on the formation of a stable complex between alkaloids and bromocresol green under acidic conditions. Alkaloids in the extract react with bromocresol green in the presence of sodium phosphate buffer (pH 4.7) to form a yellow-green complex. This complex is then extracted using chloroform, and its absorbance is measured using spectrophotometric method.

To prepare the BCG reagent, 69.8 mg of bromocresol green was mixed with 500 µL of double-distilled water and 300 µL of 2N sodium hydroxide (NaOH). The solution was gently heated for a short period and then diluted to 10 mL using distilled water.

A reaction mixture was prepared by adding 100 µL of sample extract to 100 µL of bromocresol green reagent and 100 µL of sodium phosphate buffer (pH 4.7). The mixture was thoroughly mixed, followed by the addition of 200 µL of chloroform to extract the alkaloid-bound complex. After extraction, the absorbance of the organic chloroform layer was measured at 470 nm using a UV-Vis spectrophotometer (Jasco, Japan). A reaction mixture without the sample extract was used as the blank control. Lycorine(mg/mL) was used as standard and results were expressed in mg Lycorine equivalent per gram plant extract (mg LE/g Extract). A standard calibration curve was prepared using lycorine at different concentrations (20, 40, 60, 80, 100, 120 µg/mL).

The absorbance values were plotted against the concentration, and the linear regression equation was obtained as: $y = mx + c$

Where, y = Absorbance at 248 nm

x = Lycorine concentration (µg/mL)

m = Slope and c = Intercept

Using this equation, the lycorine equivalent (LE) concentration x for the sample was calculated, and the total phenolic content was expressed as mg lycorine equivalent per gram of extract (mg LE/g Extract) using the formula:

$$\text{mg CE per g E} = \frac{x \, \mathrm{X} \, \mathrm{V}}{M}$$

Where, x = Equivalent concentration of Lycorine(mg/mL)

V = Total extract volume used (mL)

M = Mass of extract used per mL (in g)

4.2.5. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was performed using the Shimadzu Nexis GC-2030 system equipped with an SH-I-5Sil MS column (30 m × 0.5 mm, 0.25 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Sample injection was carried out using an AOC-30/20i autosampler. The oven temperature was programmed as follows: an initial temperature of 50°C was maintained for 4 minutes, followed by a gradual increase to 240°C at a rate of 5°C/min, with a 2-minute hold. The temperature was then further increased to 260°C at a rate of 5°C/min and held for 12 minutes, resulting in a total run time of 60 minutes. The MS transfer line temperature was set at 280°C, while the ion source was maintained at 200°C. The trap temperature was 190°C, with a split ratio of 1:20. Electron impact ionization was performed at 70 eV.

Data acquisition and analysis were conducted using GCMS Solutions software, with compound identification performed using the NIST 20 spectral library (National Institute of Standards and Technology, 2020).

4.2.6. RP-HPLC Analysis for Galantamine and Lycorine

4.2.6.1. Preparation of Alkaloid Sample for HPLC

For alkaloid extraction, 2 mL of crude methanolic extract of the bulb sample (100 mg/mL) was taken. The extract was dried by evaporating the supernatant at 64.7°C on a hot plate for 10 minutes. The dried residue was then dissolved in 20 mL of 2% H_2SO_4 and defatted using petroleum ether in a separatory funnel. The lower aqueous layer was collected and basified with 25% liquid ammonia to adjust the pH to 9.5–10. The alkaloid fraction was then extracted using absolute chloroform in a separatory funnel. The collected chloroform layer was dried by evaporating the supernatant at 61.2°C on a hotplate for 10 minutes. Finally, the dried residue was dissolved in 3 mL of methanol for HPLC analysis.

4.2.6.2. Preparation of Standards for HPLC

The standards used for the estimation of lycorine and galantamine were lycorine hydrochloride ($C_{16}H_{17}NO_4\cdot HCl$, molecular weight = 323.77) and galantamine hydrobromide ($C_{17}H_{21}NO_3\cdot HBr$, molecular weight = 368.27) respectively. Each compound was separately dissolved in 0.1% trifluoroacetic acid (TFA) to prepare a stock solution of known concentration. Working solutions were prepared in the ranges of 25–200 µg/mL for galantamine (GAL) and 100–1000 µg/mL for lycorine (LYC) to establish calibration curves.

Before analysis, all solutions (mixed standards, sample and spiked solutions) were filtered through a 0.22 µm nylon syringe filter and stored at 4°C to maintain stability.

4.2.6.3. Quantification of Galantamine and Lycorine using RP-HPLC

Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis of phenolic compounds was carried out using a Shimadzu Nexera Series chromatographic system (Shimadzu Corporation, Japan). The system was equipped with a binary pump, an auto-sampler, and a photodiode array (PDA) detector. Separation was achieved using a Hiber C18 column (250 × 4.6 mm, 5 µm i.d.). Data processing was performed using the built-in ChromNAV software. The mobile phase consisted of 10% acetonitrile (ACN), 20% acetate buffer (pH 3.6), and 0.025 M diethylamine (DEA), with a flow rate set at 1 mL/min. The injection volume for the extract was 20 µL. Peak detection was carried out at 240 nm, with a total run time of 30 minutes.

The qualitative analysis of galantamine and lycorine in the samples was performed by comparing their retention times with those of authentic standards. Identification of the compounds was further confirmed by spiking the samples with known concentrations of the respective standards. For quantification, calibration curves were generated for galantamine (GAL) and lycorine (LYC) alkaloid standards. The concentrations of GAL and LYC in the extracts were determined and expressed as micrograms per gram of dried extract ($\mu g\ g^{-1}$ DE). System suitability was evaluated through triplicate injections of both standard solutions and extracts. The repeatability of the method was assessed based on the peak areas obtained from three independent injections of standard solutions and extracts.

4.2.7. Antioxidant Properties

4.2.7.1 DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined following the method described by Brand-Williams *et al.* (1995), with slight modifications. DPPH is a stable free radical that exhibits a deep purple colour due to its unpaired electron. When antioxidants in the sample react with DPPH, the radical is reduced to DPPH-H, causing a colour change from purple to yellow. The decrease in absorbance at 515 nm is proportional to the radical scavenging activity of the sample.

Dissolve a known weight of dried extract (200 mg) in a 10 mL methanol. From this stock solution of 50 mg/mL. dilute to make different concentrations (0.1, 0.2, 0.5, 1, 2 mg/mL). A reaction mixture was prepared by adding 200µL of sample extract to 2.8 mL of 0.1 mM DPPH. The mixture was then incubated in the dark at room temperature for 30 minutes. The decrease in absorbance at 515 nm, due to the reduction of DPPH radicals, was recorded to assess antioxidant potential. The reduction in absorbance at 515 nm indicates the extent of radical scavenging, where a higher percentage means stronger antioxidant activity. A DPPH solution without extract served as the control.

$$\text{DPPH Scavenging Activity (\%)} = \left(\frac{\text{A control} - \text{A sample}}{\text{Acontrol}}\right) \text{X } 100$$

Where, A control = Absorbance of DPPH solution without sample at 515nm

A sample = Absorbance of DPPH solution with the sample at 515nm

To generate a calibration curve, ascorbic acid at varying concentrations (20, 40, 60, 80, 100, 120 µg/mL) was used as a reference standard. From the calibration curve obtain the linear equation: $y = mx + c$

Where, y = Percentage of scavenging activity

x = Ascorbic acid concentration (µg/mL)

m = Slope and c = Intercept.

Using the equation, the ascorbic acid equivalent concentration (x) for the sample's scavenging activity was calculated, and results were expressed as milligrams of ascorbic acid equivalent per gram of extract (mg AAE/g Extract) using the formula.

$$\text{mg AAE per g Extract} = \frac{x \text{ X V}}{M}$$

Where, x = Equivalent concentration of ascorbic acid (mg/mL)

V = Total extract volume used (mL)

M = Mass of extract used per mL (in g)

4.2.7.2. ABTS Radical Activity

The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay was performed following the protocol outlined by Re *et al.* (1999). To generate the ABTS radical ($ABTS^{\bullet+}$), 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate and incubated in the dark at room temperature for 12 hours. Before use, the solution was diluted with methanol (1:89 v/v) to obtain an absorbance of 0.70 ± 0.02 at 734 nm.

The assay was conducted by adding 200 µL of sample extract (at concentrations of 0.1, 0.2, 0.5, 1, and 2 mg/mL) to 2.8 mL of the diluted ABTS reagent. After 30 minutes of incubation in the dark, absorbance was recorded at 734 nm. A mixture of methanol and ABTS reagent without the extract served as the blank control. The percentage of ABTS radical scavenging activity was calculated using the formula:

$$\text{ABTS Scavenging Activity (\%)} = \left(\frac{\text{A control} - \text{A sample}}{\text{Acontrol}}\right) \text{X } 100$$

Where, A control = Absorbance of ABTS solution without sample at 734nm

A sample = Absorbance of ABTS solution with the sample at 734nm

The antioxidant activity was quantified using 2,6-di-tert-butyl-4-hydroxytoluene (BHT) as the standard. Prepare BHT standard solutions at different concentrations (20, 40, 60, 80, 100, 120 µg/mL). Measure their % ABTS scavenging activity and plot a graph. From the calibration curve obtain the linear equation: $y= mx + c$

Where, y = Percentage of scavenging activity

x = BHT concentration (µg/mL)

m = Slope and c = Intercept.

Using this equation, the BHT-equivalent concentration (x) for the sample's scavenging activity was determined. The results were expressed as milligrams of BHT equivalent per gram of dried extract (mg BHTE/g Extract) using the formula:

$$\text{mg AAE per g Extract} = \frac{x \text{ X V}}{M}$$

Where, x = Equivalent concentration of BHT (mg/mL)

V = Total extract volume used (mL)

M = Mass of extract used per mL (in g)

4.2.7.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) assay was conducted based on the method of Benzie and Strain (1996), with slight modifications. The FRAP assay measures the reducing power of antioxidants in a sample by reducing Fe^{3+}-TPTZ (ferric-tripyridyl-s-triazine) complex to its ferrous form Fe^{2+}-TPTZ, which exhibits a blue colour.

The FRAP reagent was freshly prepared by combining 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 300 mM acetate buffer (pH 3.6) in a 1:1:10 ratio. Before use, the reagent was incubated at 37 °C. For the assay, 200 µL of sample extract (at concentrations of 0.1, 0.2, 0.5, 1, and 2 mg/mL) was added to 2.8 mL of the FRAP reagent and incubated at 37 °C in a water bath for 30 minutes. Following incubation, the absorbance Fe^{2+} complex is recorded at 593 nm. The antioxidant activity of the sample is quantified as Fe (II)-equivalent concentration using the $FeSO_4$ calibration curve equation.

$$\text{Fe(II)eqyuivalent}(\mu\text{M}) = \frac{\text{Absorbance of sample} - \text{c}}{\text{m}}$$

Where, m = Slope of the standard curve and c = Intercept.

To express the antioxidant activity as mg Fe(II) equivalent per gram extract (mg Fe(II)/g Extract), the following formula was used:

$$\text{mg Fe(II) per g DE} = \frac{x \text{ X V}}{\text{M}}$$

Where, x = Fe(II) equivalent concentration from the calibration curve

V = Total extract volume used (mL)

M = Mass of extract per mL (g)

4.2.8. Acetylcholine Esterase Inhibition Property

The enzymatic inhibition activity assay was performed following a modified version of the *in-vitro* spectrophotometric method described by Ellman *et al.* (1961).

Requirements

1. Acetylcholinesterase (AChE) type VI-S
2. 5,5' –di thio bis [2- nitro benzoic acid] (DTNB)
3. Acetyl thio-choline iodide (AChI)
4. Tris [hydroxyl methyl] aminomethane (Tris buffer),
5. Dimethyl sulfoxide (DMSO)

Procedure

The reaction mixture was prepared by combining 500 µL of 3 mM DTNB, 100 µL of 15 mM AChI, 275 µL of 50 mM Tris–HCl buffer (pH 8), and 100 µL of ethanolic extract dissolved in ethanol, water, or DMSO. This mixture was placed in a 1 mL cuvette and used as the blank.

For the experimental reaction, 25 µL of buffer in the blank was replaced with 25 µL of enzyme solution containing 0.28 U mL^{-1}. The enzymatic reaction was monitored at 405 nm for 5 minutes, and reaction velocities were determined. Enzyme activity was expressed as a percentage of the reaction velocity compared to the control assay, where the buffer was used instead of the inhibitor (extract). The percentage inhibition was calculated as:

$$\text{Inhibition }(\%) = 100 - \text{Enzyme activity }\%$$

Each experiment was performed in triplicate, and results represent the mean values. Ethanolic extracts were reconstituted in the minimum necessary volume of DMSO or ethanol. The final concentrations of extracts in the reaction cuvettes corresponded to 0.5 mg/mL and 1 mg/mL.

4.2.9. Statistical Analysis

All values were expressed as mean ± standard error (SE). Data were analyzed using a one-way analysis of variance (ANOVA), and significant differences between mean values were determined using Duncan's multiple range test (DMRT) at a significance level of $p < 0.05$. Statistical analyses were performed using SPSS version 25.0. Multivariate statistical analyses were performed using R (R Core Team, 2024). Principal Component Analysis (PCA) was conducted using the FactoMineR package (Lê *et al.,* 2016). Hierarchical clustering was performed using the NbClust packages (Charrad, et al., 2014).

4.3. Result

4.3.1. Phytochemical Analysis

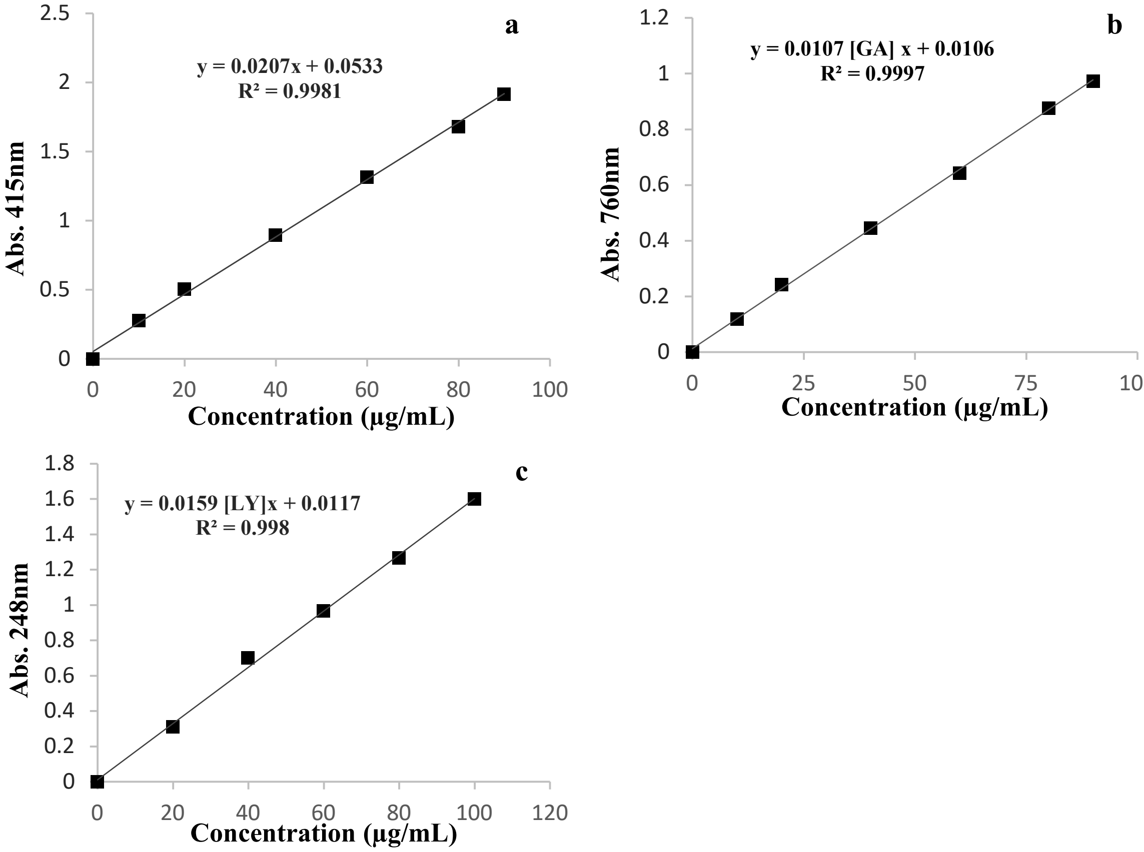
4.3.1.1. Total Phenolic Content

The total phenolic content (TPC) of the methanolic extracts of bulbs and leaves of *Crinum* genotypes are calculated as gallic acid equivalent per gram extract in compare with the standard calibration plot of gallic acid (Graph 6.a). TPC was significantly higher in methanolic extract of leaves than in bulbs of all the studied genotypes ($p < 0.001$). Among leaves, CVL (*C. viviparum* leaf) (119.40±0.14 mg GAE/g extract) showed the highest phenolic content, followed by CSL (*C. solapurense* leaf) (118.82±0.12 mg GAE/g extract) and CRL (*C. reddyi* leaf) (117.91±0.19 mg GAE/g extract). Whereas CML (*C. malabaricum* leaf) recorded 107.38 mg GAE/g extract. In contrast, bulbs exhibited substantially lower TPC, with ranging from CLTL (*C. latifolium* leaf) (93.18±0.03 mg GAE/g extract) to CSB (78.56 mg GAE/g extract) having the lowest (Table 16). For *C. malabaricum*, the growing-stage bulb extract (CMGB, 84.25 mg GAE/g extract) had a slightly lower TPC compared to the dormancy-stage bulb extract (CMDB, 91.58 mg GAE/g extract). This trend suggests that phenolic accumulation might increase as the plant enters dormancy.

4.3.1.2. Total Flavonoid Content

The total flavonoid content (TFC) of the methanolic extracts of bulbs and leaves of *Crinum* genotypes is calculated as quercetin equivalent per gram extract in comparison with the standard calibration plot of quercetin (Graph 6.b). TFC showed a variable pattern, with leaves and bulbs exhibiting comparable ranges ($p < 0.05$). The highest TFC was observed in CLTL (*C. latifolium* leaf) (80.70±0.001 mg QE/g extract) among leaves, while CLRB (*C. lorifolium* bulb) (80.14±0.04 mg QE/g extract) had the highest in bulbs. Conversely, CAL (*Crinum asiaticum* leaf) recorded the lowest TFC (61.08 mg QE/g extract).

A comparison of *C. malabaricum* extracts showed that CMGB (76.70 mg QE/g extract) and CMDB (77.17 mg QE/g extract) had nearly similar TFC values, suggesting minimal variation between the growing and dormancy phases (Table 16).



Graph 6: Standard calibration graph; a. Gallic acid; b. Quercetin; c. Lycorine

4.3.1.3. Total Alkaloid Content

The total Alkaloid Content of the methanolic extracts of bulbs and leaves of *Crinum* genotypes is calculated as lycorine equivalent per gram extract compared with the standard calibration plot of lycorine (Graph 6.c). The analysis revealed that bulbs generally exhibited a higher concentration of alkaloids compared to leaves. Statistical comparison using an independent t-test showed a significant difference between leaves and bulbs ($t = -2.83$, $p = 0.0176$), indicating that bulbs tend to accumulate significantly more alkaloids than their leaf counterparts.

Genotype	**TAC** (mg LE/g extract)	**TPC** (mg GAE/g extract)	**TFC** (mg QE/g extract)
CLRL	8.27 ± 0.02^{g}	106.26 ± 0.01^{h}	65.37 ± 0.03^{c}
CLTL	8.90 ± 0.01^{h}	112.48 ± 0.18^{k}	80.70 ± 0.00^{n}
CRL	6.27 ± 0.02^{a}	117.91 ± 0.19^{l}	71.53 ± 0.03^{g}
CSL	7.71 ± 0.06^{d}	118.82 ± 0.12^{m}	70.12 ± 0.08^{e}
CVL	7.56 ± 0.00^{c}	119.40 ± 0.14^{n}	70.47 ± 0.25^{f}
CAL	6.74 ± 0.00^{b}	110.97 ± 0.05^{j}	61.08 ± 0.07^{a}
CML	9.14 ± 0.00^{i}	107.38 ± 0.02^{i}	62.74 ± 0.02^{b}
CLRB	10.27 ± 0.02^{j}	81.20 ± 0.03^{c}	80.14 ± 0.04^{m}
CLTB	14.06 ± 0.05^{n}	93.18 ± 0.03^{g}	72.54 ± 0.05^{h}
CRB	7.66 ± 0.00^{d}	79.72 ± 0.04^{b}	78.09 ± 0.06^{l}
CSB	8.16 ± 0.02^{f}	78.56 ± 0.02^{a}	76.15 ± 0.03^{i}
CVB	7.95 ± 0.01^{e}	78.72 ± 0.04^{a}	77.36 ± 0.16^{k}
CAB	12.22 ± 0.00^{m}	85.93 ± 0.02^{e}	68.56 ± 0.08^{d}
CMGB	10.82 ± 0.00^{k}	84.25 ± 0.02^{d}	76.70 ± 0.03^{j}
CMDB	12.07 ± 0.01^{l}	91.58 ± 0.02^{f}	77.17 ± 0.11^{k}

Table 16: Phytochemical analysis (TAC, TPC, and TFC) of methanol extracts of leaf and bulb of different *Crinum* genotypes. Values are mean ± standard error (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p = 0.05

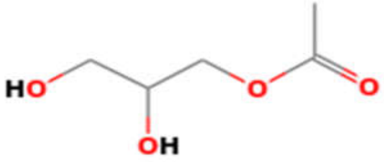
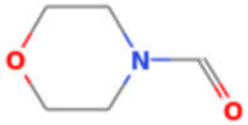
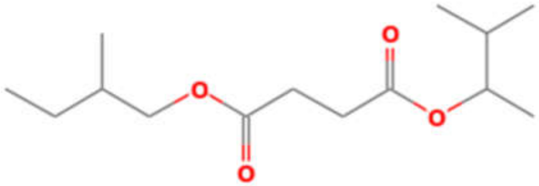
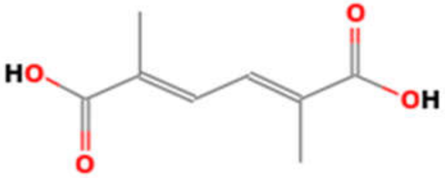
TAC varied significantly across different genotypes ($p < 0.05$). Among all genotypes, CLTB (*C. latifolium* bulb) (14.06±0.05 mg LE/g extract) exhibited the highest TAC, and in leaves, *C. malabaricum* has the highest TAC (9.14±0.003). Whereas the lowest TAC in both leaves and bulbs are in *C. reddyi*; CrL (6.27±0.02 mg LE/g E) and CrB (7.66±0.001 mg LE/g E).

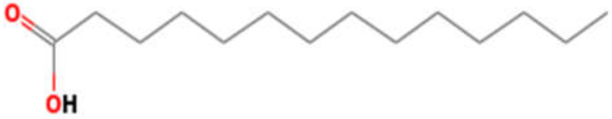
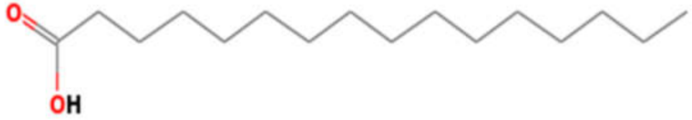
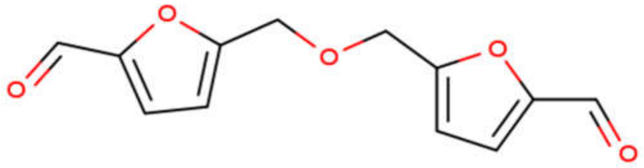
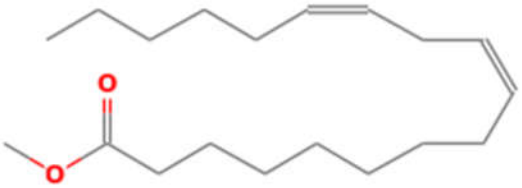
In *C. malabaricum*, significant variation was observed between its different physiological states. The TAC in CMGB (10.82±0.003 mg LE/g extract), representing the growing phase, was lower than in CMDB (12.07±0.01 mg LE/g extract), collected during dormancy (Table 16). This suggests a higher accumulation of alkaloids during dormancy, potentially linked to metabolic storage processes.

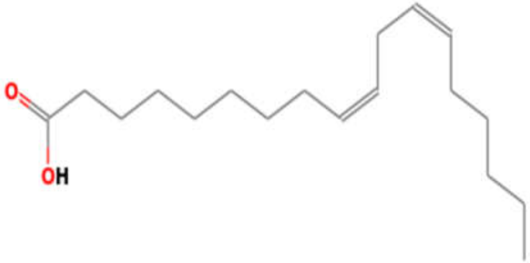
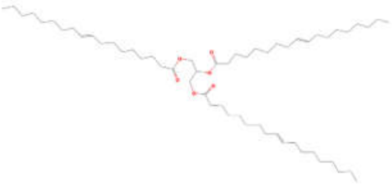
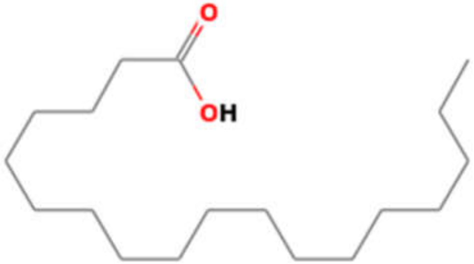
4.3.2. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was carried out on methanol extracts of *C. malabaricum* prepared from the bulbs during dormancy period (CMBD) and during growing period (CMBG). Results reveal the presence of many potentially medicinally and economically important phytocomponents. The relative percentage of each of the identified components was calculated by comparing its average peak area to the total area. The obtained mass spectrum was interpreted by comparison with the compounds present in the NIST database. The analysis detected the presence of 21 compounds in CMBD and 14 compounds in CMBG, with six compounds common to both. (Appendix I and II).

Detailed structural and functional information of the phytocompounds identified is provided in the table 17 & 18. These compounds belong to various classes, including aliphatic esters, fatty acid derivatives, aldehyde derivatives, pyrazine derivatives, alcohols, phenolic compounds, heterocyclic compounds, and alkaloids. These compounds exhibit diverse biological activities such as antidiabetic, antimicrobial, anti-inflammatory, and antitumor properties. Additionally, they have industrial applications in the pharmaceutical and food industries, serving as precursors, industrial solvents, additives, flavouring agents, and model compounds. The compound information was retrieved from the PubChem database (Kim *et al.,* 2023).

Sl.No	Compound Name	Compound Nature	Compound structure	R/T	Applications
1.	1,2,3-Propanetriol, 1-acetate	Aliphatic Ester		12.493	Food additive, fragrance ingredient, and plasticizer
2.	N-Formylmorpholine	Formamide		14.299	Antimicrobial agent
3.	Succinic acid, 3-methylbut-2-yl 2-methylbutyl ester	Aliphatic Ester		16.339	Industrial solvent and plasticizer
4.	(Z),(Z)-2,5-Dimethyl-2,4-hexadienedioic acid	Unsaturated fatty acid		17.488	Chemiluminescence detector and Photodechlorination agent

5.	Tetradecanoic acid (Myristic acid)	Saturated fatty acid		25.070	Used in cosmetics, personal care products, and as a raw material for surfactants
6.	n-Hexadecanoic acid (Palmitic acid)	Saturated fatty acid		29.258	Common dietary oil. Used in cosmetic production
7.	Cirsiumaldehyde (Knipholone)	Unsaturated aldehyde		29.591	Have cytotoxicity against tumour cells Used in the synthesize of hepatitis antiviral precursors.
8.	Methyl linoleate	Fatty acid methyl ester		31.690	Model compound in oxidation/peroxidation assays and used as a fragrance agent

9.	Linolic acid	Fatty acid		32.508	Anti-inflammatory, acne reductive, skin-lightening and moisture retentive properties
10.	Oleic Acid	Fatty acid		32.614	Used in pharmaceuticals and as an emulsifying or solubilizing agent in aerosol products.
11.	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)- (Trielaidin)	Fatty acid		32.679	Production of lubricant and emulsifier in the cosmetic and textile industries.
12.	Octadecanoic acid (Stearic acid)	Fatty acid		32.996	Used in the production of detergents, soaps, and cosmetics

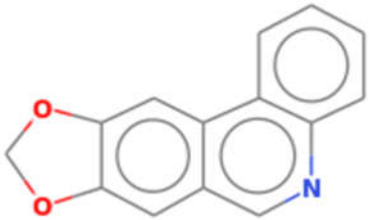
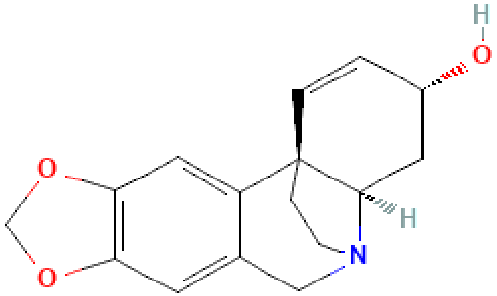
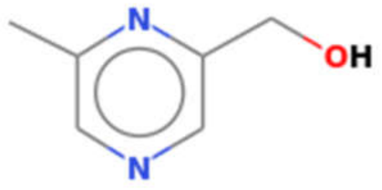
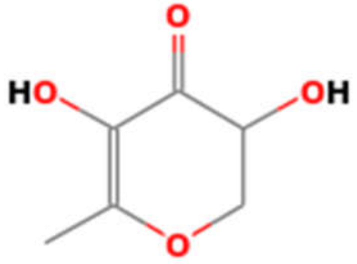
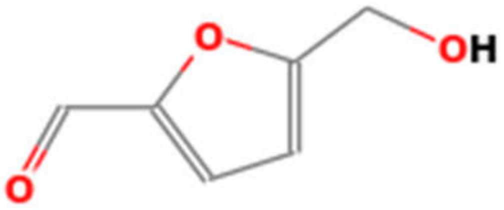
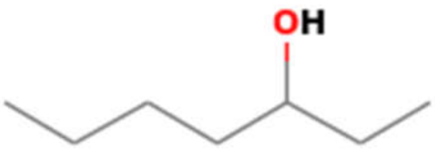
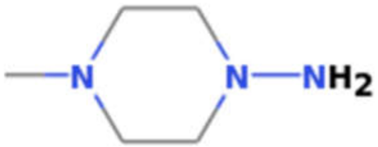
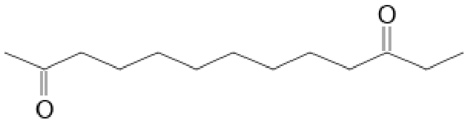
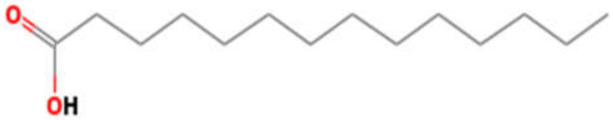
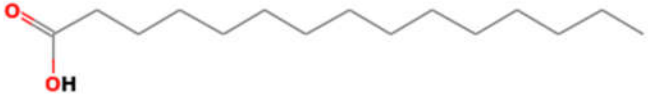
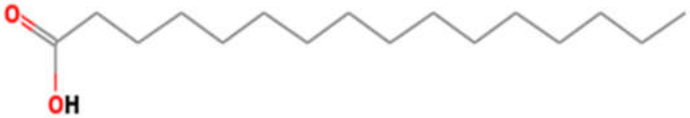
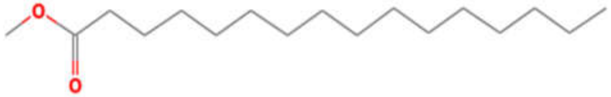
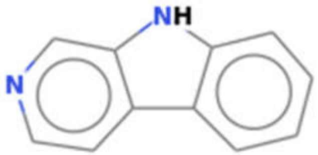
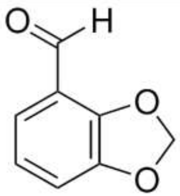
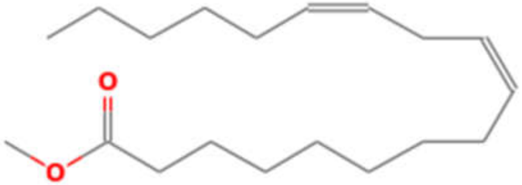
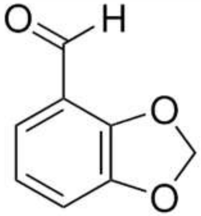
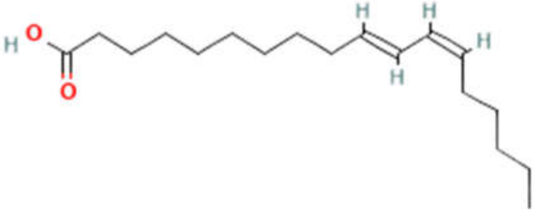
13.	[1,3]Dioxolo[4,5-j]phenanthridine	Alkaloid		34.934	Anti-cancerous agent
14.	Crinine	Alkaloid		38.765	Cytotoxic, antiviral, and acetylcholinesterase inhibitory properties

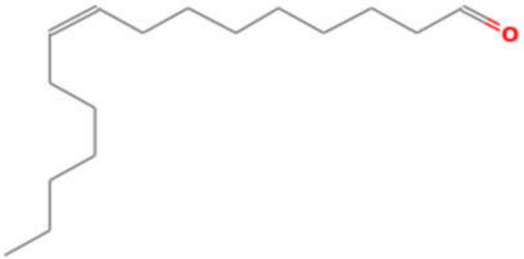
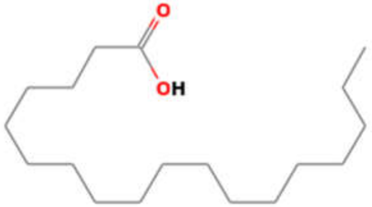
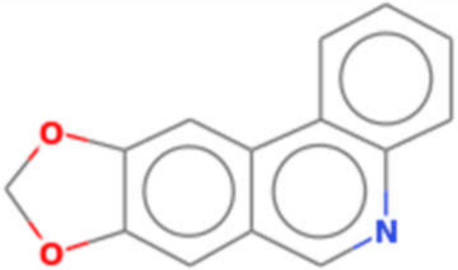
Table 17: Phytocompounds identified by GCMS analysis in the methanolic extract of *C. malabaricum* bulb (Growing period)

Sl.No	Compound Name	Compound Nature	Compound structure	R/T	Applications
1.	6-Methyl-2-pyrazinylmethanol	Pyrazine derivative		7.336	Used as a flavoring agent
2.	Pyranone	Heterocyclic organic compound		9.291	Antibacterial, antifungal, anticancer, and anti-inflammatory properties.

3.	5-Hydroxymethylfurfural	Heterocyclic organic compound		11.879	Used as food additive, biomarker and flavoring agent
4.	3-Heptanol	Alcohol		13.919	Fragrance, a flavouring agent
5.	1-Amino-4-methylpiperazine	Heterocyclic organic compound		14.113	Used in the synthesis of antidepressants, antipsychotics, and antihistamines
6.	Tridecane-2,11-dione	aliphatic di-ketones		23.430	Used in skin care products.

7.	Tetradecanoic acid (Myristic acid)	Saturated fatty acid		25.068	Used in cosmetics, personal care products, and as a raw material for surfactants
8.	Pentadecanoic acid	Saturated fatty acid		27.166	Hepatoprotective and anti-inflammatory activity
9.	n-Hexadecanoic acid (Palmitic acid)	Saturated fatty acid		29.321	Common dietary oil. Used in cosmetic production
10.	Hexadecanoic acid, methyl ester	Saturated fatty acid		28.492	Hepatoprotective and anti-microbial activity
11.	9H-Pyrido[3,4-b]indole	Alkaloid		29.755	Neuroactive properties and Potential anti-cancer agents due to its ability to inhibit specific cancer cell lines.
12.	2-Phenyl-4,5-methylenedioxybenzaldehyde	Aromatic aldehyde		30.417	Uses in pharmaceuticals, flavor & fragrance chemistry.

13.	Heptadecanoic acid	Fatty acid		31.122	anti-inflammatory and metabolic benefits
14.	Methyl linoleate	Fatty acid methyl ester		31.693	Model compound in oxidation/peroxidation assays and used as a fragrance agent
15.	2-Phenyl-4,5-methylenedioxybenzaldehyde	Aromatic aldehyde		31.859	Antibacterial, antifungal, and neuroactive properties.
16.	10E,12Z-Octadecadienoic acid	Fatty acid		32.574	

17.	cis-9-Hexadecenal (Palmitolealdehyde)	Fatty aldehydes		32.650	Used in fragrances, flavors, and surfactant production. Have antifungal activity
18.	Octadecanoic acid (Stearic acid)	Fatty acid		32.029	Used in the production of detergents, soaps, and cosmetics
19.	[1,3]Dioxolo[4,5-j]phenanthridine	Alkaloid		34.945	Anti-cancerous agent

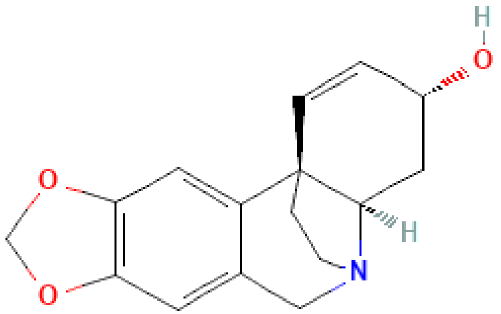
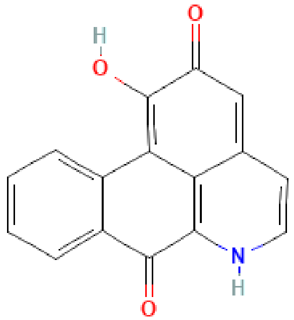
20.	Crinine	Alkaloid		38.662	Cytotoxic, antiviral, and acetylcholinesterase inhibitory properties
21.	Liriodendromine	Alkaloid		41.983	Dopaminergic activity, vasorelaxant properties, antibacterial & antifungal activity

Table 18: Phytocompounds identified by GCMS analysis in the methanolic extract of *C. malabaricum* bulb (Dormancy period)

4.3.3. HPLC Analysis for Galantamine and Lycorine

4.3.3.1. Calibration of Galantamine and Lycorine Standards

The quantification of galantamine and lycorine in the methanolic extracts of leaf and bulb of the genotypes of *Crinum* was performed using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). Galantamine hydrobromide standard exhibited a retention time of 7.307 ± 0.001 min across various concentrations (Figure 27). The peak area was used to construct a calibration curve by plotting concentration against area, yielding a linear regression equation y=16.286x−0.0008 (Graph 7). The high correlation coefficient (R^2=0.9998) indicates excellent linearity in the calibration curve.

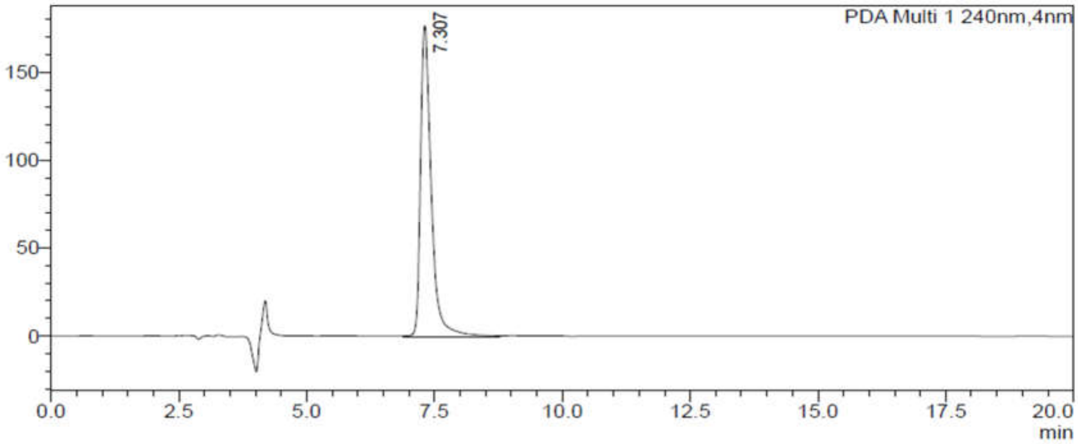
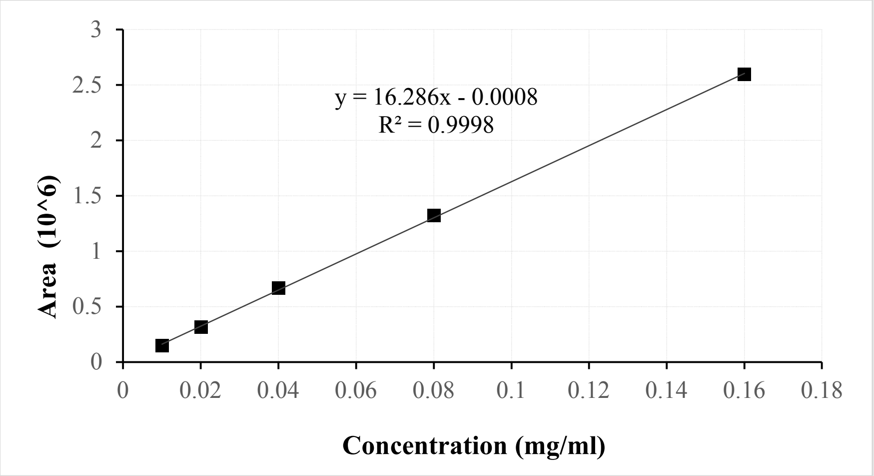


Figure 27. HPLC chromatogram of showing peak at 7.307 min for Galantamine hydrobromide (Concentration = 160 ppm)



Graph 7: Calibration curve of Galantamine hydrobromide

Similarly, the lycorine standard exhibited a retention time of 6.439 ± 0.007 min (Figure 28). A calibration curve was plotted using peak area against concentration, resulting in the equation: $y = 11.761x - 0.0926$, $R^2 = 0.9997$ (Graph 8). The strong linearity of the calibration curve enabled the quantification of lycorine in the *Crinum* extracts by comparing the sample peak areas with the standard calibration plot.

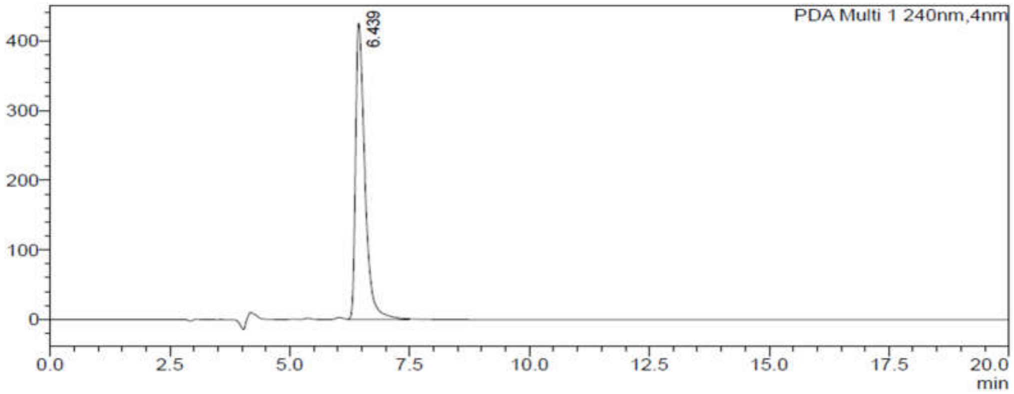
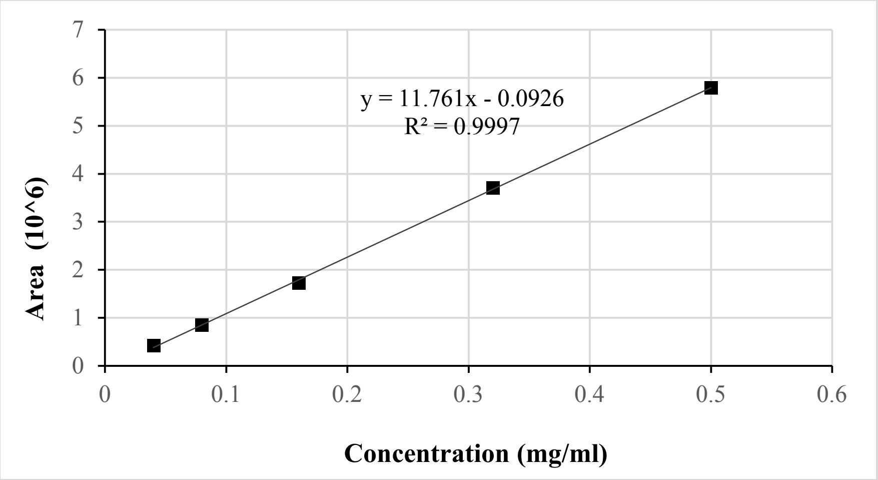


Figure28. HPLC chromatogram of showing peak at 6.439 min. for Lycorine hydrochloride (Concentration = 500 ppm)



Graph 8: Calibration curve of Lycorine hydrochloride

4.3.3.2. Quantification of Galantamine and Lycorine in *Crinum* Extracts

The concentrations of galantamine in the extracts were determined by comparing their chromatographic peak areas with the corresponding standard calibration curve (Figure 29 & 30). The RP-HPLC analysis revealed significant variations in the concentrations of galantamine and lycorine among different *Crinum* genotypes, with distinct differences between bulb and leaf extracts. The bulb extracts contained higher concentrations of galantamine compared to leaf extracts. Lycorine have comparatively high concentration in all the studied genotype than galantamine.

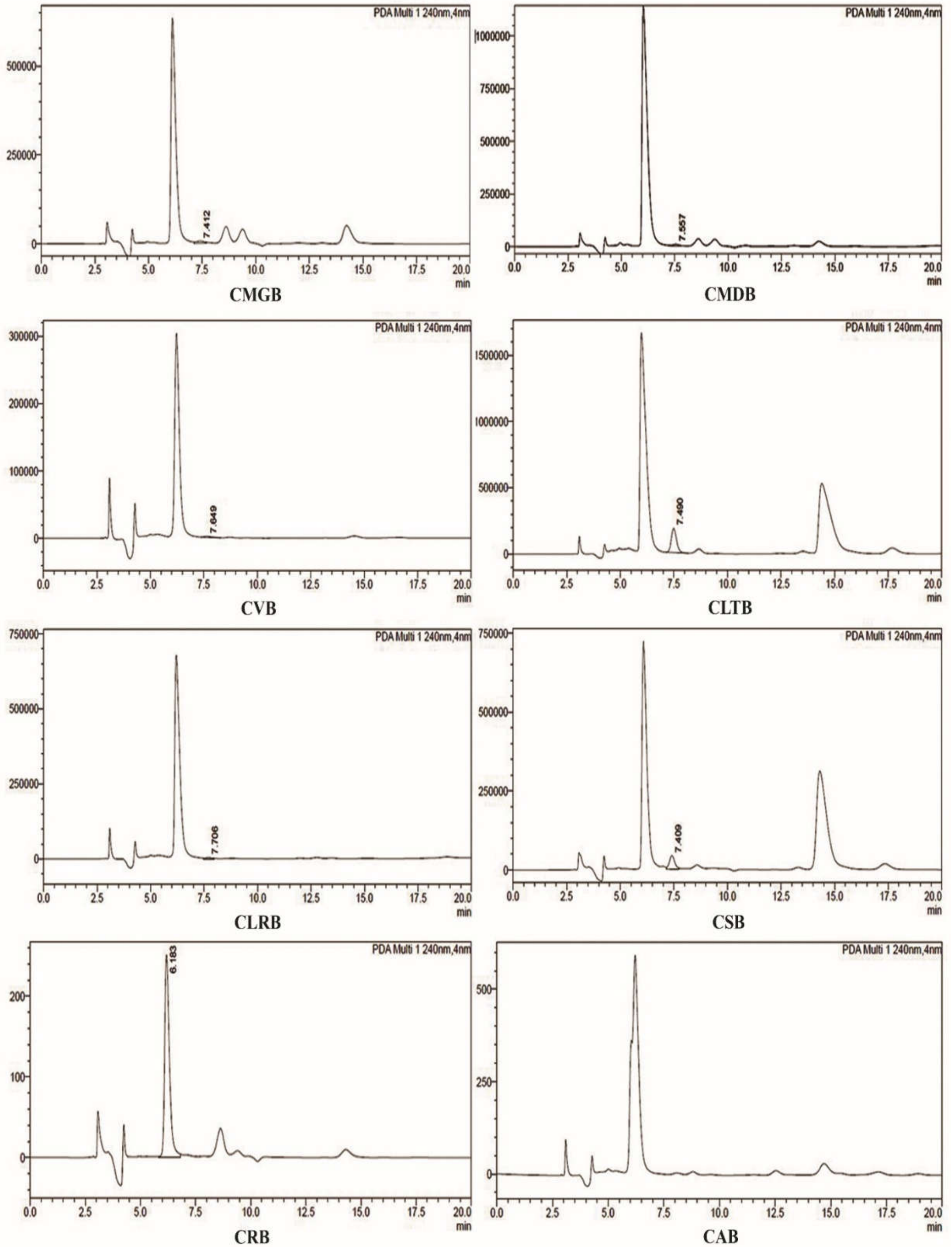


Figure 29. HPLC chromatogram of methanolic extract of bulbs of *Crinum* genotypes. Showing peak corresponding to galantamine (at 7.515±0.124 min) and lycorine (at 6.289±0.119 min). No peak was detected in CRB.

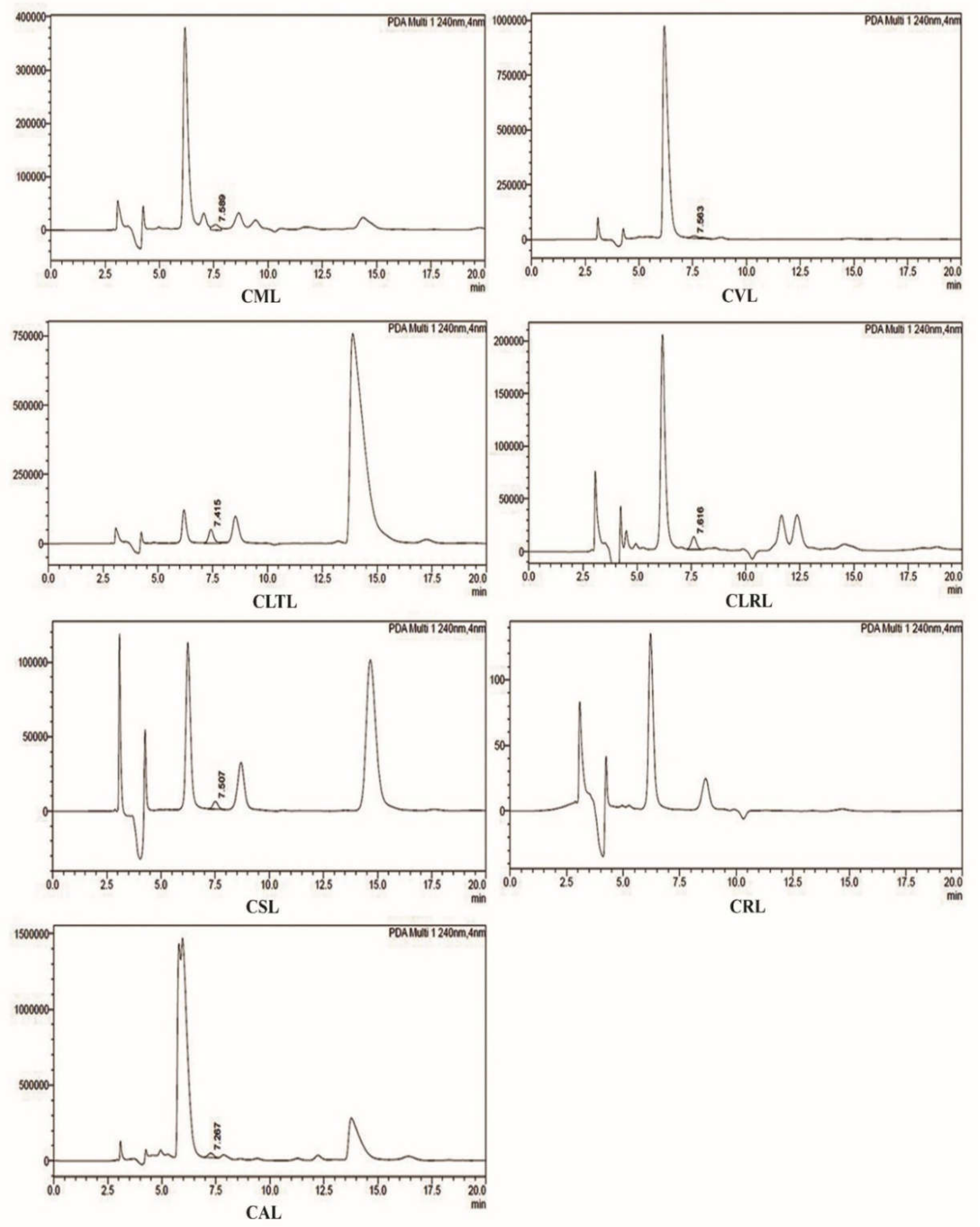


Figure 30. HPLC chromatogram of methanolic extract of leaves of *Crinum* genotypes. Showing peak corresponding to galantamine (at 7.515±0.124 min) and lycorine (at 6.289±0.119 min). No peak was detected in CRL.

Galantamine

Galantamine was detected in most samples, except for *C. reddyi* (CRB & CRL), where no peak was observed. The highest concentration of galantamine was recorded in the bulb extract of *C. latifolium* (CLTB) at 960.25±0.5 µg/g extract, followed by *C.*

solapurense bulb (CSB) (336.03 µg/g extract) and *C. asiaticum* leaf (CAL) (233.61 µg/g extract). The leaf extract of *C. latifolium* (CLTL) also exhibited a substantial amount (396.94 µg/g extract). Moderate levels of galantamine were found in *C. malabaricum* leaf (CML) (101.96 µg/g extract) and *C. viviparum* leaf (CVL) (116.54 µg/g extract), while lower concentrations were detected in *C. lorifolium* leaf (CLRL) (90.44 µg/g extract) and *C. solapurense* leaf (CSL) (38.72 µg/g extract). The bulb extracts of *C. malabaricum* (CMDB and CMFB) showed comparable concentrations (142.75±0.05 µg/g extract and 140.46±0.03 µg/g extract, respectively), whereas the bulb of *C. viviparum* (CVB) exhibited the lowest Galantamine content (13.74±0.13 µg/g extract) (Table 19).

Extract	Galantamine (µg/g Extract)	Lycorine (mg/g extract)
CMDB	142.75±0.05	30.94±0.03
CMGB	140.46±0.03	14.24±0.50
CVB	13.74±0.13	3.15±0.04
CLTB	960.25±0.50	15.17±0.13
CSB	336.03±0.15	7.98±0.03
CRB	PND*	2.46±0.02
CLRB	38.71±0.05	8.02±0.05
CAB	70.33±0.01	17.88±0.05
CML	101.95±0.03	3.78±0.03
CVL	116.53±0.51	7.97±0.01
CLTL	396.93±0.33	1.18±0.00
CLRL	90.44±0.05	1.98±0.01
CSL	38.71±0.01	1.19±0.05
CAL	233.61±0.03	14.54±0.03
CRL	PND*	30.74±0.03

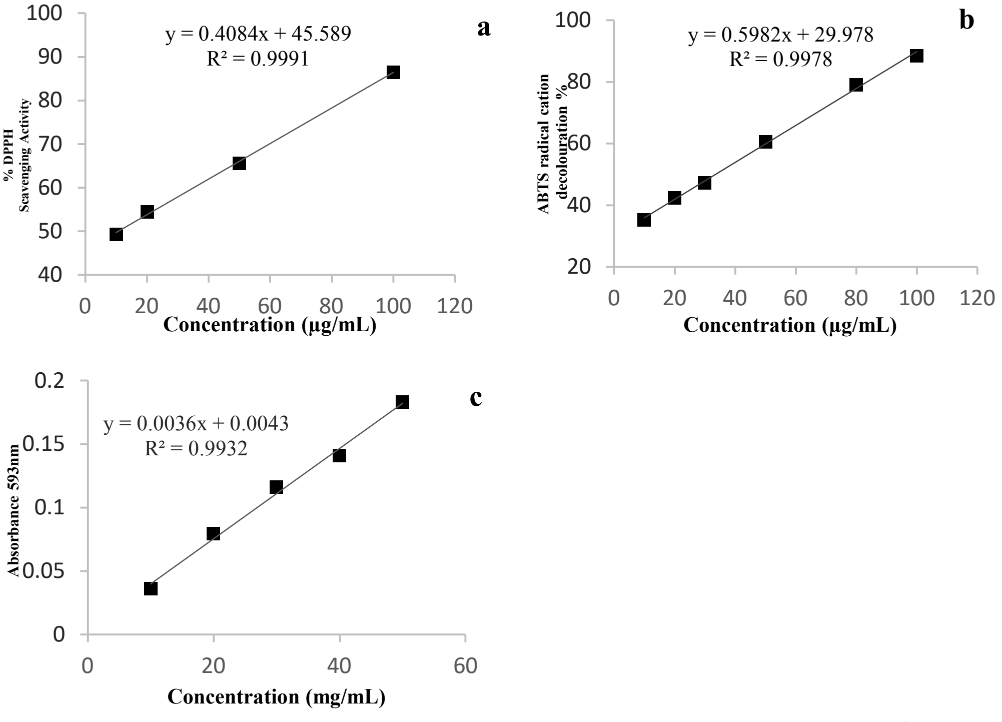
Table 19: Concentration of galantamine (GAL) and lycorine (LY) in methanolic extract of bulb and leaf of different *Crinum* genotypes. Values are mean ± standard error (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p = 0.05. *Peak not detected

Lycorine

Lycorine concentrations varied significantly among the genotypes, with the highest levels detected in *C. malabaricum* bulb during dormancy (CMDB) (30.94±0.03 mg/g extract) and *C. reddyi* leaf (CRL) (30.94 mg/g extract). The bulb of *C. asiaticum* (CAB) contained a considerable amount (17.88±0.05 mg/g extract), while moderate levels were observed in *C. latifolium* bulb (CLTB) (15.17±0.185 mg/g extract), *C. malabaricum* bulb during growing period (CMGB) (14.25 mg/g extract), and *C. asiaticum* leaf (CAL) (14.54±0.50 mg/g extract). Comparatively lower concentrations of lycorine were detected in *C. solapurense* bulb (CSB) (7.98±0.03 mg/g extract) and *C. viviparum* leaf (CVL) (7.97±0.01 mg/g extract). The lowest lycorine levels were recorded in *C. latifolium* leaf (CLTL) (1.18 mg/g extract), *C. lorifolium* leaf (CLrL) (1.98 mg/g extract), and *C. solapurense* leaf (CSL) (1.20 mg/g extract) (Table 19).

4.3.4. Antioxidant Properties

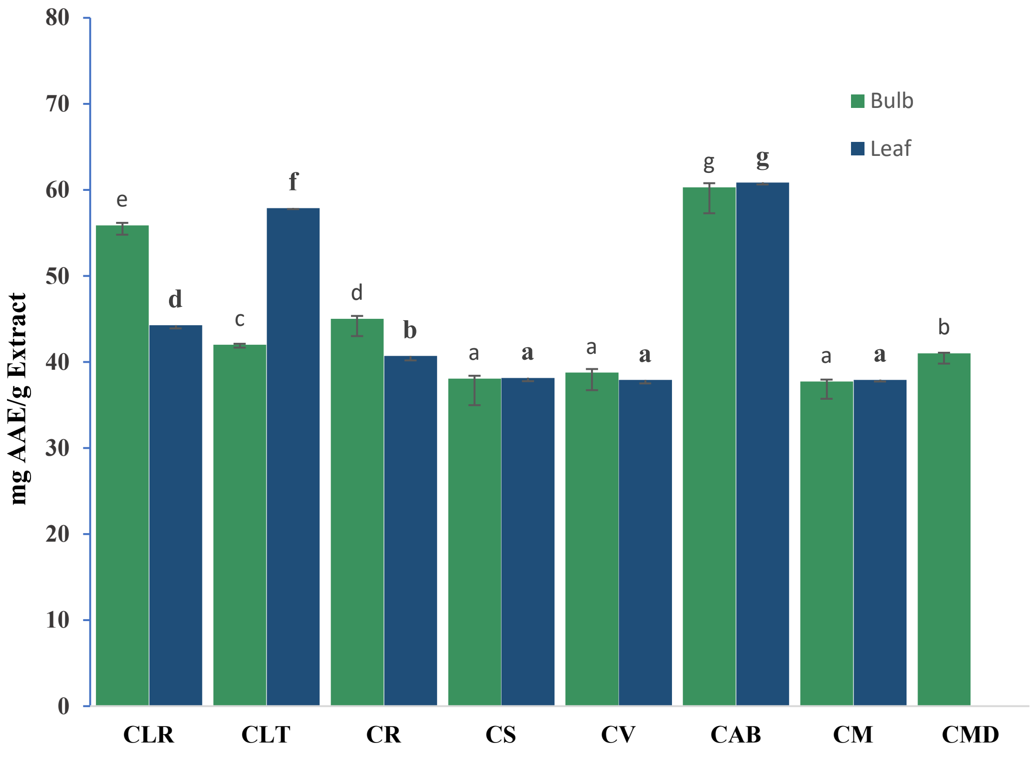
The antioxidant potential of methanolic extracts from various *Crinum* genotypes was evaluated using DPPH radical scavenging activity, ABTS radical cation decolorization assay, and FRAP assay. Based on calibration curves (Graph 9), antioxidant activity was quantified as ascorbic acid, BHT, and Fe(II) equivalents per gram of extract, respectively.



Graph 9: Standard calibration curves: a. DPPH scavenging activity of ascorbic acid; b. ABTS radical cation decolorization by BHT; c. $FeSO_4.7H_2O$

4.3.4.1. DPPH Radical Scavenging Activity.

The highest DPPH radical scavenging activity was observed in *C. asiaticum* leaf (CAL) (60.88±0.26 mg AAE/g extract), followed by *C. asiaticum* bulb (CAB) (60.32±0.46 mg AAE/g extract) and *C. latifolium* leaf (CLTL) (57.89±0.14 mg AAE/g extract). The bulb of *C. lorifolium* (CLRB) also exhibited strong DPPH activity (55.87±0.30 mg AAE/g extract). In contrast, the lowest activity was recorded in *C. malabaricum* bulb (CMGB) (37.75±0.19 mg AAE/g extract), *C. viviparum* leaf (CVL) (37.93±0.42 mg AAE/g extract), and *C. malabaricum* leaf (CML) (37.94±0.21 mg AAE/g extract). These results suggest that leaves, particularly from *C. asiaticum* and *C. latifolium*, exhibit higher free radical scavenging potential than bulbs (Graph 10, Table 20).



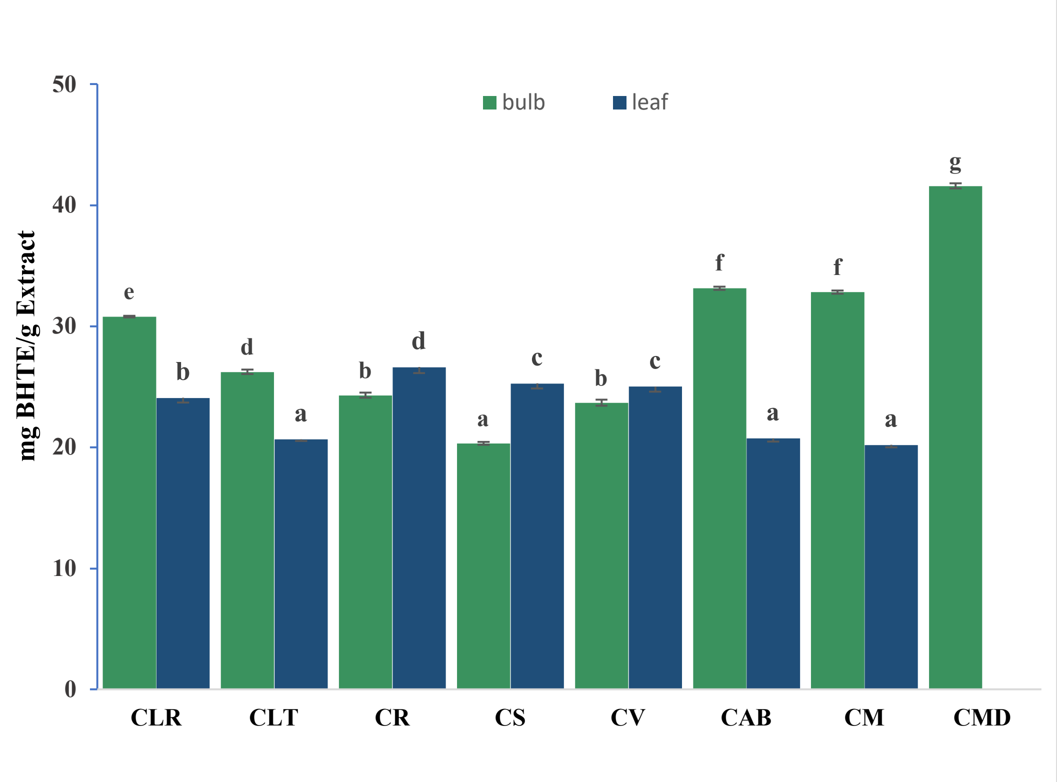
Graph 10: DPPH scavenging activity of methanolic extracts of bulbs and leaves of *Crinum* genotypes. Values are mean ± standard error (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p=0.05

GENOTYPE	**DPPH** (mg AAE/g Extract)	**ABTS** (mg BHTE/g Extract)	**FRAP** (mg Fe (II)E/g Extract)
CLRL	44.28±0.38^{d}	24.09±0.28^{b}	230.79±0.41^{j}
CLTL	57.89±0.14^{f}	20.66±0.16^{a}	252.87±0.81^{l}
CRL	40.70±0.50^{b}	26.62±0.25^{d}	270.55±0.34^{n}
CSL	38.17±0.41^{a}	25.27±0.27^{c}	265.43±0.28^{m}
CVL	37.93±0.42^{a}	25.02±0.17^{c}	278.29±0.16^{o}
CAL	60.88±0.26^{g}	20.74±0.52^{a}	240.01±0.19^{k}
CML	37.94±0.21^{a}	20.20±0.23^{a}	219.91±0.20^{h}
CLRB	55.87±0.30^{e}	30.80±0.07^{e}	200.59±0.23^{f}
CLTL	42.03±0.10^{c}	26.23±0.18^{d}	225.11±0.16^{i}
CRB	45.04±0.33^{d}	24.30±0.21^{b}	176.16±0.25^{c}
CSB	38.07±0.34^{a}	20.32±0.12^{a}	165.49±0.16^{b}
CVB	38.78±0.41^{a}	23.68±0.26^{b}	161.02±0.41^{a}
CAB	60.32±0.46^{g}	33.14±0.13^{f}	186.18±0.28^{d}
CMGB	37.75±0.19^{a}	32.82±0.14^{f}	192.04±0.70^{e}
CMDB	41.00±0.07^{b}	41.59±0.21^{g}	212.36±0.44^{g}

Table 20. Antioxidant activity of the methanolic extract of bulb and leaf of different *Crinum* genotypes. Values are Mean ± Standard error (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p=0.05

4.3.4.2. ABTS Radical Cation Decolorization Assay

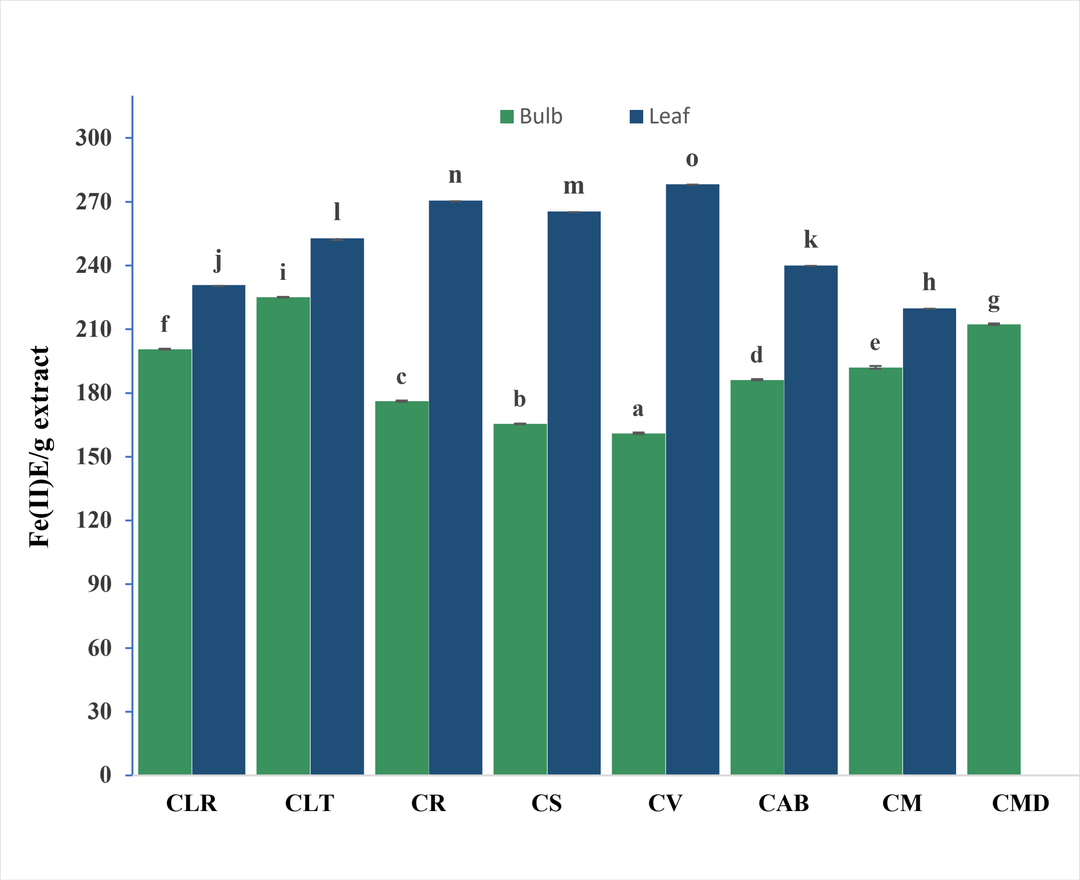
The highest ABTS scavenging activity was recorded in *C. malabaricum* bulb (CMDB) (41.59±0.21 mg BHTE/g extract), followed by *C. asiaticum* bulb (CAB) (33.14±0.13 mg BHTE/g extract) and *C. malabaricum* bulb (CMGB) (32.82±0.14 mg BHTE/g extract). *C. lorifolium* bulb (CLRB) also exhibited significant activity (30.80±0.07 mg BHTE/g extract). The lowest ABTS activity was observed in *C. malabaricum* leaf (CML) (20.20±0.23 mg BHTE/g extract), *C. latifolium* leaf (CLTL) (20.66±0.16 mg BHTE/g extract), and *C. asiaticum* leaf (CAL) (20.74±0.52 mg BHTE/g extract) indicating that antioxidant compounds correspond to ABTS radical cation activity are more concentrated in the bulb than in the leaves (Graph 11). In general, bulb extracts demonstrated superior ABTS scavenging activity compared to leaf extracts (Table 20).



Graph 11: ABTS radical cation decolouration of methanolic extracts of bulbs and leaves of *Crinum* genotypes. Values are mean ± standard (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p = 0.05

4.3.4.3. Ferric Reducing Antioxidant Power (FRAP)

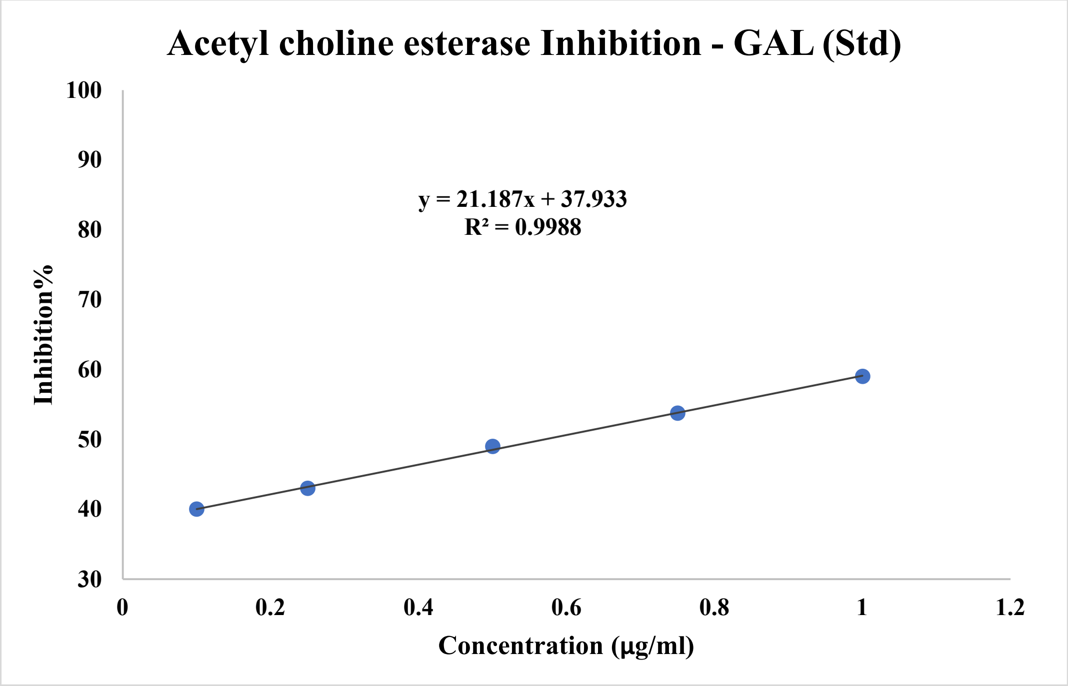
The highest ferric-reducing power was recorded in *C. viviparum* leaf (CVL) (278.29±0.16 mg Fe (II)E/g extract), followed by *Crinum reddyi* leaf (CRL) (270.55±0.34 mg Fe (II)E/g extract) and *C. solapurense* leaf (CSL) (265.43±0.28 mg Fe (II)E/g extract). *C. latifolium* leaf (CLTL) also exhibited strong FRAP activity (252.87±0.81 mg Fe(II)E/g extract) (Table 19). The lowest FRAP values were observed in *C. viviparum* bulb (CVB) (161.02±0.41 mg Fe(II)E/g extract), *C. solapurense* bulb (CSB) (165.49±0.16 mg Fe(II)E/g extract), and *C. reddyi* bulb (CRB) (176.16±0.25 mg Fe(II)E/g extract). *C. malabaricum* bulb (*CMDB*) had a ferric-reducing potential of 212.36±0.44 mg Fe(II)E/g extract, while *C. malabaricum* leaf (*CML*) recorded a slightly lower value (219.91±0.20 mg Fe(II)E/g extract) (Graph 12). Notably, leaf extracts exhibited stronger reducing power than bulb extracts, suggesting that phenolic or flavonoid compounds with ferric-reducing potential are more concentrated in the leaves (Table 20).



Graph 12: Ferric reducing antioxidant power (FRAP) of methanolic extracts of bulbs and leaves of *Crinum* genotypes. Values are mean ± standard error (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p = 0.05

4.3.5. Acetylcholine Esterase Inhibition Property

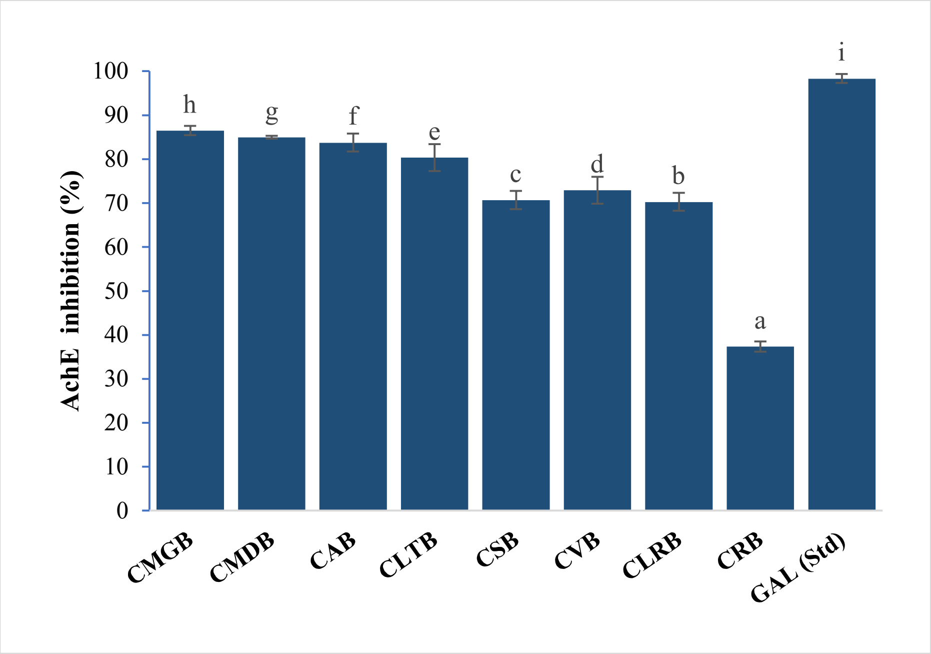
The acetylcholine esterase (AChE) inhibition activity of galantamine hydrobromide was evaluated, and the dose-response curve is presented in the graph 13. The inhibition percentage increased with increasing concentrations of galantamine, exhibiting a typical sigmoidal trend. The IC_{50} value, representing the concentration required to inhibit 50% of AChE activity, was determined to be 0.569 µg/mL, indicating strong inhibitory potential. This serves as the standard reference for comparing the inhibitory effects of *Crinum* extracts.



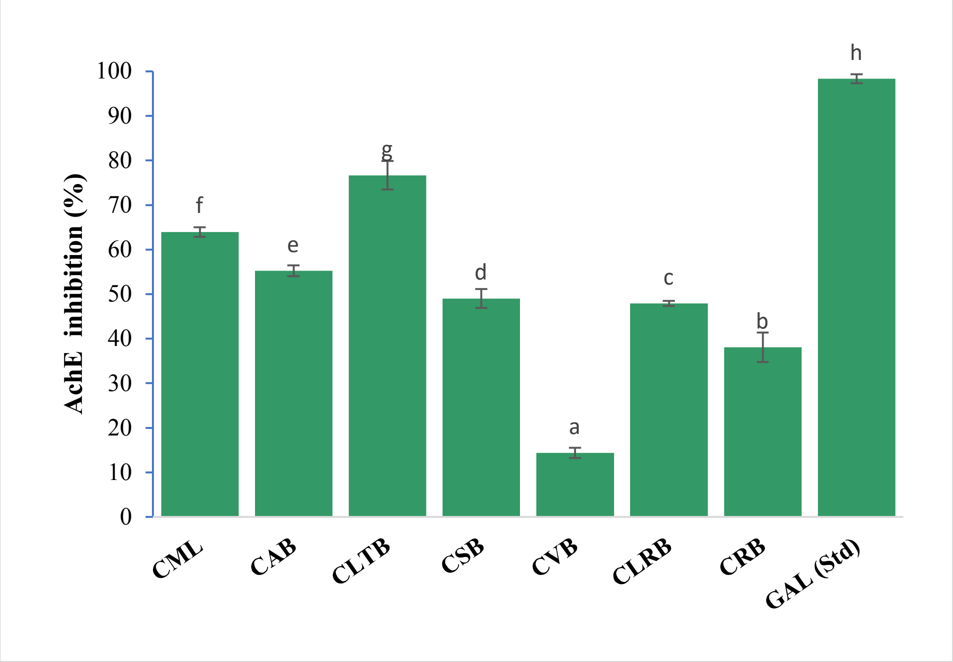
Graph 13: Acetyl choline esterase inhibition of galantamine hydrobromide. Ic50 Value = 0.569 µg/ml)

Extract	AchE Inhibition % of Bulb Extract (100 µg/ml)	IC50 Value (µg/ml)	AchE Inhibition % of Leaf Extract (100 µg/ml)	IC50 Value (µg/ml)
C.malabaricum (G)	86.47±1.06^{h}	9.20±0.017	63.92±1.065^{f}	27.77±0.051
C.malabaricum (D)	84.97±0.35^{g}	8.71±0.073	-	-
C. asiaticum	83.74±2.04^{f}	12.63±0.061	55.21±1.214^{e}	82.98±0.04
C. latifolium	80.34±3.07^{e}	14.76±0.043	76.67±3.188^{g}	38.49±0.015
C. solapurense	70.69±2.06^{c}	34.54±0.012	48.99±2.135^{d}	102.54±0.03
C. reddyii	37.33±1.175^{a}	170.42±0.05	14.34±1.136^{a}	376.2±0.05
C.viviparum	72.91±1.04^{d}	41.69±0.003	47.90±0.576^{c}	108.5±0.07
C.lorifolium	70.26±1.03^{b}	46.06±0.075	38.04±3.322^{b}	153.47±0.03
Galantamine (Std)	97.33±1.014^{i}	0.569±0.013	-	-

Table 21: Percentage of acetylcholine esterase inhibition activity and IC50 value of methanolic extracts of bulbs and leaves of *Crinum* genotype



Graph 14. AChE inhibition (%) of bulb extracts of *Crinum* genotypes at 100 µg/mL for. Values are mean ± standard error (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p = 0.05



Graph 15. AChE inhibition (%) of leaf extracts of *Crinum* genotypes at 100 µg/mL for. Values are mean ± standard error (n=3). The superscripts with different letters indicate significantly different values based on Duncan's Multiple Range Test (DMRT) at p = 0.05

The acetylcholine esterase (AChE) inhibition activity of *Crinum* extracts was assessed using methanolic extracts of bulbs and leaves. The inhibition percentage at 100 µg/mL and the corresponding IC_{50} values are summarised in the table 21. Among the bulb extracts (Graph 14), CMGB exhibited the highest inhibition (86.47±1.06%) with a corresponding IC_{50} value of 9.20±0.017 µg/ml, followed closely by CMBD (84.97±0.35%, IC_{50} = 8.71±0.073 µg/ml). Conversely, *C. reddyii* showed the lowest inhibition (37.33±1.175%) with the highest IC_{50} value (170.42±0.05 µg/ml), indicating weak inhibitory activity.

The leaf extracts generally exhibited lower inhibitory activity than the corresponding bulb extracts (Graph 15). The highest inhibition was observed in CLTL (76.67±3.188%) with an IC_{50} value of 38.49±0.015 µg/ml, followed by CML (63.92±1.065%, IC_{50} = 27.77±0.051 µg/ml). The leaf extract of *C. reddyii* also exhibited the weakest inhibition (14.34±1.136%). The bulb extracts generally exhibited stronger inhibition, as seen in *C. malabaricum* (D) and *C. malabaricum* (G), both of which had IC_{50} values below 10 µg/ml. The IC_{50} values for most leaf extracts were significantly higher, reflecting their lower potency compared to bulb extracts.

4.4. Discussion

4.4.1. Phytochemical Diversity and Conservation Implications

Understanding the phytochemical constituents of a plant requiring conservation efforts is crucial, as it highlights both its ecological and economic significance, strengthening the case for its preservation. Bioprospecting plays a direct role in conservation by creating economic incentives for habitat protection and sustainable resource management of endangered species (Barrett & Lybbert, 2000; Rausser & Small, 2000). The present study investigated the phytochemical diversity and pharmacognostic potential of critically endangered *C. malabaricum* along with other six species of *Crinum* found in India.

GCMS analysis of the methanolic extract of bulb of *C. malabaricum* highlights a dynamic shift in the phytochemical profile between growth and dormancy, with increased fatty acids and esters during growth and a higher concentration of heterocyclic and alkaloidal compounds during dormancy. The presence of bioactive alkaloids in both phases supports the medicinal importance of *C. malabaricum*, reinforcing its potential for pharmaceutical applications and conservation efforts. The composition of phytocompounds varies between the growing and dormancy periods, indicating potential metabolic shifts. The growing period exhibits a diverse range of fatty acids, alkaloids, and esters, suggesting active metabolic biosynthesis. The dormancy period shows an increased presence of heterocyclic organic compounds, pyrazines, and aromatic aldehydes, indicating potential stress-related metabolic adaptations.

Common saturated fatty acids like myristic acid, palmitic acid, and stearic acid are present in both periods, indicating their stable biosynthesis. Linoleic acid and oleic acid (anti-inflammatory agents) are predominantly growing, suggesting their role in active growth and cellular functions. Several studies (Elaiyaraja & Chandramohan, 2018; Riji *et al.*, 2023) reported the abundance of compounds with antimicrobial activity, such as hexa-decanoic acid, oleic acid, and squalene, from other *Crinum* species. The dormancy period additionally contains Liriodendromine, an alkaloid with dopaminergic and antibacterial properties, suggesting enhanced secondary metabolite accumulation during dormancy. Fatty acid methyl esters (e.g., methyl linoleate) are detected in both periods, indicating their importance in oxidative stability and bioactivity. Several esters and aldehydes in both periods have applications in cosmetics, pharmaceuticals, and the food industry. Compounds like N-Formylmorpholine and Pyranone have antimicrobial and anticancer properties, which may contribute to the plant's ecological defence mechanisms.

Crinine is present in both periods, highlighting its significance in the species' medicinal potential. It was reported in several other species of *Crinum*. It is one of the compounds contributing to the acetylcholinesterase inhibitory potential of the genus. Studies also reported the detection of several other low-molecular-weighted alkaloids with similar inhibition potential using GCMS profiling (Berkov *et al.*, 2011; Tallini *et al.,* 2021).

Trisphaeridine ([1,3] Dioxolo [4,5-j] phenanthridine), a known anti-cancerous agent, is consistently detected in both stages. Other GCMS profiling studies also reported the presence of compounds in *Crinum* species with antiproliferative effects against tumour cell lines, indicating their potential in cancer treatment (Berkov *et al.*, 2011; Alawode & Lajide, 2024).

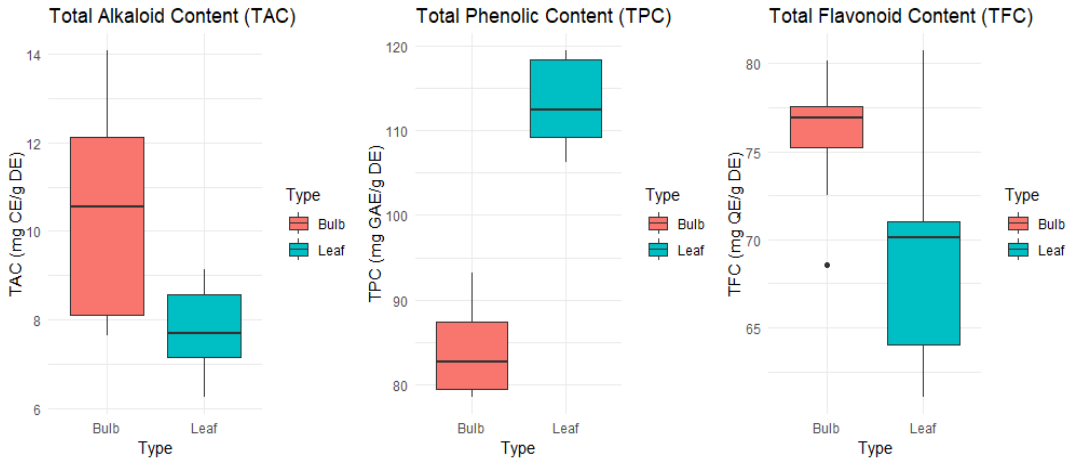
Gas Chromatography-Mass Spectrometry (GC-MS) profiling is a powerful analytical technique used to identify and quantify compounds from different plant species, which would help in accessing and harnessing the potential medicinal value of documented and most importantly unexplored plants like *C. malabaricum*.

4.4.2. Comparative Analysis of Phytochemical Constituents of *Crinum*

The phytochemical profiling of *C. malabaricum* and related species reveals notable variations in total antioxidant capacity (TAC), total phenolic content (TPC), and total flavonoid content (TFC) across different genotypes, plant parts, and physiological stages. These variations likely reflect metabolic adaptability, influenced by environmental stress responses, growth phases, and tissue-specific functional differentiation of metabolites (Elgorashi *et al.*, 2002; Moodley *et al.*, 2021).

Among the studied genotypes, *C. malabaricum* exhibits a moderate TPC compared to other *Crinum* species. Ghane *et al.* (2018) reported the highest TPC in *C. asiaticum* (86.18 ± 0.61 mg TAE/g extract), while the present study records a comparable phenolic content in *C. malabaricum* (107.38 ± 0.02 mg GAE/g extract), reaffirming its phytochemical richness. Prior studies on *C. bulbispermum* and *C. ornatum* (Adewusi & Steenkamp, 2011; Mohammed *et al.*, 2014) reported significantly higher TPC values (202.38 mg/g and 271.5 mg/g, respectively), primarily in root extracts. In *C. malabaricum*, root extracts also exhibit moderate phenolic levels (Lekhak *et al.*, 2022).

A distinct variation is observed between leaf and bulb extracts, where leaves consistently show higher TPC than bulbs across *Crinum* species, a trend previously reported by Ghane *et al.* (2018). However, in *C. malabaricum*, bulb extracts exhibit higher TAC than leaves, suggesting a greater concentration of bioactive compounds in underground storage organs. This trend is also reflected in TFC, where bulbs (CMGB: 76.70 mg QE/g extract; CMDB: 77.17 mg QE/g extract) surpass leaves (CML: 62.74 mg QE/g extract) (Graph 16). The higher phenolic and flavonoid content in bulbs suggests a role in protective storage mechanisms and long-term metabolic defence, as bulbs serve as critical survival structures in seasonal environments.



Graph 16: Phytochemical variations in bulbs and leaves of *Crinum* spp.: Total Phenolic Content (TPC) is highest in leaves, whereas Total Alkaloid Content (TAC) and Total Flavonoid Content (TFC) are higher in bulbs

Physiological stages significantly influence phytochemical properties in *C. malabaricum*, impacting TAC, TPC, TFC, GC-MS profiling, and alkaloid concentrations. Dormancy is associated with greater phenolic accumulation, likely as a defence against oxidative stress and microbial threats. TPC is higher during dormancy (91.58 ± 0.02 mg GAE/g extract) compared to the growing stage (84.25 ± 0.02 mg GAE/g extract), indicating a physiological regulation of phenolic biosynthesis. Similarly, TAC and TFC follow a comparable trend, with CMDB (dormant bulb) exhibiting higher TAC (12.07 mg LE/g extract) than CMGB (growing bulb: 10.82 mg LE/g extract). This growth stage decline suggests resource allocation towards active metabolism and development rather than chemical defence.

The total alkaloid content (TAC) in *C. malabaricum* varies across plant parts and physiological stages, reflecting species-specific metabolic adaptations. The highest alkaloid concentration is found in the dormant-stage bulb extract (CMDB), with galantamine at 142.75 ± 0.05 µg/g extract and lycorine at 30.94 ± 0.03 mg/g extract.

In contrast, the growing-stage bulb (CMGB) maintains a similar galantamine level (140.46 ± 0.03 µg/g) but shows a significantly lower lycorine concentration (14.24 ± 0.50 mg/g). This suggests that alkaloid biosynthesis, particularly lycorine accumulation, may be developmentally regulated.

Comparing *C. malabaricum* to other *Crinum* species, *C. latifolium* bulb (CLTB) exhibits the highest galantamine content (960.25 ± 0.50 µg/g), followed by its leaf extract (CLTL: 396.93 ± 0.33 µg/g). Meanwhile, *C. asiaticum* and *C. solapurense* show moderate TAC values. However, in terms of lycorine, *C. malabaricum* (CMDB) surpasses most species except *C. reddyi* leaf (CRL: 30.74 ± 0.03 mg/g), indicating its potential as a lycorine-rich source. Bulb extracts generally contain higher galantamine concentrations than leaf extracts, except in *C. latifolium* leaves (CLTL), which show relatively high levels. Conversely, lycorine is more abundant in the leaf extracts of *C. malabaricum*. The absence of galantamine in *C. reddyi* bulbs (CRB) highlights potential species-specific variations in alkaloid biosynthesis.

While phenolic compounds significantly contribute to antioxidant capacity, alkaloids such as galantamine and lycorine provide additional pharmacological relevance. The moderate TPC in *C. malabaricum* suggests a balanced secondary metabolite profile, supporting both antioxidant and neuroprotective properties. The observed variations in TAC and TPC across species and physiological stages highlight the complex interplay of genetic and environmental factors regulating phytochemical biosynthesis.

These findings emphasize the importance of tissue type and developmental stage in determining phytochemical richness in *C. malabaricum*. The relatively lower flavonoid content in leaves but higher TAC in bulbs suggests distinct functional roles of plant parts in metabolic adaptation. Given the conservation priority of *C. malabaricum*, understanding these variations is essential for optimizing its sustainable use, particularly for medicinal applications. The bulb's superior phytochemical profile, especially during dormancy, highlights its potential as a bioactive compound source, while the moderate antioxidant properties of leaves may contribute to ecological stress adaptation.

Furthermore, *Crinum* species contain phytochemicals with established applications in medicine, cosmetics, and the food industry due to their antioxidant, anti-inflammatory, and antimicrobial properties. These findings reinforce the medicinal relevance of *C. malabaricum*, particularly its bulb extracts, for pharmaceutical applications.

4.4.3. Antioxidant Assay: Correlation with Phytochemical Composition

Antioxidants are compounds that inhibit oxidation, a chemical reaction that can produce free radicals leading to cellular damage. They have the ability to chelate metals, inhibit different enzymes, and scavenge free radicals. The antioxidant assays demonstrated that the extracts from different *Crinum* species exhibit varying degrees of free radical scavenging activity and reducing potential. The highest DPPH activity was recorded in *C. asiaticum* leaf, while *C. malabaricum* bulb showed the strongest ABTS scavenging activity. In terms of reducing power (FRAP), *C. viviparum* leaf exhibited the highest activity. The general trend suggests that leaf extracts possess higher reducing power, whereas bulb extracts are more effective in ABTS radical scavenging. These variations may be attributed to differences in phenolic, flavonoid, and alkaloid content among genotypes and plant parts.

Presence of phytochemicals like phenolic compounds and flavonoids contribute to antioxidant potential of plants (Rice-Evans, 1997; Pietta, 2000). The correlation analysis between antioxidant assays (DPPH, ABTS, FRAP) and phytochemical components (TPC, TFC) based on Pearsons's correlation coefficient in *C. malabaricum* extracts provides insights into the antioxidant potential and the role of phenolic and flavonoid compounds in scavenging free radicals (Figure 31).

DPPH - TPC: r= -0.156 (No correlation)
DPPH - TFC: r= 0.480 (Moderate correlation)
ABTS - TPC: r= -0.742 (Negative correlation)
ABTS - TFC: r= 0.924 (Positive correlation)
FRAP - TPC: r= 0.893 (Positive correlation)
FRAP - TFC: -0.689 (Negative correlation)

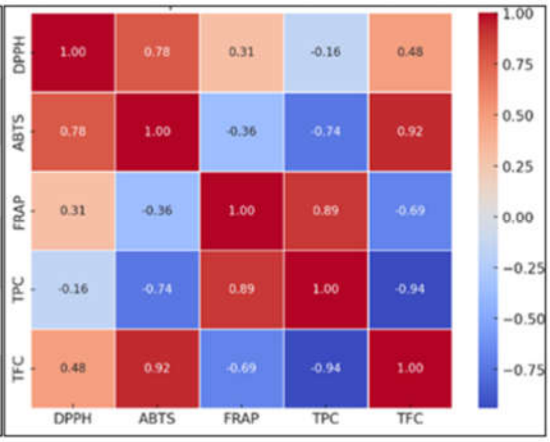


Figure 31. Correlation plot of antioxidant activity and phytochemical concentration in the methanolic extract of *Crinum malabaricum*. The values represent Pearson's correlation coefficient (r), with significance at $p < 0.05$

C. malabaricum exhibits a strong positive correlation between TPC and FRAP (0.89), suggesting that phenolic compounds contribute significantly to reducing power (FRAP). TFC shows a strong positive correlation with ABTS (0.92), suggesting that flavonoids play a significant role in ABTS radical scavenging in *C. malabaricum*. In other *Crinum* species, TPC and TFC generally show a strong correlation with DPPH and ABTS, indicating that polyphenolic compounds are major contributors to antioxidant activity. However, in *C. malabaricum*, the DPPH assay does not significantly correlate with TPC or TFC, suggesting that different phytochemical constituents (possibly alkaloids or non-phenolic compounds) may be involved in its antioxidant defence mechanism.

4.4.4. Principal Component Analysis

The Principal Component Analysis (PCA) provided an insightful dimensionality reduction approach to assess the relationship between antioxidant activity (DPPH, ABTS, FRAP) and phytochemical content (TAC, TPC, TFC) across different *Crinum* genotypes. The first two principal components (PC1 and PC2) accounted for a significant proportion of the total variance, enabling the differentiation of genotypes based on their biochemical profiles (Figure 32).

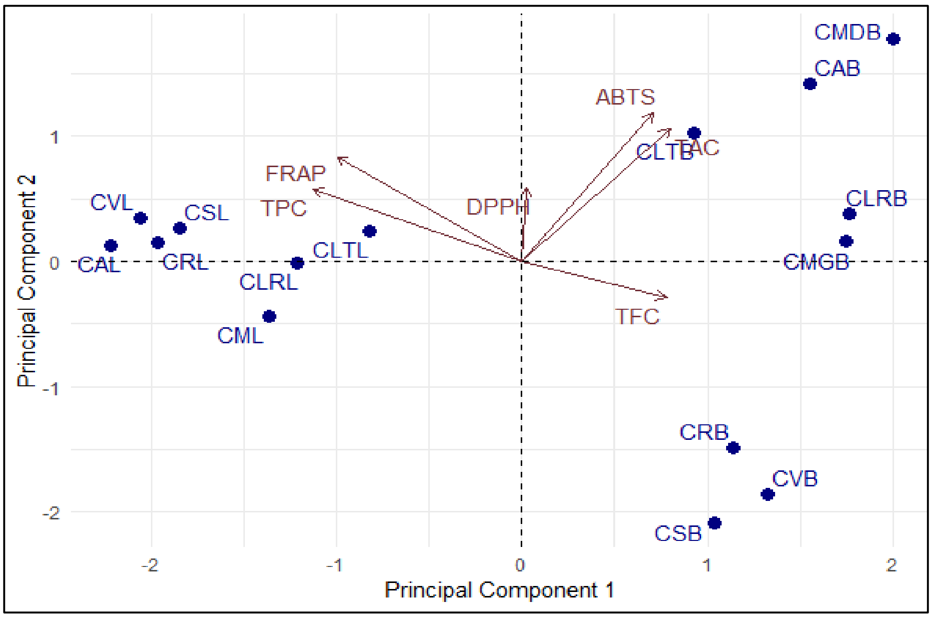


Figure 32. Principal components analysis (PCA) based on different phytochemical compounds analyzed in methanol extract of *Crinum* genotypes and their antioxidant activities (DPPH, ABTS and FRAP). TPC: total phenolics content; TFC: total flavonoid content, TAC: total alkaloid content

PC1 exhibited a high positive loading for total phenolic content (TPC) and total flavonoid content (TFC), suggesting that these phytochemicals contribute strongly to the variance observed in antioxidant activity. On the other hand, PC2 displayed a high correlation with FRAP values, indicating that iron-reducing potential may behave somewhat independently from free radical scavenging abilities (DPPH and ABTS). The PCA biplot revealed that genotypes with higher TPC and TFC content, such as CLTB, CRL, and CLRL, clustered towards the positive PC1 axis, suggesting a direct association between phenolic and flavonoid content with antioxidant activity. Conversely, genotypes such as CMGB and CSB, which exhibited lower antioxidant potential, were positioned towards the negative PC1 axis. The weaker correlation between ABTS and TPC/TFC, as indicated by the PCA loadings, suggests that different antioxidant assays may emphasize distinct antioxidant mechanisms.

The clustering pattern of genotypes within the PCA space demonstrated that certain genotypes exhibit similar biochemical compositions. For instance, CAB and CMDB formed a distinct cluster, indicating their higher antioxidant potential across multiple assays. In contrast, genotypes such as CVL and CML were positioned further away from this cluster, reflecting their relatively lower antioxidant activity and phytochemical content.

The differentiation of *Crinum* genotypes based on PCA highlights the biochemical diversity within the species, which has important implications for conservation strategies and potential pharmaceutical applications. The strong contribution of phenolic and flavonoid content to antioxidant activity reinforces their role in medicinal properties, particularly in neuroprotection and anti-inflammatory applications. Genotypes with higher antioxidant capacity may be prioritized for further pharmacological studies and conservation efforts to preserve their bioactive potential. Overall, the PCA analysis effectively summarized the relationships between antioxidant potential and phytochemical content, demonstrating that while total phenolic and flavonoid contents are key contributors to antioxidant activity, variations exist among different assays. These findings enhance our understanding of biochemical diversity of the genotypes of *Crinum* supporting targeted conservation and utilization strategies.

4.4.5. Acetylcholine Esterase Inhibition

Acetylcholinesterase (AChE) plays a crucial role in regulating neurotransmission by hydrolyzing acetylcholine (ACh), thereby terminating synaptic transmission. This enzymatic activity is essential for maintaining cholinergic signaling balance, which underpins cognitive functions such as memory and attention. Modulation of AChE activity has significant implications for cognitive health, particularly in neurodegenerative conditions such as Alzheimer's disease and mild cognitive impairment (Colović *et al.*, 2013; Padmor, 2024).

The bulb extract of *C. malabaricum* exhibits higher AChE inhibitory activity than the leaf extract. This corresponds to the higher alkaloid content, particularly galantamine concentration, in bulb extracts, as estimated by HPLC analysis. A similar trend is observed across the genus (Adewsusi and Steenkamp, 2011; Ghane *et al.*, 2018). Notably, the lowest AChE inhibition is exhibited by *C. reddyi* extracts, which lack detectable levels of galantamine in RP-HPLC analysis.

The AChE inhibitory activity of the bulb extract remains relatively stable across different physiological conditions, with minimal variation. However, the highest inhibition (lowest IC_{50} value) is observed during the dormancy phase, with an IC_{50} of 8.71 ± 0.073 µg/ml—making it the most potent AChE inhibitor among all studied genotypes. Adewsusi and Steenkamp (2011) reported that the ethyl acetate root extract of *Crinum* species had an IC_{50} value of 39.3 µg/ml, while the methanolic bulb extract showed 14.8 µg/ml, both of which are significantly higher than that of *C. malabaricum*. This study further supports that methanolic extraction is the most effective method for isolating AChE inhibitors.

Several studies have evaluated the AChE inhibition potential of *Crinum* species. Lekhak *et al.* (2022) reported the highest AChE inhibition in methanolic root extracts of *C. asiaticum* and aqueous root extracts of *C. malabaricum*. Ghane *et al.* (2018) demonstrated that methanolic leaf extracts of *C. latifolium* exhibited maximum AChE inhibition (70%), while *C. malabaricum* showed 41.25%. In contrast, our study reports a higher inhibition activity of *C. malabaricum* leaf extracts at 63.92 ± 1.065%. This variation may be attributed to differences in experimental conditions or extraction methods. The IC_{50} value of the *C. malabaricum* leaf extract (CML) was determined to be 27.77 ± 0.051 µg/ml, indicating its potential as a viable source of AChE inhibitors.

Chapter 5

Conservation Strategies for *C. malabaricum*

Chapter 5

Conservation Strategies for *C. malabaricum*

5.1. Ecological Niche Modelling and Species Translocation

5.1.1. Introduction

Species endemic to specific geographical ranges faces significant conservation challenges due to their restricted distribution and vulnerability to environmental changes. Understanding the ecological and evolutionary factors driving endemism is crucial for their conservation (Kruckeberg & Rabinowitz, 1985). One approach to mitigate the risks associated with endemism is understanding its causes and spreading the species beyond its occurrence through species translocation (a generic term that refers to transplanting plants for conservation purposes). This strategy ensures long-term population viability by maintaining genetic diversity and enhancing adaptive potential.

Species translocation can be categorized into four main concepts: augmentation (reinforcement of existing populations), reintroduction (restoring populations within their historical range), within-range introduction (establishing populations in areas with no historical records but within the species' natural range), and conservation introduction (introducing species beyond their known range, also termed assisted colonization) (Seddon, 2010; Godefroid *et al.,* 2011; Dalrymple *et al.,* 2012; IUCN, 2013). Identifying ecologically suitable landscapes for translocation requires integrating field surveys with predictive modelling approaches such as ecological niche modelling.

Ecological niche modelling (ENM), or habitat suitability modelling, is a computational approach that combines species occurrence data with environmental variables to predict potential species distributions (Hutchinson, 1957). ENM helps define the ecological niche of a species- the range of environmental conditions that support its survival and reproduction. By analysing species-environment relationships, ENM can estimate current habitat suitability and predict future distribution shifts in response to environmental changes (Drake *et al.,* 2006; Dolci & Peruzzi, 2022). This predictive capacity is essential for conservation planning, particularly for identifying and prioritizing areas for species translocation.

Ecological niche modelling utilizes various statistical, clustering, and machine-learning algorithms to estimate species distributions. Statistical methods such as generalised linear models (GLM) and generalised additive models (GAM) use logistic regression to correlate species presence with environmental predictors (McCullagh & Nelder, 1989; Hastie & Tibshirani, 1990; Guisan *et al.,* 2002). Clustering-based approaches, including kernel density estimation (KDE) and Marble (MA), assess niche structure by analyzing the density of presence points in environmental space (Blonder *et al.,* 2014; Qiao *et al.,* 2015). Machine-learning algorithms, such as genetic algorithms for rule-set prediction (GARP), boosted regression trees (BRT), and maximum entropy modeling (Maxent), provide flexible niche estimations with minimal assumptions (Stockwell, 1999; Phillips *et al.,* 2004, 2006; Elith *et al.,* 2008).

Among these, Maxent is one of the most influential and widely used machine-learning algorithms. It estimates species distributions by finding the probability distribution that maximizes entropy while remaining constrained by known presence data. Maxent is ideal for modelling species with limited distribution, as it can work with a few occurrence records (Phillips and Dudik, 2008; Elith *et al.,* 2011). In addition, Maxent modelling requires presence-only datasets. It applies regularisation to prevent overfitting and improve model generalisation (Merow *et al.*, 2013), making it effective for species distribution modelling under varying environmental conditions.

Effective conservation strategies require biological and ecological knowledge of a species and understanding its potential distribution in current and future environmental contexts (Pulparambil & Pradeep, 2013). ENM is a powerful tool in conservation planning, offering insights into species' physiological thresholds, land cover preferences, and environmental suitability. By integrating ecological parameters with remote sensing data, ENM predicts areas suitable for species establishment, particularly for critically endangered species (Adhikari *et al.,* 2019). Additionally, it aids in assessing habitat suitability under projected climate change scenarios, thereby informing long-term conservation strategies (Thuiller, 2024). ENM-guided translocation efforts help identify microsites with optimal survival conditions, improving the success of conservation initiatives (Elliott *et al.,* 2024). Combining predictive modelling with field-based habitat assessments allows conservationists to enhance species translocation while mitigating habitat degradation and climate change risks (Schwartz, 2012; Ashraf *et al.,* 2017).

To develop effective conservation measures for *Crinum malabaricum*, assessing its potential distribution across suitable phytogeographical regions is essential. This study employs Maxent modelling to predict habitat suitability for *C. malabaricum* across the laterite plateaus of the Malabar Plains in the Western Ghats. By identifying ecologically suitable habitats, this research aims to facilitate species translocation, mitigate the risks of endemism, and establish sustainable populations in favourable environments. Additionally, the study examines key environmental variables influencing the species' distribution and projects future range shifts under climate change scenarios. The integration of ENM with conservation strategies provides a scientific basis for decision-making, ensuring the long-term survival of *C. malabaricum* in changing environmental conditions.

5.1.2. Materials and Methods

5.1.2.1. Study Area

C. malabaricum is endemic to the seasonal streams on the laterite plateaus of northern Kerala. These laterite plateaus lie within the Malabar Plains of the Western Ghats (21.31° N - 8.09° N, 72.73° E - 77.5° E; Area ≈ 66,490 km^2). The Malabar Plains form the lowland region of the Western Ghats, bounded by the mountains of Western Ghats to the east and the Western Coastal Plains to the west. Due to their proximity to the Arabian Sea, these plains experience high humidity and heavy monsoon rainfall from June to September. The Malabar Plains extend from Tapi district in Gujarat to Kanyakumari district in Tamil Nadu (Figure 33).

The laterite plateaus of the Malabar Plains are unique landforms, remnants of ancient erosion surfaces that play a crucial role in the region's ecology, hydrology, and biodiversity. These plateaus are found in northern Kerala (Kannur, Kasaragod, Kozhikode, Malappuram, and parts of Palakkad districts), Karnataka (Uttara Kannada, Udupi, and Dakshina Kannada districts), as well as in Goa and Maharashtra (Sindhudurg and Ratnagiri districts).

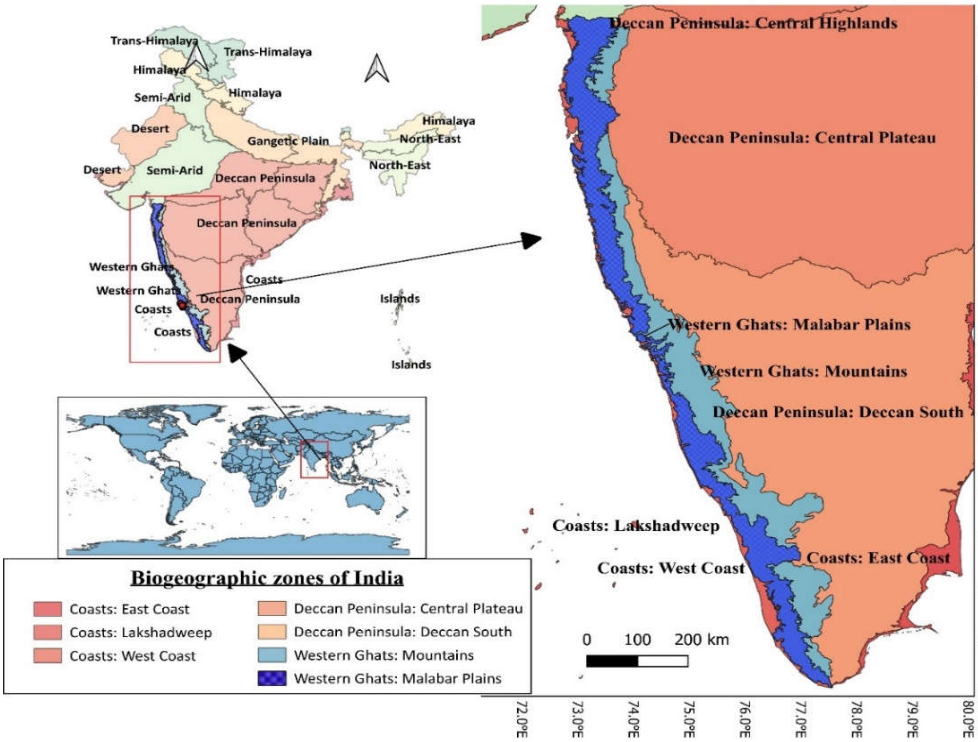


Figure 33. Biogeographic zones of India, highlighting the Malabar Plains of Western Ghats

Geologically, the laterite plateaus are composed primarily of laterite, a highly weathered rock rich in iron and aluminium, formed due to prolonged leaching under tropical humid conditions, which results in an iron-oxide-rich crust. The soil is porous, acidic, and nutrient-deficient, supporting a unique set of plant species adapted to harsh conditions. Despite heavy rainfall, high porosity leads to poor water retention, causing seasonal water scarcity. However, these plateaus act as groundwater recharge zones, influencing the hydrology of the surrounding lowland regions.

Topographically, these plateaus consist of elevated landforms rising 50–300 meters above sea level, interspersed with seasonal wetlands, rock outcrops, and ephemeral streams. They are often referred to as "sky islands" of the Malabar Plains, hosting rare and endemic flora highly adapted to seasonal water availability and lateritic soil. The region has a tropical climate, and the vegetation primarily depends on monsoon distribution.

5.1.2.2. Species Occurrence Data

The distribution data of *C. malabaricum* was recorded using GPS (Garmin Montana 360) from their natural habitats in the Kannur and Kasaragod districts of Kerala during the field survey (June to November 2021) (Table 22).

Sl.No.	Habitat	District	Latitude	Longitude
1.	Aravanchalkavu, Aravanchal	Kannur	12° 12' 32.00"N	75° 17' 0.42"E
2.	Nedumkunnu, Aravanchal	Kannur	12° 12' 54.32"N	75° 16' 53.65"E
3.	Pukkal, Aravanchal	Kannur	12° 13' 13.30"N	75° 17' 5.46"E
4.	Mudikkanam, Embate	Kannur	12° 4' 21.04"N	75° 18' 5.00"E
5.	Deavamatha, Embate	Kannur	12° 4' 30.72"N	75° 18' 32.26"E
6.	Pariyaram, Embate	Kannur	12° 4' 41.30"N	75° 18' 40.68"E
7.	Nalakra, Periye	Kasaragod	12° 24' 41.69"N	75° 6' 46.62"E
8.	Periyanam, Periye	Kasaragod	12° 24' 15.44"N	75° 6' 23.62"E
9.	Niduvottupara, Periye	Kasaragod	12° 24' 24.30"N	75° 6' 55.76"E
10.	Mooriyanam, Periye	Kasaragod	12° 24' 33.12"N	75° 6' 43.42"E
11.	Thottuvally, Cheemeni	Kasaragod	12° 13' 31.26"N	75° 14' 6.47"E
12.	Kankole-Cheemeni	Kasaragod	12° 13' 25.68"N	75° 13' 59.81"E

Table 22: Geographical details of the populations of *Crinum malabaricum*

Prepared the species presence data in CSV (comma-delimited) format using MS Excel. Out of 23 occurrence points, 11 were selected for the modelling by removing the duplicate presence points in the same grid in the study area (within 1 Km^2 area), leaving only one occurrence point per cell (Figure 34).

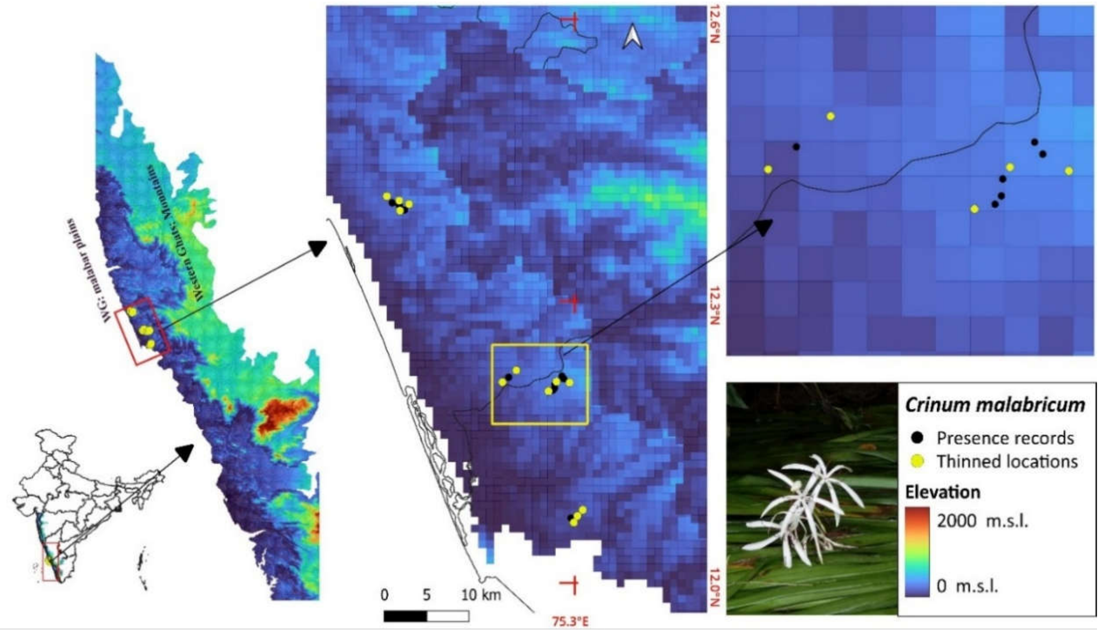


Figure 34. Locations of *Crinum malabaricum* used for ENM. Presence locations are thinned by leaving only one occurrence point per cell

5.1.2.3. Environmental Data

5.1.2.3.1. Collection of Environmental Variables

The 19 standard bioclimatic variables representing current climatic conditions were obtained from the WorldClim database (https://www.worldclim.org/). These variables provide long-term climatic averages for the period 1970–2000, with a spatial resolution of 30-arc seconds (~1 km²). They were derived from monthly climate data, including minimum and maximum temperatures as well as precipitation patterns (Fick & Hijmans, 2017). These bioclimatic variables are widely used in ecological and species distribution modeling, as they capture critical aspects of climate, such as temperature seasonality, annual precipitation, and temperature extremes, which influence species distribution and habitat suitability.

In addition to the bioclimatic variables, two topographical factors—elevation and soil type were included (Table 23). Elevation data were sourced from the USGS EROS Archive, which provides high-resolution digital elevation models, ensuring a consistent resolution of 30-arc seconds (~1 km²). The soil type data were acquired from the UN Food and Agriculture Organisation (FAO) database (FAO-UNESCO, 1977), a globally recognized dataset that classifies soil properties based on their physical and chemical characteristics. To ensure consistency across datasets, all environmental and topographical raster layers were re-sampled and clipped to match the spatial extent and resolution of the study area. These preprocessing steps were performed using QGIS version 3.28.4 (QGIS Development Team, 2022).

Code	Variable Description	Unit	Type	Source
Bio1	Annual Mean Temperature	°C	Continuous	WorldClime
Bio2	Mean Diurnal Range	°C	Continous	WorldClime
Bio3	Isothermality (Bio2/Bio7) (100)	Percent	Continous	WorldClime
Bio4	Temperature Seasonality (sd 100)	°C	Continous	WorldClime
Bio5	Max. Temperature of Warmest Month	°C	Continous	WorldClime
Bio6	Min. Temperature of Coldest Month	°C	Continous	WorldClime
Bio7	Temperature Annual Range	°C	Continous	WorldClime
Bio8	Mean Temperature of Wettest Quarter	°C	Continous	WorldClime
Bio9	Mean Temperature of Driest Quarter	°C	Continous	WorldClime
Bio10	Mean Temperature of Warmest Quarter	°C	Continous	WorldClime
Bio11	Mean Temperature of Coldest Quarter	°C	Continous	WorldClime
Bio12	Annual Precipitation	Mm	Continous	WorldClime
Bio13	Precipitation of Wettest Month	Mm	Continous	WorldClime
Bio14	Precipitation of Driest Month	Mm	Continous	WorldClime
Bio15	Precipitation Seasonality (CV)	Percent	Continous	WorldClime
Bio16	Precipitation of Wettest Quarter	Mm	Continous	WorldClime
Bio17	Precipitation of Driest Quarter	Mm	Continous	WorldClime
Bio18	Precipitation of Warmest Quarter	Mm	Continous	WorldClime
Bio19	Precipitation of Coldest Quarter	Mm	Continous	WorldClime
Elevation	Topographic Elevation	msl	Continous	USGS
Soil	Type of Soil	-	Categorical	FAO

Table 23. Details of the environmental variables employed in maxent ENM of *Crinum malabaricum* in the study area

5.1.2.3.2. Selection of Variables

In this modelling, variables are selected so that highly correlated variables are not used simultaneously to avoid multicollinearity in the model. Pearson's correlation coefficient (r) was calculated to select variables, and a threshold of r = ±0.8 was chosen to remove variables (Xu *et al.,* 2019). In addition, the permutation importance of each variable (>3%) was also considered, which was analyzed from the Maxent model run with 21 variables using the default setting. Between correlated variables, the one with the highest permutation importance was chosen. The permutation approach idea is that the training performance would degrade if the values of an essential predictor in the training data were randomly permuted (Greenwell, 2020). Seven variables were selected for this study, including two precipitation-based variables and five temperature-based variables.

5.1.2.4. Ecological Niche Modelling

5.1.2.4.1. Model Construction and Estimations

This study employed the maximum entropy approach to construct an ecological niche model. For this, the R package ENMTools version 1.0.6 (Warren *et al.,* 2021) and the modelling software Maxent Version 3.4.4. (Phillips *et al.,* 2006) were used (Figure 35). Maxent uses georeferenced occurrence records and grid-based environmental variables to apply machine learning to predict the habitat suitability of the species in each grid within its distributional range.

In Maxent, the model complexity was controlled by adjusting two parameters: model feature combinations (FC) and regularisation multiplier (RM). Using the default parameters may overfit the model prediction (Warren *et al.,* 2011). Regularization is crucial to control overfitting. It is essential to use suitable regularization multipliers and feature combinations to obtain the optimal model in Maxent for different species and research goals (Phillips, 2005). Maxent uses a set of features like linear (L), hinge (H), quadratic (Q), threshold (T), and product (P), that help to determine the predicted probability of presence or environmental suitability of the study area based on the site's environmental values. R packages “dismo” and “SDMtune” are used to analyse FC and RM values for optimum Maxent model. The combinations of feature classes L, H, LH, LQ, LQH, LQHP, and LQHPT and the regularisation multiplier ranging from 0.5 to 5 with a difference of 0.5 were used. To reduce the sampling bias, a bias file was created using R package “ENMeval” and used in the modelling. Maxent modelling was performed using the combination LQPTH features and a regularisation multiplier of 1. Adjust the model settings to 10000 randomly selected background points as pseudo-absence in the studied area. The cross-validation test was done with 10 replications (*k*-fold cross-validation) and 5000 iterations. The logistic output was generated with an estimation of the habitat suitability (Habitat suitability index; HSI) of *C. malabaricum* that ranged between 0 (unsuitable) and 1 (the most suitable). The area was calculated from the HSI using DIV-GIS version 7.5.0 (Hijmans, *et al.,* 2012).

5.1.2.4.2. Assessment of Model Performance

The model's performance was assessed based on five measures; AUC, TSS, p-ROC-AUC, AUC ratios, and Kappa statistics (Khan *et al.,* 2022). True skill statistics (TSS) is a threshold-dependent metric whose value ranges from -1 to +1 and a TSS value > 0.6 indicates good model prediction (Allouche *et al.,* 2006). The area under the receiver operating curve (AUC) metric value ranges from 0 to 1, where a value less than 0.5 indicates poor model prediction and a value closer to 1.0 indicates robust model prediction (Phillips *et al.,* 2008). As suggested by Lobo *et al.* (2006); Peterson *et al.* (2008); and Leroy *et al.* (2018), considering AUC and TSS alone for assessing a predictive model may leads to error. To eliminate the possible error using the AUC value and TSS we calculated partial-area ROC (P-ROC AUC) to estimate the predictive accuracy of the maximum entropy model using "pROC" R package (Robin *et al.,* 2011). Also, AUC ratio was calculated as suggested by Gao *et al.* (2022). Kappa and TSS are calculated using the "evaluate" function in the "ENMeval" R package (Muscarella *et al.,* 2014).

5.1.2.4.3. Analysing the Variable Importance

To compute the contribution of each variable in the modelling a Jack-knife test was conducted. It is a resampling method that is used to estimate variance and bias. Jack-knife estimation is done by excluding each observation one by one from a set of data, finding its estimate, and then calculating the average. The output of Jack-knife is a bar graph showing the AUC of the model with all variables and the AUC of the model with and without each variable (Burch *et al.,* 2020).

5.1.2.4.4. Habitat Status Assessment and Species Translocation

To validate the model, niche suitability prediction maps indicating the suitability index were converted to KMZ format using QGIS version 3.28 (QGIS Development Team, 2022). These KMZ files were overlaid on Google Earth Ver. 7.3.6 (www.google.com/earth) to assess actual habitat conditions (Adhikari *et al.,* 2012). Additionally, QGIS Open Street Map (OpenStreetMap contributors, 2024) was used to analyse terrain features and identify potential streams in suitable areas. A suitability threshold of 0.85 and above was considered for field observations.

Based on the model output, repeated field surveys were conducted across highly suitable predicted areas to verify habitat suitability. Prior knowledge of the species' habitat preferences was utilized to locate potential new habitats (Souravi, 2017). Study sites were selected based on the presence of at least one stream, as identified using QGIS OpenStreetMap and Google Earth Pro (Google Earth Pro, 2024). The selected areas required Nitisol soil; a substrate favourable for the species.

Streambed characteristics were assessed to ensure the presence of gravel with fine grit and small to medium-sized laterite stones. The streams needed to have a well-defined middle course, with a minimum extent of 50 meters, a width of at least 2 meters, and a depth ranging between 0.5 and 1 meter. Vegetation analysis confirmed the absence of dominant competing vegetation within the streams.

The seasonal streams identified through field surveys, exhibiting ecological conditions similar to those of the natural habitats of *C. malabaricum*, were selected for species translocation. A total of 30 bulbils of *C. malabaricum* were planted at these sites during the summer (April–May 2022) and monitored throughout the subsequent monsoon season to assess their growth. Observations continued into the following growing seasons in 2023 and 2024 to evaluate their long-term responses and establishment success.

5.1.2.5. Future Climate Projection of the Model

Eight future climatic scenarios were analysed to project future habitat suitability under predicted climate change scenarios. Downscaled future climate projections from the

Coupled Model Intercomparison Project, Phase 6 (CMIP6), specifically the ACCESS-CM2 global climate model (GCM), were used. The projections covered two future periods: the 2050s (2041–2060) and the 2070s (2061–2080). Four Shared Socio-economic Pathways (SSPs) were considered to account for different climate change trajectories: SSP1-2.6, SSP2-4.5, SSP3-7.0, and SSP5-8.5. These future climate datasets, with a spatial resolution of 30 arc seconds, were obtained from the WorldClim dataset available at https://www.worldclim.org/data/cmip6.html (Fick & Hijmans, 2017). The climatic layers were then resampled to match the extent of the study area. The predicted variations in habitat suitability under future climate conditions were estimated and compared to the current climate model to assess potential shifts in suitable habitats.

5.1.2.6. Outline of the Work

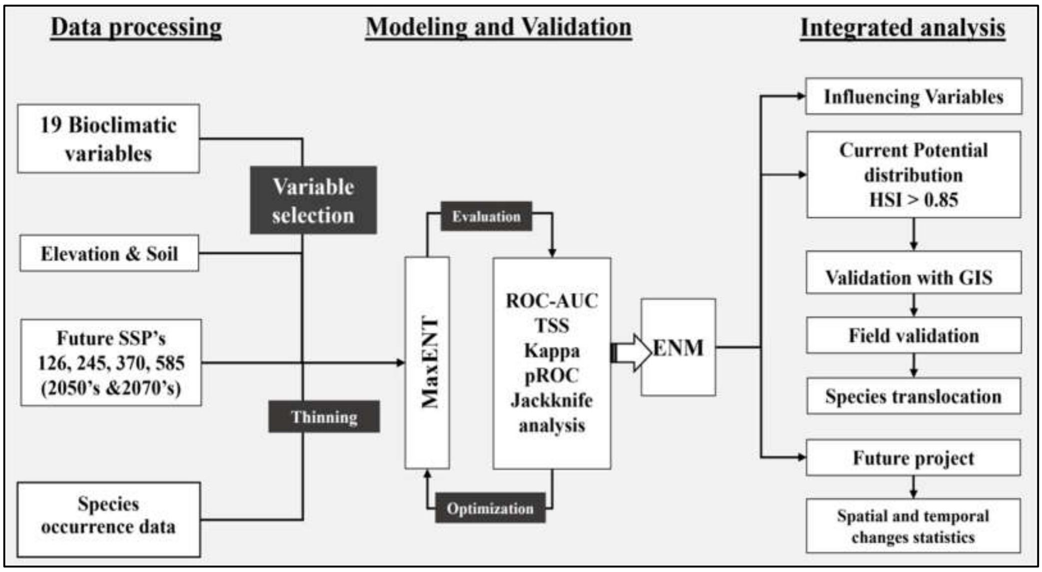
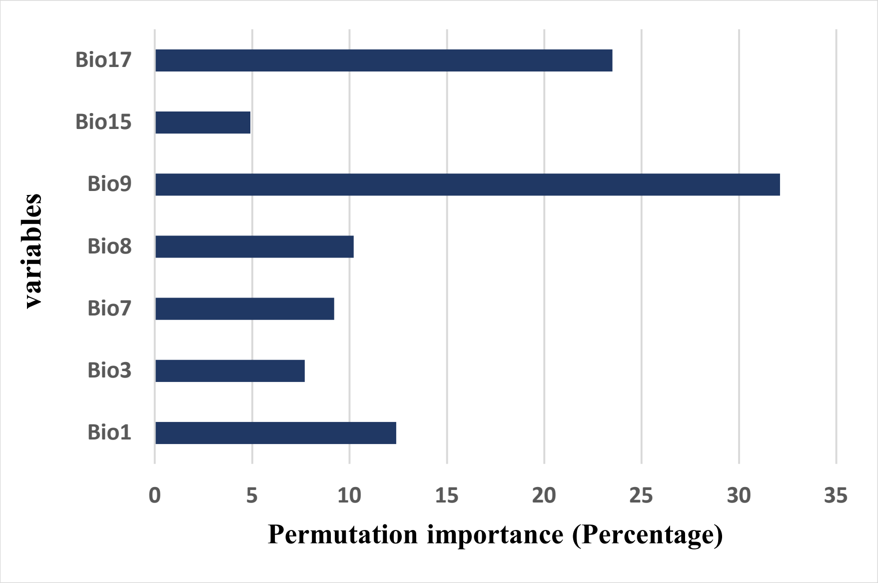


Figure 35. Workflow of ecological niche modelling and species translocation

5.1.3. Results

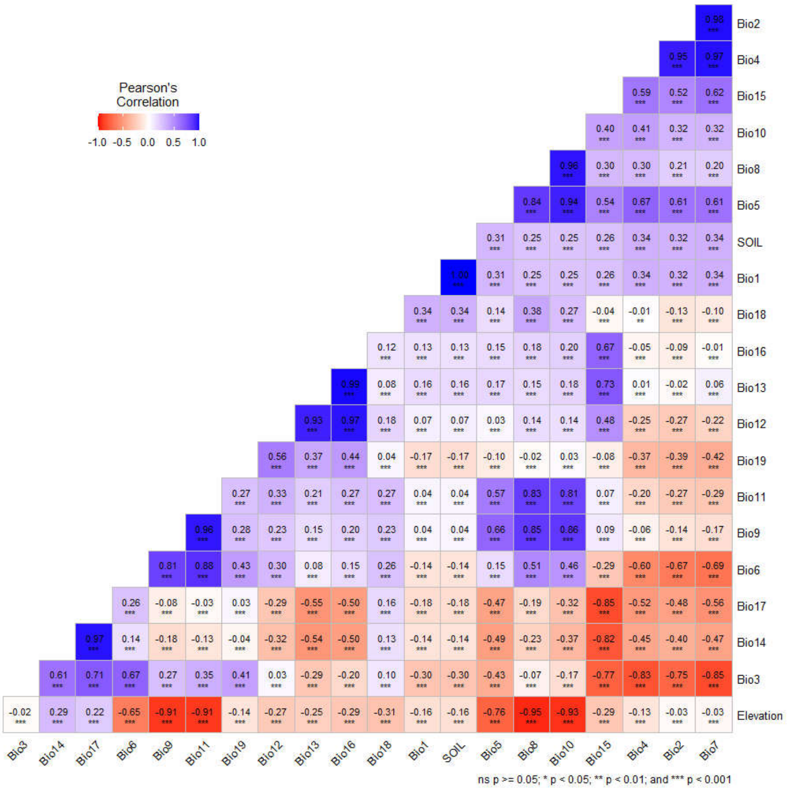
5.1.3.1. Selection of Variables

Seven variables were chosen from a pool of 19 bioclimatic and two topographic variables following a variable selection process. The variables that met the selection criteria were annual mean temperature (Bio1), isothermality (Bio3), temperature annual range (Bio7), mean temperature of the wettest quarter (Bio8), mean temperature of the driest quarter (Bio9), precipitation seasonality (Bio15), and precipitation of the driest quarter (Bio17). In the initial analysis, Bio9 exhibited the highest permutation importance, followed by Bio1, Bio17, and Bio7. Retained these variables, while the variables showing collinearity with them were eliminated. Although Bio14 (precipitation of the driest month) demonstrated significant permutation importance (7.2%), it was excluded due to its strong collinearity with Bio7 (97%) (Figure 36).



Graph 17: Permutation importance of the variables using AUC metric. The permutation importance is presented as a percentage

Other variables without collinearity issues and with permutation importance, viz., Bio3, Bio8, and Bio15, were included, while those without significant permutation importance were excluded. The topographic variables, Elevation and soil type, were excluded from the analysis due to their lack of permutation importance. Additionally, Elevation exhibited collinearity with Bio9 and Bio8, while soil type showed collinearity with Bio1. The permutation importance of the selected variables in the final model is depicted in graph 17, illustrating their relevance after undergoing the variable selection process.



Bio3	Bio14	Bio17	Bio6	Bio9	Bio11	Bio19	Bio12	Bio13	Bio16	Bio18	Bio1	SOIL	Bio5	Bio8	Bio10	Bio15	Bio4	Bio2	Bio7	
																			0.98 ***	Bio2
																		0.95 ***	0.97 ***	Bio4
																	0.59 ***	0.52 ***	0.62 ***	Bio15
																0.40 ***	0.41 ***	0.32 ***	0.32 ***	Bio10
															0.96 ***	0.30 ***	0.30 ***	0.21 ***	0.20 ***	Bio8
														0.84 ***	0.94 ***	0.54 ***	0.67 ***	0.61 ***	0.61 ***	Bio5
													0.31 ***	0.25 ***	0.25 ***	0.26 ***	0.34 ***	0.32 ***	0.34 ***	SOIL
												1.00 ***	0.31 ***	0.25 ***	0.25 ***	0.26 ***	0.34 ***	0.32 ***	0.34 ***	Bio1
											0.34 ***	0.34 ***	0.14 ***	0.38 ***	0.27 ***	-0.04 ***	-0.01 **	-0.13 ***	-0.10 ***	Bio18
										0.12 ***	0.13 ***	0.13 ***	0.15 ***	0.18 ***	0.20 ***	0.67 ***	-0.05 ***	-0.09 ***	-0.01 ***	Bio16
									0.99 ***	0.08 ***	0.16 ***	0.16 ***	0.17 ***	0.15 ***	0.18 ***	0.73 ***	0.01 ***	-0.02 ***	0.06 ***	Bio13
								0.93 ***	0.97 ***	0.18 ***	0.07 ***	0.07 ***	0.03 ***	0.14 ***	0.14 ***	0.48 ***	-0.25 ***	-0.27 ***	-0.22 ***	Bio12
							0.56 ***	0.37 ***	0.44 ***	0.04 ***	-0.17 ***	-0.17 ***	-0.10 ***	-0.02 ***	0.03 ***	-0.08 ***	-0.37 ***	-0.39 ***	-0.42 ***	Bio19
						0.27 ***	0.33 ***	0.21 ***	0.27 ***	0.27 ***	0.04 ***	0.04 ***	0.57 ***	0.83 ***	0.81 ***	0.07 ***	-0.20 ***	-0.27 ***	-0.29 ***	Bio11
					0.96 ***	0.28 ***	0.23 ***	0.15 ***	0.20 ***	0.23 ***	0.04 ***	0.04 ***	0.66 ***	0.85 ***	0.86 ***	0.09 ***	-0.06 ***	-0.14 ***	-0.17 ***	Bio9
				0.81 ***	0.88 ***	0.43 ***	0.30 ***	0.08 ***	0.15 ***	0.26 ***	-0.14 ***	-0.14 ***	0.15 ***	0.51 ***	0.46 ***	-0.29 ***	-0.60 ***	-0.67 ***	-0.69 ***	Bio6
			0.26 ***	-0.08 ***	-0.03 ***	0.03 ***	-0.29 ***	-0.55 ***	-0.50 ***	0.16 ***	-0.18 ***	-0.18 ***	-0.47 ***	-0.19 ***	-0.32 ***	-0.85 ***	-0.52 ***	-0.48 ***	-0.56 ***	Bio17
		0.97 ***	0.14 ***	-0.18 ***	-0.13 ***	-0.04 ***	-0.32 ***	-0.54 ***	-0.50 ***	0.13 ***	-0.14 ***	-0.14 ***	-0.49 ***	-0.23 ***	-0.37 ***	-0.82 ***	-0.45 ***	-0.40 ***	-0.47 ***	Bio14
	0.61 ***	0.71 ***	0.67 ***	0.27 ***	0.35 ***	0.41 ***	0.03 ***	-0.29 ***	-0.20 ***	0.10 ***	-0.30 ***	-0.30 ***	-0.43 ***	-0.07 ***	-0.17 ***	-0.77 ***	-0.83 ***	-0.75 ***	-0.85 ***	Bio3
-0.02 ***	0.29 ***	0.22 ***	-0.65 ***	-0.91 ***	-0.91 ***	-0.14 ***	-0.27 ***	-0.25 ***	-0.29 ***	-0.31 ***	-0.16 ***	-0.16 ***	-0.76 ***	-0.95 ***	-0.93 ***	-0.29 ***	-0.13 ***	-0.03 ***	-0.03 ***	Elevation

ns p >= 0.05; * p < 0.05; ** p < 0.01; and *** p < 0.001

Figure 36. Correlation chart of the variables considered in this study. The red-to-blue bar represents the correlation coefficient between variables, and the values in the squares are Pearson's correlation coefficient (r)

5.1.3.2. Validation of Model Performance

Model performance was significantly changed with different feature combinations and regularization multiplier settings. Combination of feature class LQPTH and a regularisation multiplier of 1 were employed, leading to a high-performing model. In this study, the ten replicate runs of the model yielded an average train and test AUC metrics of 0.996 ± 0.0002 and 0.998 ± 0.002 respectively. In addition, this study recorded the model accuracy values of 0.9215, 0.892, 1.63, and 0.82 for TSS, Kappa statistics, AUC ratio and p-AUC-ROC, respectively (Table 24).

Metrices	**Train AUC**	**Test AUC**	**TSS**	**Kappa**	**AUC Ratio**	**pROC-AUC**
Value	0.905	0.901	0.9215	0.892	1.63	0.82

Table 24. Average value train and test AUC, TSS, Kappa statics, AUC ratio and partial ROC-AUC of ten replicates of the mode. These results indicate strong model performance, demonstrating the effectiveness of the chosen features and regularisation settings

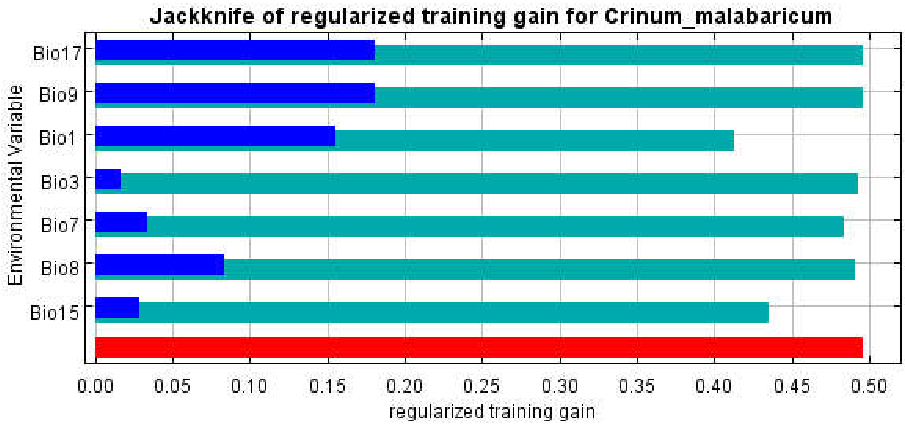
5.1.3.3. Contribution of Environmental Variables

The Jack-knife analysis was conducted to understand the importance of environmental variables on the distribution of *C. malabaricum.* The longer blue bar shows the influence of the variable to the species distribution, and the shorter green bar indicates that the variable has more information than others do (Graph 18).

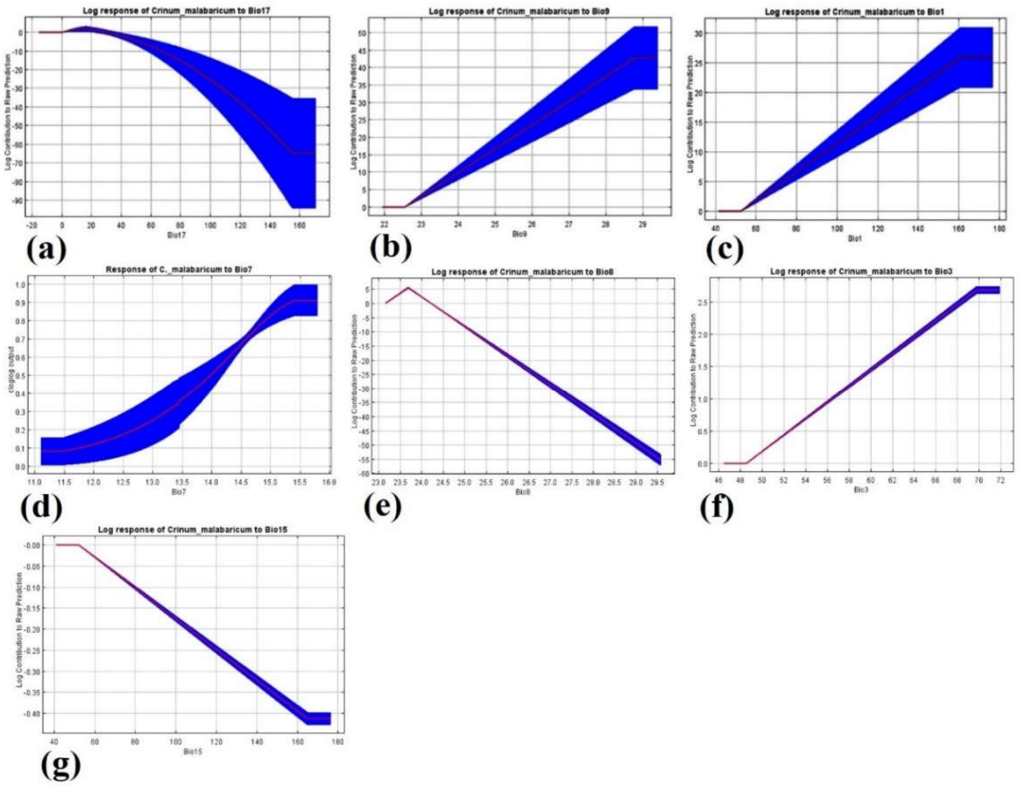
The current habitat prediction model of *C. malabaricum* highly depends on the variable Bio 17 (precipitation of the driest quarter) with 35.6% percent contribution. Followed by Bio 9 (mean temperature of the driest quarter, 24.6%) and Bio1 (annual mean temperature, 14%) (Table 25).

Code	**Variable**	**Percent Contribution**
Bio 17	Precipitation of the driest quarter	35.6
Bio 9	Mean temperature of the driest quarter	24.6
Bio 1	Annual mean temperature	14
Bio 7	Temperature annual range	10.3
Bio 8	Mean temperature of the wettest quarter	9.7
Bio 3	Isothermality	3.7
Bio 15	Precipitation seasonality	2.1

Table 25. Percent contribution of the environmental variables in the ecological niche modelling of *C. malabaricum* in the study area



Graph 18. Jack-knife analysis of regularized training gain showing the contribution of each bioclimatic variable. Light blue bars indicate the training gain when the corresponding variable is excluded, dark blue bars show the gain when the model is run using only that variable, and the red bar indicates the gain when all variables are included



Graph 19. The relationship of environmental variables in this model; a. Bio17: Precipitation of the driest quarter; b. Bio9: Mean temperature of the driest quarter; c. Bio1: Annual mean temperature; d. Bio7: Temperature annual range; e. Mean temperature of the wettest quarter; f. Bio3: Isothermality; g. Bio15: Precipitation seasonality

Graph 19 shows the relationship of these variables with the habitat suitability index. The environmental variable that exhibits the highest gain when used in isolation is Bio17. In addition, when Bio17 is omitted, it results in the most significant decrease in gain compared to the other variables. This suggests that Bio17 contains unique information not present in the different variables, making it particularly informative in the analysis.

5.1.3.4. Potential Habitat Suitability Prediction and Species Translocation

The predicted map of the model shows that the suitable habitats are in the Kannur, Kasaragod, and Palakkad districts of Kerala; the Uttara Kannada, Udupi, and Dakshina Kannada districts of Karnataka; the Sindhudurg district of Maharashtra; and the Surat district of Gujarat (Figure 37). Most of the predicted area lies within the Nitisol-distributed area, except in Surat. The total predicted area of the highly suitable region was 2393 Km^2 (3.6 % of the study area). The areas predicted under medium and low suitable regions were 10771 Km^2 (16.2%) and 8178 Km^2 (12.3%), respectively. The remaining area (~70%) was found unsuitable. The extent of occurrence (EOO) of *C. malabaricum* was estimated as 283 Km^2 based on the standard method suggested by IUCN. This indicates that, under the current climatic conditions, the predicted highly suitable habitat area (2393 km^2) is significantly larger than the EOO of the species.

The selection of potential areas for the translocation of the species considered the presence of laterite streams. This determination was made based on observations made on the Q-GIS open street map and a comparison with the terrain of the natural habitats of *C. malabaricum*. Similar seasonal streams were identified in areas with high-predicted suitability (Suitability index ≥ 0.85), including Sindhudurg district in Maharashtra, Uttara Kannada, Udupi and Dakshina Kannada districts in Karnataka, and Kasaragod and Kannur districts in Kerala (Table 26).

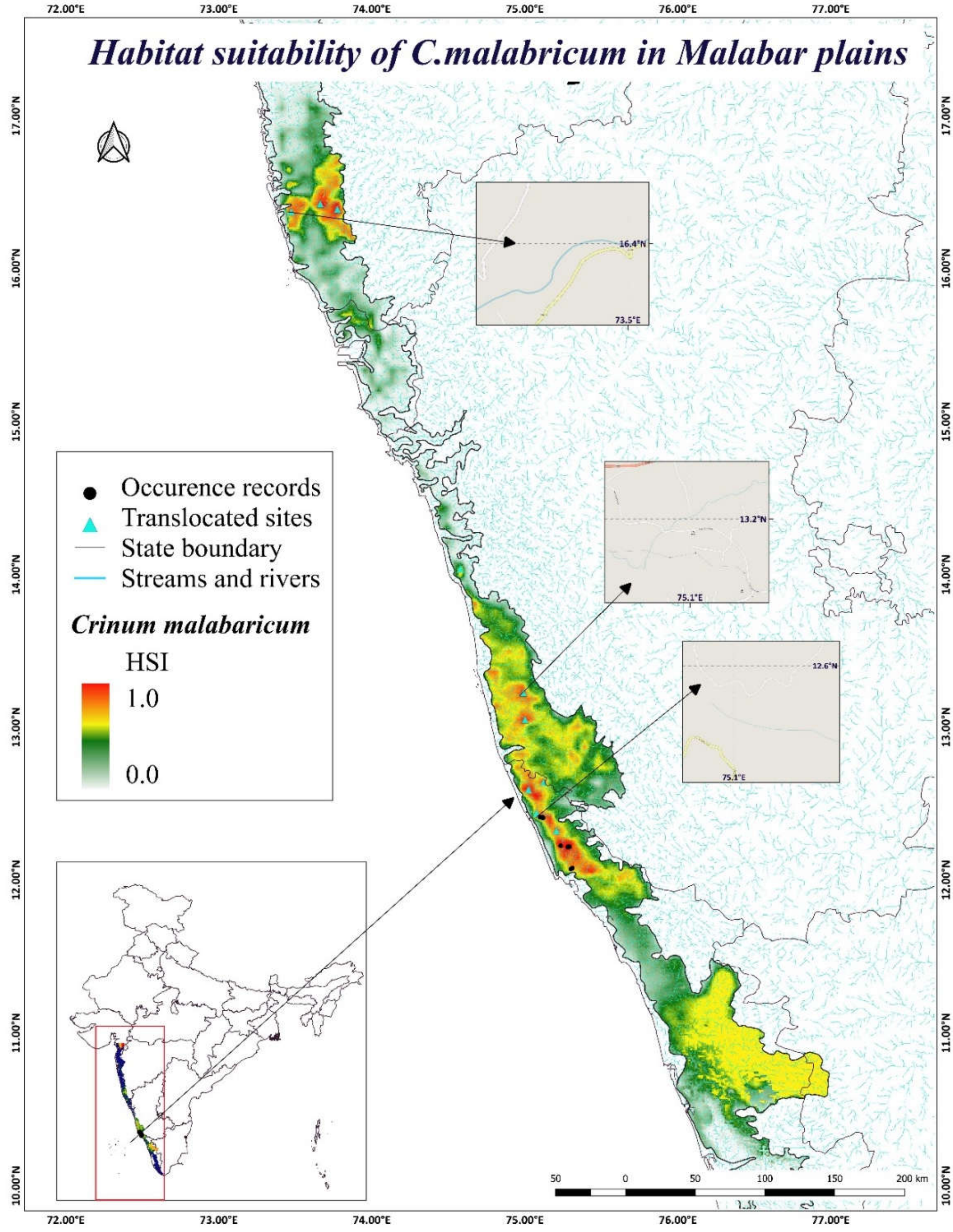


Figure 37. Habitat suitability map of *Crinum malabaricum* in the study area with natural habitats and translocated sites. Highlighting some of the suitable areas where laterite streams are located in GIS mapping

Bulbs of *C. malabaricum* were translocated to these ten suitable sites across Kerala, Karnataka, and Maharashtra. Post-translocation survival was assessed following the monsoon season of 2023, and the plant responded positively in six translocated sites. The highest survival rates were recorded in Panayal (84%) and Perla (72%) in

Kasaragod, Kerala, followed by Nandgaon (70%) in Maharashtra. Lower survival rates were observed in Mudbidri (65%) and Karkala (55%) in Karnataka, while the lowest survival was recorded in Devgad, Maharashtra (40%) (Figure 38). All surviving individuals successfully established by rooting and initiating leaf sprouting. Vegetative reproduction through bulbils was observed in all sites; however, no flowering occurred during the first post-translocation season. With the onset of the dry summer season, all plants entered dormancy as streams in the translocated sites dried up.

Sl. No.	Location	District & State	Latitude	Longitude
1.	Karkala	Udupi, Karnataka	13.189028°N	75.057858°E
2.	Bhatkal	Uttara Kannada, Karnataka	13.991708°N	74.566009°E
3.	Moodbidri	Dakshina Kannada, Karnataka	13.049497°N	75.001577°E
4.	Perla	Kasaragod, Kerala	12.636339°N	75.091584°E
5.	Panayal	Kasaragod, Kerala	12.439394°N	75.076079°E
6.	Seethangoli	Kasaragod, Kerala	12.573932°N	75.000271°E
7.	Kalichanadukkam	Kannur, Kerala	12.280895°N	75.171910°E
8.	Nandgaon	Sindhudurg, Maharashtra	16.388534°N	73.642227°E
9.	Devgad	Sindhudurg, Maharashtra	16.314469°N	73.455658°E
10.	Lore	Sindhudurg, Maharashtra	16.387371°N	73.774730°E

Table 26: Location details of suitable habitats for the species translocation of *Crinum malabaricum* predicated with Habitat suitability index (> 0.85) and validated by field exploration

Following the monsoon in 2024, resprouting was observed across all sites, indicating successful re-establishment after dormancy. Flowering was recorded at Perla and Panayal in Kerala and Karkala in Karnataka (Figure 39). These observations suggest that the species can acclimate to the new environments, with monsoonal rainfall playing a crucial role in post-translocation survival and growth.

Figure 38. Translocated populations of *C. malabaricum*; a. Nandgaon, and b.Naringre (Maharashtra); c. Karkala, and d. Mudbidri (Karnataka); e. Perla, and f. Panayal (Kerala)

Figure 39. Flowering and fruit setting were observed in the translocated populations in Perla and Panayal in Kasaragod

5.1.3.5. Future Projection of Habitat Suitability

This study used eight projected climate change scenarios (SSPs 126, 245, 370, and 585 for both 2050 and 2070) to assess the potential distribution shifts of *C. malabaricum*. The results indicate significant changes in habitat suitability across all scenarios, with a general trend of declining highly suitable habitats and increasing moderately suitable areas (Table 27, Figure 40 & 41).

The total potential habitat suitability (HSI > 0.25) in the study area (Malabar Plains of the Western Ghats) is projected to undergo substantial changes. Under SSP126 (2050), the suitable habitat expands to 46,135 km², reflecting a 69.67% increase from the current distribution. A similar expansion is observed under SSP245 (2050) (58,629 km², +94.79%) and SSP370 (2050) (52,703 km², +80.91%). However, under SSP585 (2050), the total suitable area declines slightly to 55,556 km² (+72.93%), indicating moderate stability. By 2070, the trend becomes more pronounced, with SSP126 (2070) reaching 60,120 km² (+97.52%), while SSP245 (2070) achieves the highest expansion to 63,955 km² (+105.32%). However, SSP370 (2070) shows a more moderate increase (48,589 km², +70.62%), and SSP585 (2070) exhibits the lowest expansion (49,325 km², +71.53%).

Climate Scenario	HSC1 (HSI < 0.25)	HSC2 (0.25 ≤ HIS < 0.5)	HSC3 (0.5 ≤ HSI < 0.75)	HSC4 (HSI ≥ 0.75)
Current	45208	8178	10711	2393
SSP126 2050	10355	5665	31564	8906
SSP245 2050	7861	20265	36372	1992
SSP370 2050	13787	15359	35637	1707
SSP585 2050	10934	18440	36378	738
SSP126 2070	6370	16414	41879	1827
SSP245 2070	2535	25173	38226	556
SSP370 2070	17901	9473	36778	2338
SSP585 2070	17165	29614	19432	279

Table 27: Habitat suitability classification (HSC) by using predicted probability of species occurrence values (Four equal-sized intervals of habitat suitability index -HSI) and potential spatial distribution (area in Km^2) of *Crinum malabaricum* under current and future climate change scenarios (HSC-1 = unsuitability; HSC-2 = least suitability; HSC-3 = moderate suitability; HSC-4 = high suitability)

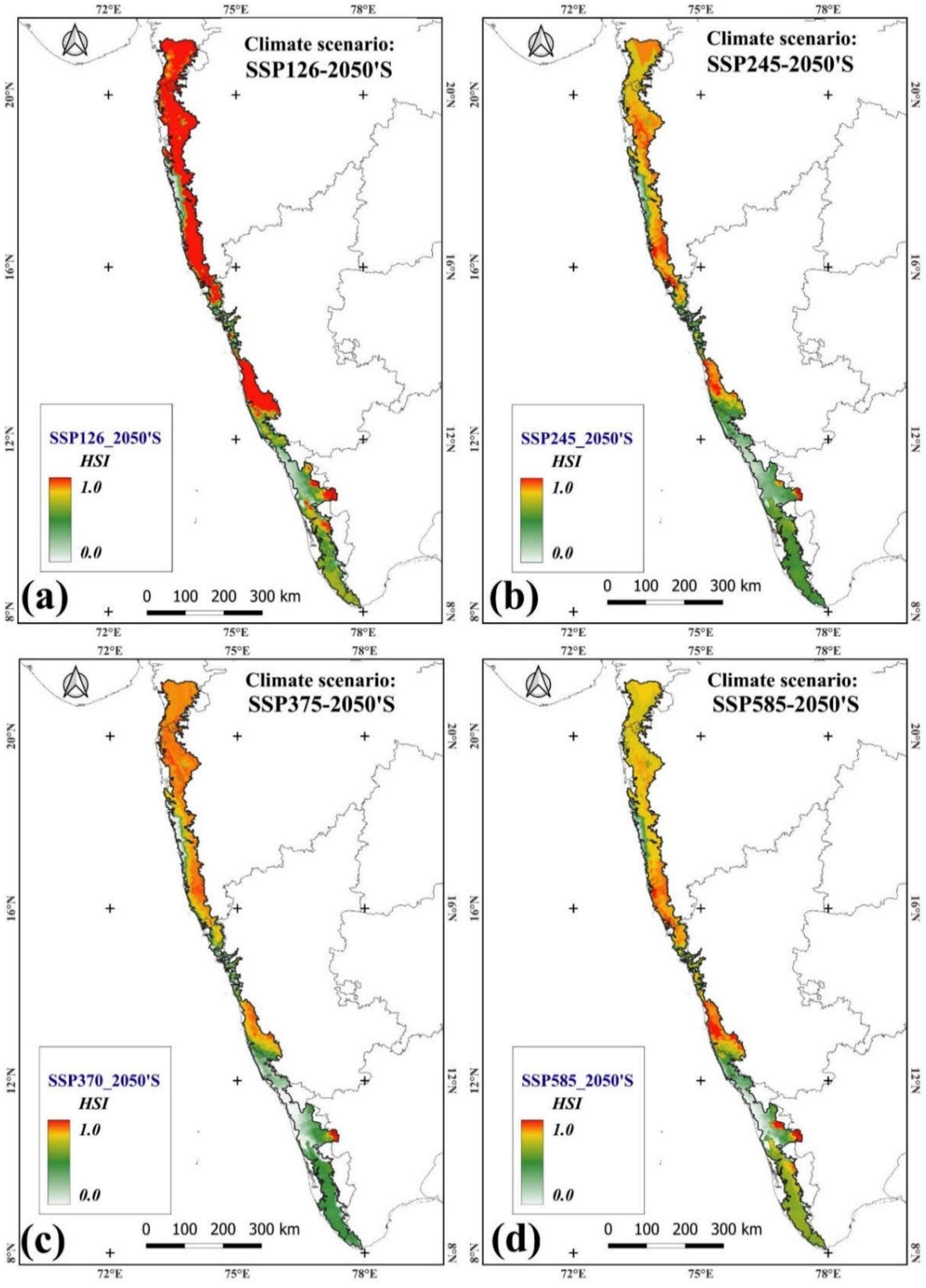


Figure 40. Variations in the predicted habitat suitability of *C. malabaricum* under projected future climate change scenarios of 2050's (2041 to 2060). (Colour coding: No colour = No suitability; Green= Least suitability; Yellow= Moderate suitability; Red= High suitability) ; A. SSPs 126; B. SSPs 245; C. SSP. 370 and D. SSP 585

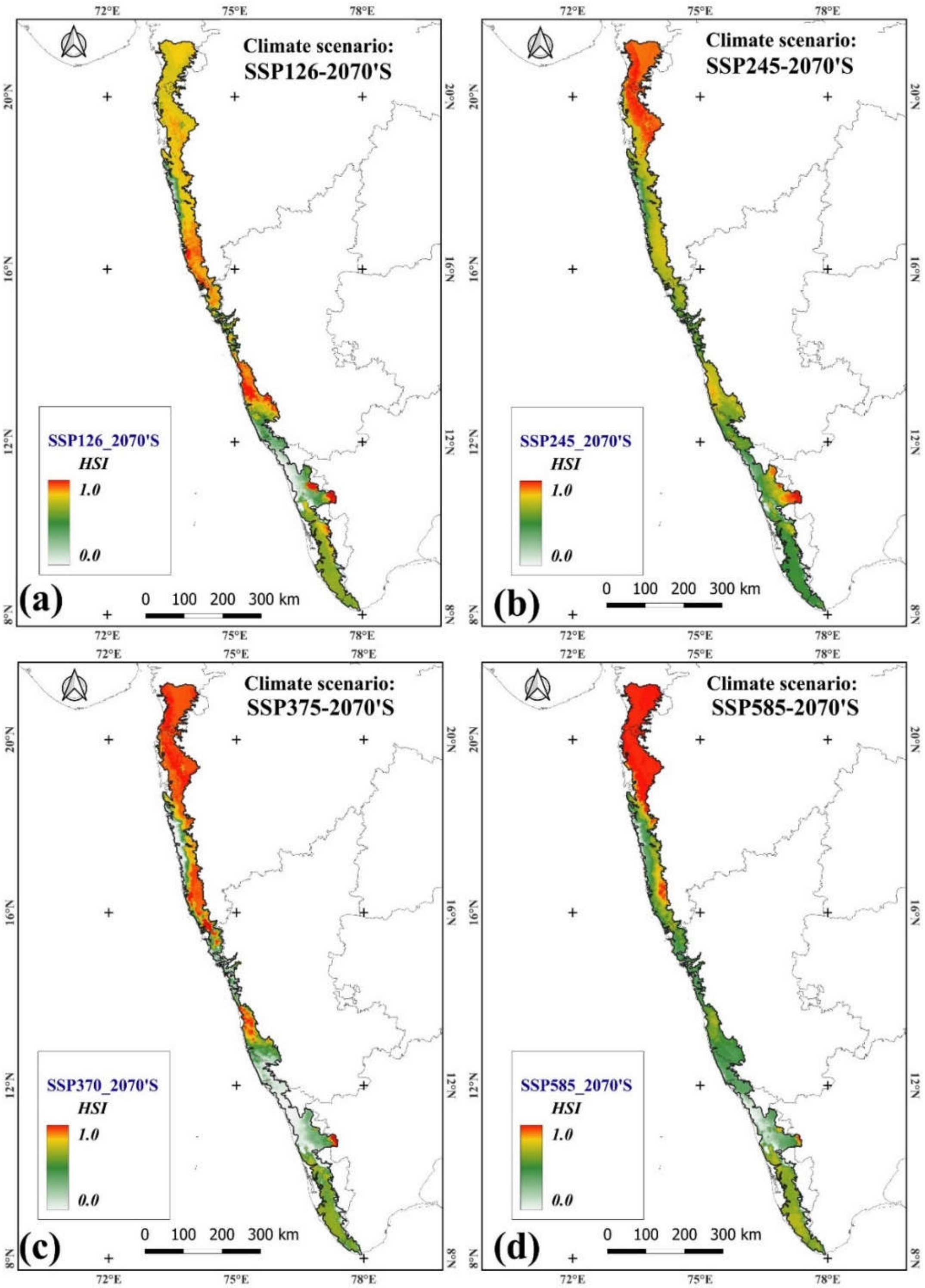


Figure 41. Variations in the predicted habitat suitability of *C. malabaricum* under projected future climate change scenarios of 2070's (2061 to 2080). (Colour coding: No colour = No suitability; Green= Least suitability; Yellow= Moderate suitability; Red= High suitability) ; A. SSPs 126; B. SSPs 245; C. SSP. 370 and D. SSP 585

The habitat suitability classes (HSCs) reveal critical shifts. Currently, highly suitable habitats (HSC4) cover 2,393 km², while unsuitable areas (HSC1) dominate with 45,208 km². By 2050, under SSP126, the highly suitable area expands to 8,906 km², a 272.17% increase, while unsuitable areas shrink significantly to 10,355 km² (-77.09%). Similarly, SSP245 (2050) sees a 16.39% increase in HSC4 (1,992 km²) but a -82.61% decline in HSC1 (7,861 km²). However, extreme climate scenarios (SSP370 and SSP585 for 2050) show a drastic reduction in highly suitable habitat, with HSC4 shrinking to 1,707 km² (-28.64%) and 738 km² (-69.14%), respectively.

By 2070, habitat losses become even more severe. Under SSP126, the highly suitable area decreases to 1,827 km² (-23.65%), while SSP245 records a further contraction to 556 km² (-76.76%). The most extreme losses occur under SSP370 and SSP585, where HSC4 shrinks to 2,338 km² (-2.30%) and 279 km² (-88.34%), respectively, signalling a near-total loss of highly suitable habitat. A critical finding is that habitat suitability for *C. malabaricum* is predicted to decrease in most future scenarios, except SSP126, posing a severe threat to its survival. However, the translocated sites show favourable suitability under SSP126, SSP245, and SSP370, emphasizing the importance of translocation as a conservation strategy. This indicates that translocation efforts are well-aligned with future climatic conditions, underscoring the urgent need for habitat protection and strategic translocation to mitigate climate-driven habitat loss and ensure the species' long-term survival.

5.1.3.6. Significance Testing of Maxent Future Prediction

The predicted probability values for the 12 occurrence points of *C. malabaricum* were extracted from nine models, totalling 60 observations. ANOVA analysis revealed a significant difference among the five climate scenarios ($F = 18.17$; $p < 0.001$). Post-hoc analysis showed a significant decline in occurrence probability for *C. malabaricum* under the SSP585 and 370 scenarios for both the 2050s and 2070s, indicating a potential negative impact of extreme climate change on the species' habitat suitability.

5.1.4. Discussion

The endemism of laterite plateaus in the Malabar Plains of the Western Ghats has been extensively addressed by many studies (Watve & Thakur, 2006; Lekhak & Yadav, 2012; Rao *et al.,* 2012; Pramod *et al.,* 2014; Gude *et al.,* 2021). The Malabar River lily (*Crinum malabaricum*) is an endemic aquatic species in this region. This endemism is primarily driven by unique climatic conditions and monsoon-driven seasonality, compounded by the fragmentation of laterite plateaus, which restricts species to limited areas. Conservation measures such as species translocation are crucial to mitigate human impact on these endemic species. The primary challenge in this process is the identification of suitable habitats for translocation. This study demonstrates that Ecological Niche Modelling (ENM), particularly Maxent modelling combined with field exploration, can effectively address this issue by identifying suitable habitats for *C. malabaricum* translocation.

Successful translocation guided by ENM has been documented in other species, such as the critically endangered *Gymnobelideus leadbeateri*, endemic to Australian alpine forests (Gallerani *et al.,* 2023). Similarly, for the translocation of *Narcissus cavanillesii*, species distribution models (SDMs) were employed alongside *in situ* validation trials to identify suitable receptor sites, with germination trials conducted at sites with varying suitability values (Draper *et al.,* 2019). These studies underscore the importance of innovative strategies in conserving biodiversity in regions with high endemism.

The selection of appropriate environmental variables is critical in Maxent modelling. However, identifying relevant variables can be challenging, as irrelevant variables may lead to inaccurate predictions. A thorough understanding of the target species' ecology is essential for modelling variable selection. Studies have highlighted the significance of seasonal precipitation and temperature variations in the distribution of seasonal aquatic plants, particularly in the laterite plateaus of the Malabar Plains (Bowman *et al.,* 2010; Kumar *et al.,* 2011; Rao *et al.,* 2012; Pramod *et al.,* 2014). In this study, topographic variables were deprioritised in favour of precipitation and temperature variables, as *C. malabaricum* is highly influenced by seasonal fluctuations in these factors. The key variables contributing to its distribution were the mean temperature of the driest quarter (Bio9) and the precipitation of the driest quarter (Bio17). *C. malabaricum* is sensitive to the summer season, entering dormancy during the driest period (December to March) and remaining dormant until the onset of monsoon (Lekhak & Yadav, 2012). Precipitation during this period is critical for its survival.

Five model accuracy parameters were evaluated in this study. The model achieved accuracy exceeding threshold values, with TSS, Kappa, pROC-AUC, and AUC values ranging from 0.89 to 0.94, and AUC ratios of 1.6, indicating high precision and reliability (Gao *et al.,* 2021; Allouche *et al.,* 2006). To avoid multicollinearity, the least correlated and most contributing variables were selected, as Maxent assumes variable independence, which may not always hold in real-world scenarios (Feng *et al.,* 2019). Additionally, overfitting was minimized by using the fewest possible variables, balancing model complexity with available data (Kramer-Schadt *et al.,* 2013). A Pearson's correlation coefficient threshold of ±0.8 and a variable contribution of >3% were used for variable selection.

The limited occurrence records of *C. malabaricum* pose a challenge for developing reliable species distribution models. However, Maxent has demonstrated high modelling capability even with minimal observations, as shown by Pearson *et al.* (2007) in cryptic geckos in Madagascar using just five occurrence records. This study further confirms that Maxent can effectively model ecological niches with high accuracy for endemic species with restricted distributions.

The model identified over 2,393 km² of suitable habitats with an HSI > 7.5 for *C. malabaricum* in the study area, demonstrating strong predictive capabilities. Given the species' aquatic nature, incorporating water bodies would refine habitat suitability predictions. However, including topographic variables related to all water body types would be impractical due to the species' seasonal growth patterns. *C. malabaricum* is highly sensitive to water level fluctuations and stream flow rates, making field exploration more effective for identifying suitable translocation sites than modelling these variables. The model predicted high-suitability areas in Sindhudurg district of

Maharashtra, Uttara Kannada, Udupi, and Dakshina Kannada districts of Karnataka, and Kannur and Kasaragod districts of Kerala. These regions contain potential laterite seasonal streams, as documented in previous studies (Chandran *et al.,* 2012; Pramod & Pradeep, 2021; Sarkar & Soman, 1986). Given that the four natural habitats of *C. malabaricum* are associated with laterite seasonal streams in northern Kerala (Lansdown, 2016), the model's predictions strongly suggest the likelihood of identifying similar habitats in these regions, highlighting its potential efficacy in guiding targeted field explorations for translocation.

Maxent maps a species' fundamental niche based on provided bioclimatic variables, representing the complete set of environmental conditions under which a species can survive and reproduce (Pulliam, 2000). Consequently, Maxent's predictions may overestimate suitable areas, necessitating field validation to distinguish the realized niche. This approach is most effective for well-studied species, and increased survey efforts or novel methods are required to refine site selection. This study identified survey areas using QGIS, Open Street Map, and Google Earth, which helped locate streams within highly suitable predicted regions falling within the Nitisol-Nd soil type. Additionally, field surveys incorporated ecological knowledge of *C. malabaricum*, considering factors such as terrain, topography, water level fluctuations, seasonal drying patterns, species occurrence, water flow, and bed material (Lansdown, 2016).

Endemic species, characterized by restricted distributions, require special attention due to their vulnerability to environmental changes and habitat loss (Maunder, 1992). Translocation is widely employed to mitigate risks associated with small population sizes and limited geographic ranges. However, establishing viable populations in new habitats presents numerous challenges, from initiating pilot populations to assessing ecological impacts. The primary constraint is selecting suitable receptor sites. This study addresses this challenge in translocating *C. malabaricum* through a methodological approach integrating ecological niche modelling with field assessments. ENM is a valuable tool in conservation biology, facilitating the identification of new populations and suitable sites for species reintroduction and translocation. However, its limitations must be acknowledged, as models may deviate from real-world scenarios and are prone to overfitting (Qiao *et al.,* 2015). Additionally, statistical methods like Maxent prioritize predictive accuracy over understanding underlying ecological processes. Complementing ENM with systematic field studies is crucial to bridge this gap. This study follows the framework Costa *et al.* (2010) suggested, integrating ecological niche modelling with field assessments for conservation planning and prioritisation. By incorporating field knowledge, this methodology enhances the identification of potential habitats similar to the natural habitat of *C. malabaricum*, ensuring a more comprehensive and effective conservation strategy.

Maxent modelling of *C. malabaricum* indicates a dynamic and complex shift in habitat suitability under future climate scenarios. It predicts an initial increase in highly suitable habitats under SSP126 (2050), expanding by 272.17%, suggesting that moderate climate change may temporarily benefit the species. However, by 2070, a significant contraction of high-suitability areas is evident across all SSPs, with the most severe declines observed under SSP245 (-76.76%) and SSP585 (-88.34%). This highlights the transient nature of favourable climatic conditions and the long-term risks of high-emission scenarios. Although moderately suitable habitats (HSC3) expand in SSP126 and SSP370, acting as potential habitat refugia, the reduction in highly suitable areas suggests that *C. malabaricum* may experience greater physiological stress and reduced reproductive success. The shift towards marginally suitable habitats underscores the necessity of proactive conservation measures to mitigate population declines.

Species translocation has emerged as a viable conservation approach for endangered plants, particularly those facing habitat loss due to climate change and anthropogenic pressures. Translocation has been widely adopted to establish new populations in ecologically suitable areas, with studies suggesting that approximately 72% of endangered plant species require some form of translocation for survival (Volis & Blecher, 2021). The success of translocation is influenced by ecological compatibility, genetic diversity, and management interventions (Tojibaev *et al.,* 2019; Weeks *et al.,* 2011). In the case of *C. malabaricum*, translocation efforts align well with future projections, particularly under SSP126 and SSP245, validating the effectiveness of this strategy. By relocating populations to predicted moderate-suitability habitats (HSC3), the study provides the species with additional habitat refugia that may support long-term survival despite climatic shifts. Similar studies on translocated plant populations highlight the importance of site selection and adaptive management in ensuring long-term success (Abeli & Dalrymple, 2023).

The advantages of translocation extend beyond immediate conservation benefits. It enables species persistence in the face of environmental change, facilitates genetic exchange, and mitigates the risk of localized extinctions (Volis *et al.,* 2011). However, translocation success is not guaranteed, and several challenges must be addressed. Environmental mismatches between donor and recipient sites, competition from invasive species, and unforeseen ecological interactions can limit establishment success (Grange *et al.,* 2015). Additionally, translocated populations may suffer genetic bottlenecks if not adequately sourced from diverse genetic backgrounds (Pacioni *et al.,* 2019). Long-term monitoring and adaptive management are crucial, as emphasized by Gomes *et al.* (2018), who highlight the role of continuous assessment in evaluating translocation outcomes. Experimental approaches, such as controlled irrigation and protective measures like fencing, have improved seedling establishment in translocated populations (Dillon *et al.,* 2018), underscoring the importance of multi-faceted conservation strategies. Studies on conservation translocation, such as those on the Houbara bustard (*Chlamydotis undulata*), have successfully linked niche suitability with individual survival, reinforcing the relevance of ENMs in guiding conservation interventions (Monnet *et al.,* 2015).

The conservation of *C. malabaricum* presents significant challenges due to its restricted range, habitat specificity, and vulnerability to climate change. Our findings indicate that while some climatic scenarios may initially enhance habitat suitability, long-term projections predict a substantial loss of optimal habitats, necessitating proactive conservation interventions. Species translocation, validated by both our study and broader conservation literature, emerges as a promising strategy for ensuring the persistence of *C. malabaricum*. However, its success depends on careful site selection, genetic considerations, and adaptive management.

By integrating translocation with habitat protection and leveraging ENM tools for predictive conservation, this study provides a framework for safeguarding not only *C. malabaricum* but also other endangered aquatic species within laterite plateaus. Continued research, long-term monitoring, and adaptive strategies will be essential to refine and enhance translocation efforts, ultimately contributing to the sustainable conservation of this critically endangered species.

5.2. Pollen cryopreservation of *Crinum malabaricum*

5.2.1. Introduction

Cryopreservation is a crucial technique for the long-term *ex situ* conservation of plant genetic resources, offering a reliable solution for maintaining pollen viability over extended periods. This method involves the storage of biological material at ultra-low temperatures, typically in liquid nitrogen (-196°C), ensuring genetic preservation with minimal viability loss. It plays a vital role in overcoming reproductive constraints, particularly for rare, endemic, and threatened species, caused by habitat loss, climate change, and asynchronous flowering, supporting breeding programs and conservation initiatives (Rajasekharan & Rohini, 2023).

Pollen cryopreservation is essential for maintaining genetic diversity in endangered species. This method particularly valuable for species with recalcitrant or short-lived seeds that cannot be conserved through traditional seed banking methods. Pollen cryopreservation extends the availability of viable pollen beyond natural flowering cycles, overcoming challenges such as asynchronous flowering and limited reproductive windows. This technique enhances hybridisation efforts, maintains genetic diversity, and serves as an effective *ex situ* conservation tool. Cryopreserved pollen is also useful for haploid culture, *in vitro* propagation, and genetic research, making it indispensable for both conservation and breeding efforts (Benelli *et al.,* 2024; Rajasekharan & Ganeshan, 2019).

Crinum malabaricum, a critically endangered aquatic plant endemic to the laterite plateaus of northern Kerala, is under severe threat due to habitat degradation, restricted distribution, and climatic fluctuations. Classified as Critically Endangered (CR) by the IUCN Red List (Lansdown, 2016), its reproductive success is highly dependent on monsoon-driven flowering between July and September. Any variations in rainfall patterns can disrupt pollination and seed production. Given these challenges, pollen cryopreservation provides a reliable means of preserving the species' genetic material for future restoration and breeding efforts.

Beyond its ecological significance, *C. malabaricum* possesses ornamental potential due to its striking inflorescence and adaptability to aquatic environments, making it suitable for water gardens and aqua scaping. However, its limited natural population and short reproductive window hinder commercial propagation and hybridization. Cryopreservation facilitates year-round pollen availability, enabling controlled cross-breeding programs to develop cultivars with enhanced ornamental and ecological traits.

As a conservation and breeding tool, pollen cryopreservation offers multiple advantages for *C. malabaricum*. It safeguards genetic material from threats such as habitat loss, climate change, and disease, while also enabling hybridization efforts and international collaborations by ensuring pollen availability across geographic and temporal barriers. Additionally, it supports scientific research on pollen viability, fertilization mechanisms, and genetic studies, including DNA analysis and marker-assisted selection. In restoration ecology, cryopreserved pollen plays a crucial role in species reintroduction and population establishment. The effectiveness of cryopreserved pollen in fertilization and seed production has been demonstrated in various plant species, reinforcing its potential for the conservation of *C. malabaricum*.

Pollen cryopreservation is a vital strategy for the conservation and breeding of *C. malabaricum*, offering several key benefits. It ensures long-term genetic conservation by safeguarding endangered species and protecting valuable genetic material from threats such as habitat loss, climate change, and diseases. This technique also facilitates breeding programs by allowing year-round availability of pollen, enabling hybridization efforts, and overcoming geographical barriers through international collaborations. Additionally, it supports scientific research by enabling studies on pollen viability, germination, fertilization mechanisms, and genetic research, such as DNA analysis and marker-assisted selection. Furthermore, it plays a crucial role in restoration ecology by aiding in the reintroduction of *C. malabaricum* into its natural habitat or establishing new populations. The effectiveness of cryopreserved pollen in fertilization and seed production has been demonstrated in multiple plant species.

To ensure successful pollen cryopreservation, critical factors such as moisture content, viability assessment methods, and germination media composition must be optimized. Moisture content plays a crucial role in determining pollen viability post-thaw, as excessive dehydration or inadequate drying can lead to loss of cellular integrity. Studies emphasize the importance of optimizing dehydration conditions to maintain high viability post-cryopreservation. Furthermore, the choice of storage conditions, such as the use of cryovials, protects pollen from direct exposure to liquid nitrogen, preserving its genetic integrity and ensuring long-term stability.

Assessing the viability of cryopreserved pollen is essential for evaluating the effectiveness of storage protocols. Various methods, including *in vitro* germination tests and staining techniques like Alexander's stain, are employed to determine pollen viability. Alexander's stain effectively differentiates viable and non-viable pollen grains, providing a reliable assessment of pollen health and fertility (Hister & Tedesco, 2016). The simplicity and accuracy of this technique make it particularly useful for conservation and breeding programs, enabling efficient screening of stored pollen samples.

In *in vitro* pollen germination assays, the composition of the culture medium significantly impacts pollen viability and tube elongation. Brewbaker and Kwack (1963) demonstrated that calcium and boron are essential for successful pollen germination, with calcium playing a pivotal role in pollen tube growth. Boric acid further enhances pollen viability by stabilizing cellular structures and facilitating metabolic processes. Additionally, in species where high sucrose concentrations disrupt osmotic balance, polyethylene glycol (PEG) serves as an alternative osmoticum, maintaining the necessary osmotic pressure for sustained pollen tube elongation.

Pollen cryopreservation represents a valuable conservation strategy for *C. malabaricum*, ensuring the long-term preservation of its genetic material, facilitating hybridization efforts, and supporting ecological restoration initiatives. By optimizing cryopreservation protocols and addressing critical factors such as moisture content, viability assessment, and germination media composition, this technique can significantly contribute to the conservation and sustainable utilization of *C. malabaricum* and other threatened plant species.

5.2.2. Materials and Methods

5.2.2.1. Selection of Flowers

Optimized the pollen collection period based on the flowering cycle of *C. malabaricum*. Pollen quality is highly influenced by the environment, and the floral stage. To ensure contamination-free pollen, flower buds were bagged one day before anthesis to prevent external pollen or insect interference. Flowers were harvested on the first day of anthesis and transported to the laboratory under controlled conditions.

5.2.2.2. Pollen Extraction

Anthers were carefully excised from fresh flowers and placed on butter paper in a Petri dish. Pollen was collected at one-hour intervals post-anthesis to determine the optimal collection period for maximum viability. Pollen grains were extracted using sterile needles and immediately subjected to desiccation.

5.2.2.3. Desiccation and Moisture Content Determination

Pollen grains were desiccated using activated silica gel in a desiccator until the optimal moisture content was achieved. Drying durations were tested at 20, 40, 60, and 80 minutes intervals. Moisture content was determined using the gravimetric method suing the equation:

$$\mathrm{MC} = \frac{(\mathrm{w} - \mathrm{d}\,)}{\mathrm{w}} \mathrm{x}100$$

Where, MC = Moisture content of pollen (%)

w = Fresh weight of pollen.

d = Dry weight of pollen.

5.2.2.4. Viability Assessment

Pollen viability was assessed using Alexanders stain (Alexander, 1969) and *in vitro* germination by hanging drop method (Sivanna & Rangaswamy, 2012). Viable pollen stained purple, while non-viable pollen appeared light blue when treated with Alexander's stain.

The pollen germination medium was optimized by modifying Brewbaker & Kwack medium (1963) with varying concentrations of Sucrose (1%, 2.5%,5 %, 10%, 15%) and addition of polyethylene glycol (PEG). The combinations of pollen germination media are listed in the table 28. Hanging drop cultures were incubated at 25ºC in a moisture chamber and germination was observed at 10, 30, and 60 minutes intervals under a compound microscope. All experiments followed a Completely Randomized Design (CRD) with three replications per species. For each replication, a minimum of 500 to 600 pollen grains were examined across at least ten microscopic fields of view.

PGM	Sucrose (%)	PEG MW-6000 (%)	Other salts
M1	15	-	B&K salts (Brewbaker and Kwack, 1963)
M2	10	-	B&K salts (Brewbaker and Kwack, 1963)
M3	5	-	B&K salts (Brewbaker and Kwack, 1963)
M4	1	10	H_3BO_3: 100 ppm, $CaCl_2$: 200 ppm
M5	5	10	H_3BO_3: 100 ppm, $CaCl_2$: 200 ppm
M6	10	10	H_3BO_3: 100 ppm, $CaCl_2$: 200 ppm
M7	2.5	10	H_3BO_3: 100 ppm, $CaCl_2$: 200 ppm

Table 28. Pollen germination media used for invitro pollen viability assessment.

5.2.2.5. Cryopreservation and Post-Thaw Assessment

Desiccated pollen grains were enclosed in parchment paper, transferred into 1.8 mL cryogenic vials, and stored in liquid nitrogen (-196ºC) for 24 hours in cryogenic container (Heir, India). After the cryostorage period, cryovials were retrieved and thawed gradually at room temperature by dipping in water bath (30°C for 2 min). Post-thaw viability was evaluated through *in vitro* germination assay and controlled pollination experiments using fresh and cryopreserved pollen. For pollination, 40 flowers were selected from ten plants and are emasculated and half of them pollinated with fresh pollen grains from another plant (xenogamy) while the other half pollinated with the cryopreserved pollen carried to the natural habitat in a dry shipper (IX2, Inox, India).

5.2.2.6. Scanning Electron Microscopy (SEM) Imaging

Fresh and cryopreserved pollen samples were dehydrated using activated silica gel for one hour to remove moisture content. Dehydrated pollen was mounted on adhesive carbon tape fixed to aluminium stubs, coated with gold-palladium in a sputter coater (sc7620, Quorum, UK) and examined under a scanning electron microscope (EVO-18, Carl-Zeiss, Germany). Morphological parameters, including polar length (µm) and equatorial width (µm), were measured using SmartSEM software (Carl Zeiss, Germany). SEM micrographs were analysed for morphological changes in pollen grains following cryopreservation.

3.2.2.7 Statistical Analysis

Data were analysed using statistical software, and results were expressed as mean ± standard deviation. Germination percentages were compared using one-way ANOVA followed by post-hoc tests to determine significant differences between treatments. A significance level of $p < 0.05$ was considered statistically significant. The variation in pollen viability before and after cryopreservation was analysed using a *t*-test. The *p*-value was determined based on the degrees of freedom (*df*) and the corresponding critical *t*-value from the *t*-distribution table. A *p*-value above 0.01, indicating that the calculated *t*-value did not exceed the critical *t*-value at *df*, was considered statistically insignificant. All the statical analysis were conducted using SPSS 25.0 (IBM).

5.2.3. Results and Discussion

5.2.3.1. Optimization of PGM & Pollen Collection Period

The study identified M7 (2.5% sucrose, 10% PEG MW-6000, 100 ppm H_3BO_3, and 200 ppm $CaCl_2$) as the most effective pollen germination medium, achieving the highest germination rate of 92.63% at 10:00 AM. M4 (1% sucrose, 10% PEG MW-6000, 100 ppm H_3BO_3, and 200 ppm $CaCl_2$) also supported high germination, reaching 80.84% at 10:00 AM, making it a viable alternative (Table 28).

The results suggest that Polyethylene glycol (PEG) plays a crucial role in the *in vitro* pollen germination medium of *C. malabaricum*, functioning as an osmotic regulator that enhances germination. Media without PEG (M1, M2, and M3) showed little to no germination, indicating that PEG is essential for maintaining osmotic balance and preventing rapid dehydration of the pollen grains. Among PEG-supplemented media, exhibited the highest germination, suggesting that a lower sucrose concentration with PEG creates an optimal osmotic environment for pollen hydration and tube growth.

In contrast, M6 (10% sucrose, 10% PEG) resulted in no germination, implying that an excessive sucrose concentration may create a hyperosmotic condition, negatively affecting pollen viability. M4 (1% sucrose, 10% PEG) also supported high germination, reinforcing that PEG enhances germination when combined with a lower sucrose concentration. This suggests that while sucrose provides an energy source, its optimal concentration must be carefully balanced with PEG to prevent osmotic stress and ensure successful pollen tube growth.

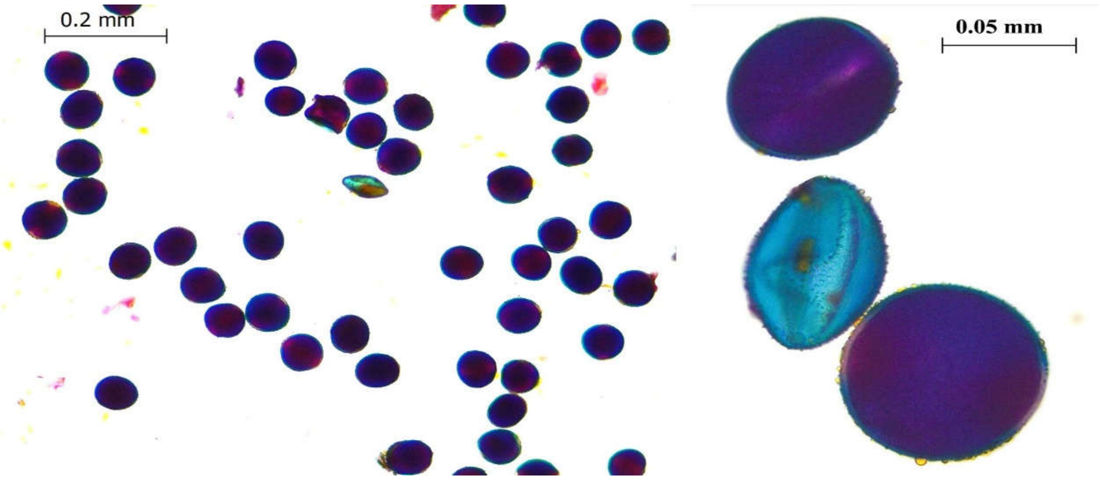


Figure 42. Pollen grains of *C. malabaricum* stained with Alexanders stain, showing viable (purple) and non-viable (light blue) pollen. Left panel: 10X view; Right panel: 40X view

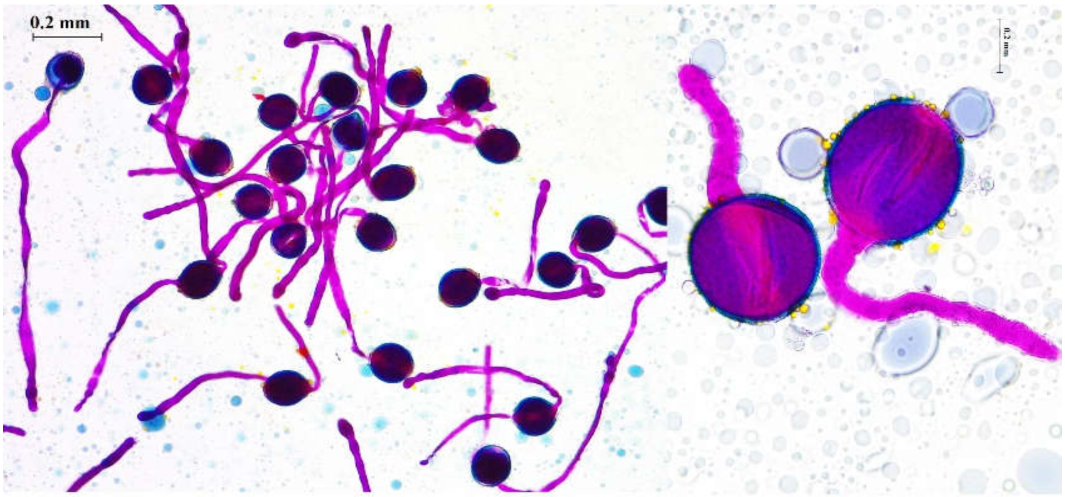


Figure 43: *In vitro* germinated fresh pollen grains of *C. malabaricum.* Left panel**:** 10X view; Right panel**:** 40X view

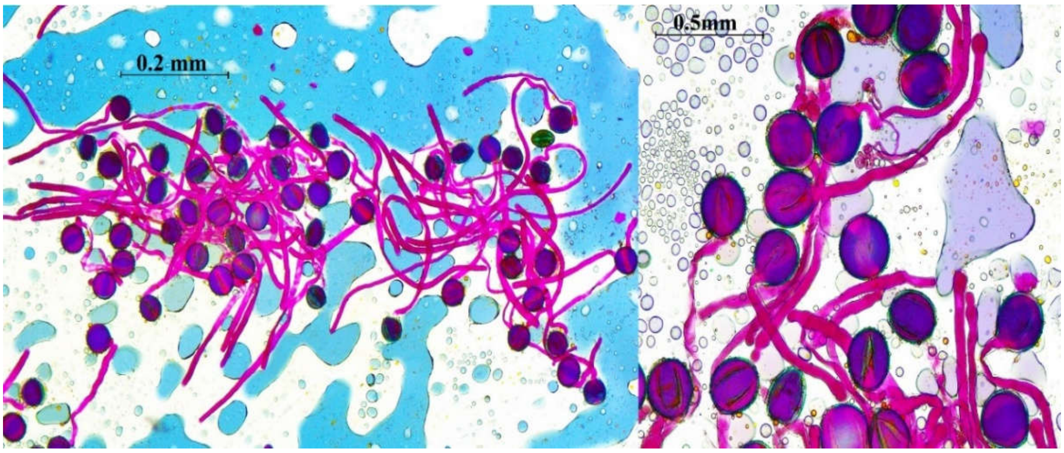


Figure 44: *In vitro* germinated pollen grains of *C. malabaricum*, after cryopreservation. Left panel: 10X view; Right panel: 40X view

Pollen viability peaked at 10:00 AM, three hours after anthesis, indicating this is the optimal collection time, while a sharp decline was observed after 12:00 PM, suggesting that pollen should be collected in the morning for maximum viability (Table 29). This is the ideal time to collect pollen grains for cryopreservation which ensure the quality of pollen grains (Figure 42 & 43).

PGM	8:00 AM	10:00 AM	12:00 AM	2:00 PM	4:00 PM	6:00 PM
M1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M2	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M3	12.96 ± 0.27^{b}	18.28 ± 0.24^{b}	9.88 ± 0.13^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	19.37 ± 0.6^{b}
M4	66.32 ± 0.32^{d}	80.84 ± 0.25^{d}	63.27 ± 1.7^{d}	19.99 ± 0.3^{b}	41.31 ± 0.3^{c}	61.60 ± 0.9^{d}
M5	21.07 ± 0.43^{c}	56.78 ± 0.5^{c}	41.01 ± 0.4^{c}	0.00 ± 0.00^{a}	20.41 ± 0.4^{b}	39.65 ± 0.2^{c}
M6	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M7	74.03 ± 1.25^{e}	$\mathbf{92.63\pm0.27^{e}}$	69.57 ± 0.3^{e}	26.30 ± 1.7^{c}	44.61 ± 1.3^{d}	69.06 ± 0.5^{e}

Table 29: *In vitro* pollen germination percentages at different time points in various pollen germination media. Values represent mean ± standard error. Different letters indicate significant differences ($p < 0.05$) among treatments

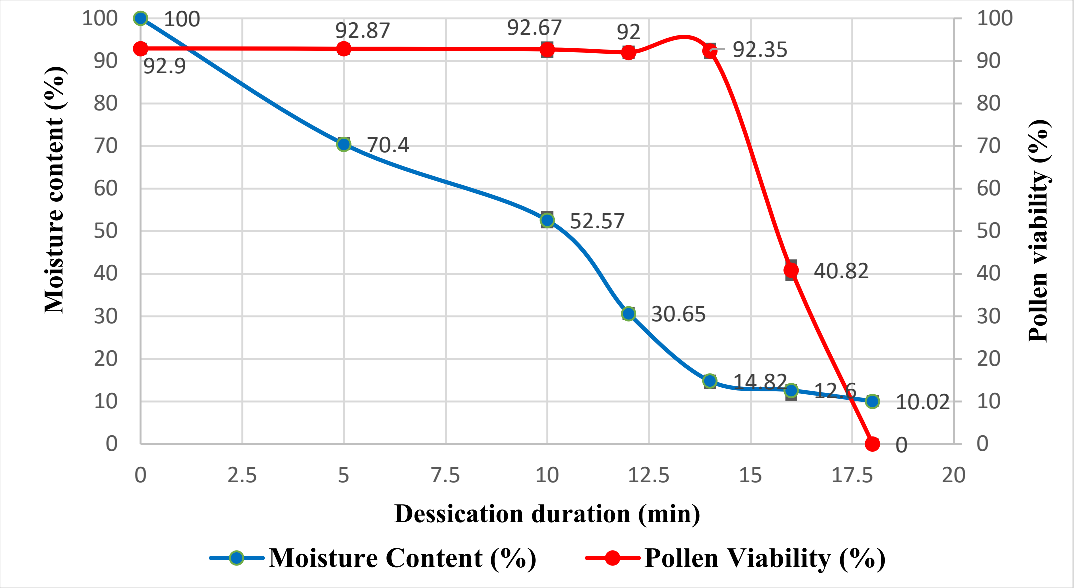
5.2.3.2. Optimizing Moisture Content

The optimum moisture content was identified as 14 to 15% for *Crinum malabaricum*. Beyond this critical moisture content, the germination rate was drastically declined and the at ten percentage of relative moisture content all the pollens become non-viable (Graph 20).

Desiccation period (min.)	Moisture Content	Pollen Viability
0	100.00 ± 0.00^{g}	92.90 ± 0.12^{c}
5	70.40 ± 0.44^{f}	92.87 ± 0.14^{c}
10	52.57 ± 0.84^{e}	92.67 ± 0.64^{c}
12	30.65 ± 0.33^{d}	92.00 ± 0.29^{c}
14	14.82 ± 0.17^{c}	92.35 ± 0.62^{c}
16	12.60 ± 0.12^{b}	40.82 ± 1.25^{b}
18	10.02 ± 0.18^{a}	0.00 ± 0.00^{a}

Table 30. Effect of desiccation on pollen viability. The values represent the mean ± standard error of replications. Different superscript letters indicate significant differences at $P < 0.05$ based on Duncan's Multiple Range Test (DMRT)

The significant difference was noticed beyond the moisture level of 14% (Table 30). This is due to the lack of water content that is necessary for the survival of the cells of the pollen grains. Many studies also suggested that moisture content of the pollen grains prior to cryopreservation should be ranges from 5 to 14 percentage, which will prevent the damaging of pollen cells by Ice formation during cryopreservation while maintaining the highest germination rate (Ganeshan & Alexander, 1991; Rajashekaran *et al.,*1995).



Graph 20: Effect of desiccation on pollen viability; A drastic drop in pollen viability occurs when moisture content falls below the critical point (14–15%)

5.2.3.3. Post Thaw Pollen Viability and Fertility Assessment

Highest pollen viability of *C. malabaricum* assessed pre and post cryopreservation indicates the success of cryopreservation. During the peak period the cryopreserved pollen shows a viability of 92.913 ±0.3% and 92.47±0.39 % based on staining method and *in vitro* germination method respectively. While fresh pollen has shown a viability of 93.26±0.25% and 92.627±0.27%. There was no significant variation in the pollen viability before and after cryopreservation both in *in vitro* pollen germination method and in staining method (Figure 44). The calculated t-values for staining method and invitro germination methods are 1.53 and 0.57 respectively. Which are lower than their critical t-values (±4.303 at DF=2). This indicating a that there was no statistically significant difference between both stages. Pollen fertility assessment shows that the fruit set of 84.5±2 % was set in cryopreserved pollen grains used hybridisation whereas 86.25 ± 4.8% was observed in fresh pollen pollinated flowers (xenogamy). These tests confirm the effectiveness of cryopreservation in maintaining pollen viability and fertility over time.

5.2.3.4. SEM Imaging

SEM images of pollen grains before and after cryopreservation show no significant morphological changes in terms of polar length and equatorial width. Fresh pollen of *C. malabaricum* has an average polar length of 101.11 ± 1.2 µm, an equatorial width of 45.11 ± 0.07 µm, and a length-to-width ratio of 0.44 ± 0.07. Cryopreserved pollen also retains these dimensions, indicating that short-term cryostorage does not alter pollen morphology (Figure 45).

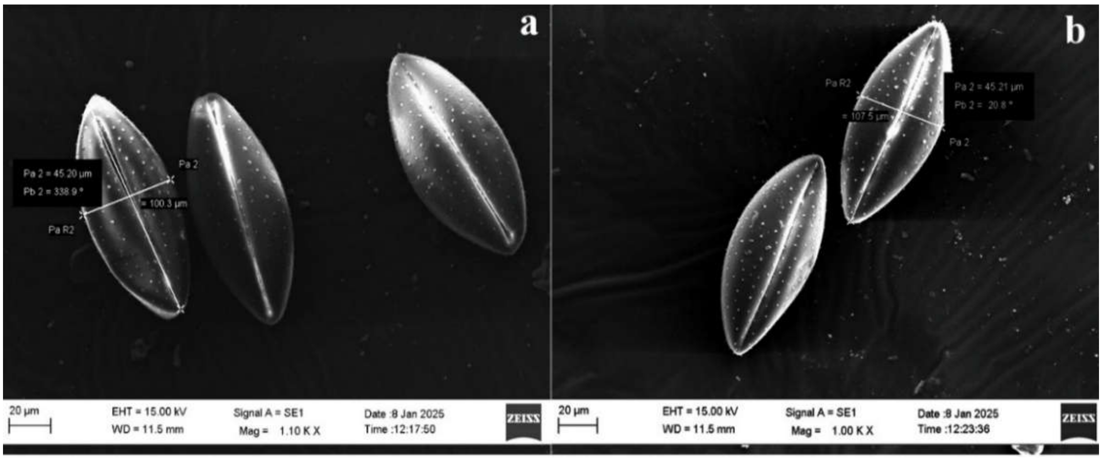


Figure 45. SEM image of pollen grains of *Crinum malabaricum* showing polar length and equatorial width; a. before cryopreservation; b. after cryopreservation

5.2.4 Conclusion

Pollen cryopreservation is a viable conservation strategy for *C.malabaricum*, ensuring genetic preservation and enabling year-round breeding efforts. Optimized moisture content (14-15%) and germination media (M7: 2.5% sucrose, 10% PEG) significantly improved pollen viability. Post-thaw assessment confirmed that cryopreservation effectively retains pollen fertility and viability, making it a promising tool for *ex situ* conservation and breeding programs. Future studies should explore long-term storage effects on genetic stability.

5.3. Other Conservation Measures

5.3.1. *Ex situ* Conservation at KSCSTE- MBGIPS

Ex situ conservation of critically endangered plants is essential to safeguard their genetic diversity and ensure their survival outside their natural habitats. This conservation strategy is particularly crucial for species facing threats such as habitat loss, climate change, and human activities. *Ex situ* conservation not only preserves genetic material but also facilitates research, restoration, and potential reintroduction in to native environments. This method aims to generate large quantities of seedlings for genotype maintenance and act as a resource for the species germplasm.

Ex situ conservation provides a controlled environment where optimal growth conditions can be maintained. Understanding the specific requirements of a species is essential for successful *ex situ* conservation. For *Crinum malabaricum*, an endemic and critically endangered aquatic plant, factors such as potting material, water depth, and flow dynamics play a crucial role in survival and growth.

To develop *C. malabaricum* in *ex situ* conditions, the pot-in-pot method was employed (Figure 46.a). This approach ensures that environmental conditions mimic the species' natural habitat as closely as possible. Mature bulbs were collected during the dormancy period and planted in 16 x 30 cm (diameter x height) pots. Several holes were made in the walls and bottom of the pots to facilitate drainage and prevent silt accumulation. Because increased silt content prevents the growth of the species or it causes rotting of the roots, in nature condition it continues stream flow prevents the sedimentation of silt.

Laterite gravels were used as the primary potting medium. These gravels were washed thoroughly to remove dirt and excess silt, mixed with river sand, and enriched with potash. The prepared medium was sun-dried for a day before use. Medium-sized laterite stones were placed in the pots to secure the bulbs, ensuring that the collar of each bulb remained at the top level of the pot.

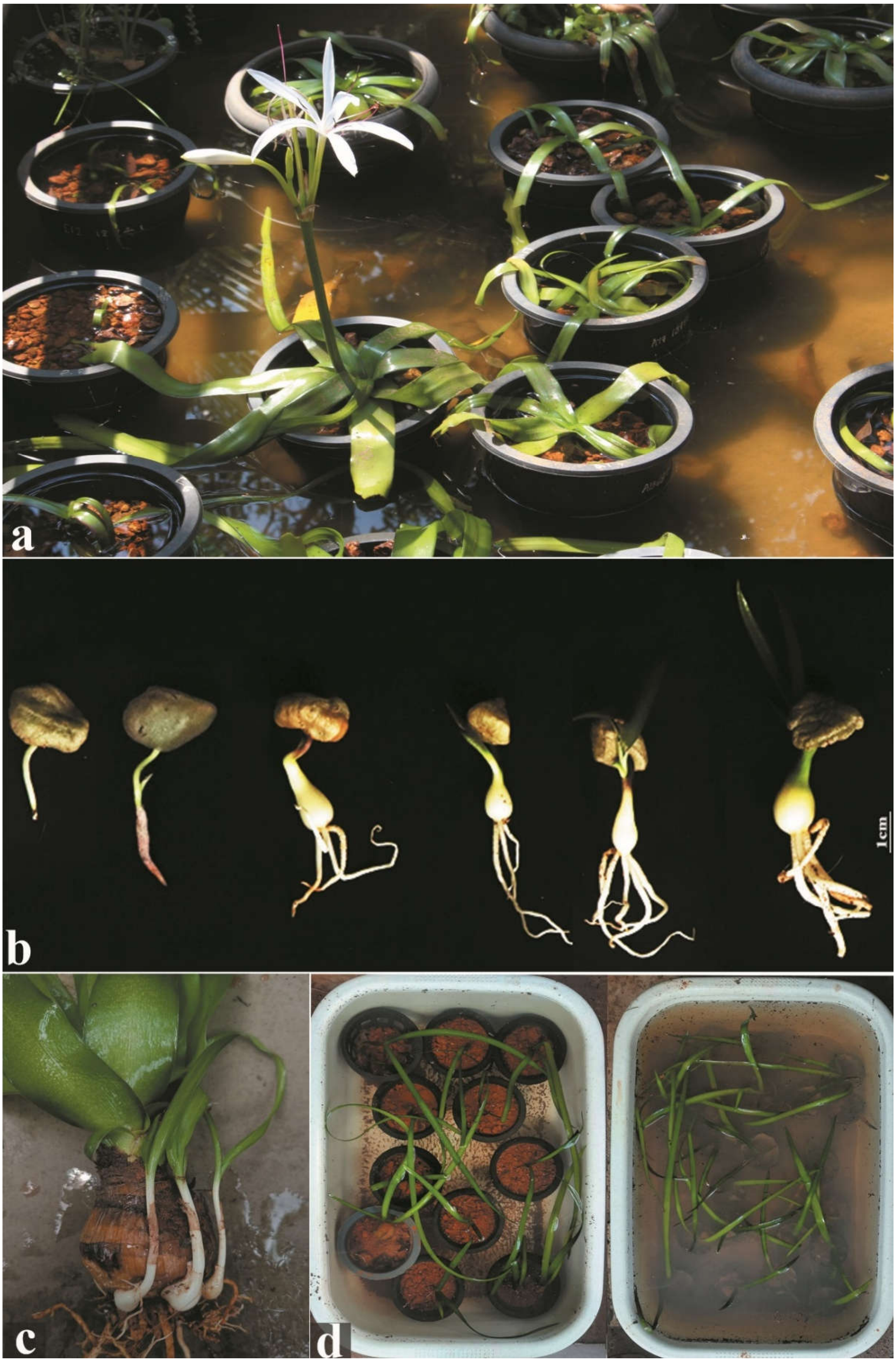


Figure 46: *Crinum malabaricum*; a. In *ex situ* condition; b. Seed germination stages; c. Bulblet development; d. Seedlings

The pots were then placed in a pond or a larger water container with a depth of less than 0.5 m to mimic natural conditions. Continuous pumping or controlled overflow was employed to simulate natural running water conditions, preventing silt deposition.

The species adapted well to the *ex situ* environment, though leaf length remained shorter than in the wild. Decayed leaves were removed regularly to prevent water quality deterioration and pest infestation. The Indian lily moth (*Polytela gloriosae*) was observed as a potential pest in *ex situ* conditions. Early detection and manual removal were practiced. In case of severe infestations, 0.03% Azadirachtin was used as a control measure. Repotting was conducted after each growing season (June to October) to prevent silt accumulation and promote healthy growth. Simulated dormancy (January to April) was crucial for inducing flowering in *ex situ* conditions.

The species was successfully propagated through both vegetative and seed propagation methods. Vegetative propagation was achieved through bulb division. Drying the bulbs outside of water induced sprouting, which, upon re-immersion, led to the growth of multiple new bulbs. A single mother plant produced 2–10 bulbs using this method (Figure 46.c). Seed propagation was also effective, as *C. malabaricum* seeds exhibited high germination rates but were recalcitrant, making long-term storage difficult.

Germination was successfully achieved in coir pith and vermiculate, providing an ideal medium for rapid growth. Seedlings developed identifiable bulbs within 25 to 30 days and were then transferred to small (5 x 5 cm) pots filled with laterite gravel. These pots were placed in water trays at a depth of approximately 10 cm. Once the seedlings reached a leaf length of over 30 cm, they were transplanted into larger ponds for further growth (Figure 46.b&d).

By implementing these *ex situ* conservation strategies, *Crinum malabaricum* can be effectively safeguarded, ensuring its genetic preservation and providing a reliable source for future restoration efforts. While *ex situ* conservation is vital for protecting endangered species, it is important to recognize that it should complement in-situ conservation efforts. The ultimate goal is to restore natural habitats and promote biodiversity in their original ecosystems.

5.3.2. Protection of Natural Habitat as Local Biodiversity Heritage Sites (LBHS)

5.3.2.1 Biodiversity Heritage Sites (BHS)

Biodiversity Heritage Sites (BHS) are designated areas recognized for their ecological significance, characterized by unique and fragile ecosystems. These areas may encompass a variety of biodiversity components, including high species richness, endemism, and the presence of rare or keystone species. BHS designation aims to protect these ecologically important sites while promoting conservation ethics within local communities.

Key provisions under the Biological Diversity Act, 2002:

- Under Section 37, State Governments can notify ecologically significant areas as BHS in consultation with local bodies.
- The National Biodiversity Authority (NBA) provides guidance on site selection and management.
- Section 37(3) mandates State Governments to frame compensation or rehabilitation schemes for individuals affected by BHS designation.
- The creation of BHS aims to install conservation ethics, prevent over-exploitation of resources, and maintain biodiversity while allowing voluntary community participation.
- BHS status does not impose restrictive regulations on local communities beyond those they voluntarily adopt, ensuring sustainable coexistence between conservation and traditional land use practices.

5.3.2.2. Legal framework for Local Biodiversity Heritage Sites (LBHS)

Local Biodiversity Heritage Sites (LBHS) are ecologically significant areas identified and declared at the local level by Biodiversity Management Committees (BMCs) constituted under local self-governments, based on the ecological significance and biodiversity richness of a particular habitat. Such declarations must be formally sanctioned by the Kerala State Biodiversity Board (KSBB), in accordance with

Government Order No. G.O. (MS) No. 05/2020/P dated 03.03.2020 issued under the authority granted by Section 37 and 41 of the Biological Diversity Act, 2002 (Amendment act, 2023), and Rule 20 of the Kerala Biological Diversity Rules, 2008 (Appendix III).

This legal provision empowers the KSBB to recognize and protect ecologically fragile areas, particularly in a state like Kerala, which is characterized by high population density, rich biodiversity, and sensitive ecosystems. LBHS are recognized as a form of Other Effective Area-Based Conservation Measures (OECMs), a globally accepted conservation mechanism under the Convention on Biological Diversity (CBD). As such, they contribute meaningfully to the achievement of international conservation goals, including those outlined in the Kunming-Montreal Global Biodiversity Framework, 2022, which aims to ensure the protection and sustainable management of biodiversity-rich areas beyond conventional protected zones.

5.3.2.3. Protection of the Habitats of *C. malabaricum* as LBHS

The conservation of natural habitats should be prioritized for critically endangered species like *C. malabaricum*. The destruction of its habitat poses a significant threat to its survival, necessitating legal and administrative actions for habitat protection. Since all four known natural habitats of *C. malabaricum* are not located within any officially protected areas such as reserved forests, wildlife sanctuaries, national parks, or Ramsar sites, it is imperative to secure legal recognition and protection for these sites.

A critical challenge is that most of the fringes of these habitats are located on private lands. Therefore, conservation efforts must integrate the interests of landowners and local communities. A comprehensive strategy should be developed that ensures habitat protection while addressing the socio-economic interests of the local communities. Community participation and awareness programs are essential for garnering support from landowners, emphasizing the ecological importance of *C. malabaricum* and the benefits of conservation.

To facilitate habitat protection, KSCSTE-MBGIPS has proposed to the concerned Biodiversity Management Committees (BMCs) through the Kerala State Biodiversity Board to designate the natural habitats of *C. malabaricum* as Local Biodiversity Heritage Sites (LBHS) and the process are ongoing (Appendix IV). This designation would provide legal backing to restrict destructive activities and promote sustainable conservation initiatives.

5.3.2.4. Recommended conservation practice in LBHS with Community Participation and Management

The management of each Local Biodiversity Heritage Site (LBHS) is primarily overseen by the Biodiversity Management Committee (BMC) of the respective local governing body. In cases where an LBHS extends across multiple administrative jurisdictions, a dedicated Biodiversity Heritage Site Management Committee is constituted. This committee comprises local stakeholders, conservation experts, and government representatives, and is responsible for monitoring conservation activities, promoting community involvement, and implementing sustainable management practices.

Since local communities are key stakeholders in conservation, their active involvement is essential in maintaining and protecting LBHS sites. Strategies to foster participation may include incentivized conservation programs, eco-tourism initiatives, and the promotion of alternative livelihood opportunities that align with conservation goals. The integration of traditional ecological knowledge and indigenous conservation practices into site management further strengthens the sustainability and cultural relevance of these efforts.

The 13th Working Group of Local Self-Governments (LSGs) should also be actively involved in the planning and implementation of LBHS management strategies. Dedicated Plan Funds must be allocated for the effective maintenance and development of these sites. Additionally, BMCs should establish and maintain a Local Biodiversity Fund (LBF) to support ongoing conservation, awareness, and restoration efforts. The Technical Support Groups (TSGs) at the district and state levels should provide scientific and technical guidance to assist BMCs in achieving their conservation objectives.

The protection of *C. malabaricum* through the declaration of its natural habitat as an LBHS represents a vital conservation intervention. Leveraging the existing legal framework, encouraging community-based stewardship, and ensuring robust site management will not only help secure the long-term survival of *C. malabaricum*, but also contribute to regional biodiversity conservation. The collaborative efforts of policy makers, governmental agencies, BMCs, TSGs, academic institutions, and local communities are essential for the successful implementation and sustainability of these conservation measures.

5.3.3. Declaration of District Flower: *Crinum malabaricum* in Kasaragod and its Conservation Implications.

On October 14, 2023, the Kasaragod District administration, in collaboration with the Kerala State Biodiversity Board (KSBB), declared *Crinum malabaricum* as the district flower. This marks the first instance in India where a district has designated a tree, flower, bird, and animal, thereby setting a significant precedent for decentralized biodiversity conservation. The initiative aims to promote conservation awareness by assigning symbolic biodiversity representatives to each district.

The choice of *C. malabaricum* was influenced by its endangered status as an endemic species in the laterite wetlands of Kasaragod district, northern Kerala, where it faces threats from habitat loss due to laterite mining. The initiative is in line with the objectives of the section 41 (1A) of the Biological Diversity Act, 2002 (Amendment act, 2023) and the Kerala Biological Diversity Rules, 2008. These legal frameworks empower local self-governing bodies and Biodiversity Management Committees (BMCs) to participate in activities focused on biodiversity conservation and public awareness. According to the Kerala Biological Diversity Rules of 2008, the State Biodiversity Board (KSBB) is authorized under Rule 23, and BMCs under Rule 24, to facilitate the documentation of biodiversity through People's Biodiversity Registers, promote public education, and support community-driven conservation efforts.

The designation of *C. malabaricum* as the district flower is crucial for its conservation through several mechanisms:

- **Increased Public Awareness:** The species' symbolic status enhances community recognition, pride, and a sense of responsibility toward its protection.
- **Highlighting the Ecological Significance:** As a keystone species in its ecosystem, *C. malabaricum* supports the breeding and feeding of diverse fauna such as odonates, fishes, and amphibians, serving as a bioindicator of wetland health.
- **Heritage Value:** Its designation imbues the species with cultural and natural heritage significance, aiding in its long-term conservation narrative.
- **Educational Value:** Featuring in local biodiversity education and outreach programs, it raises awareness of the region's unique and fragile ecosystems.
- **Policy Prioritization:** Recognized symbols are more likely to receive attention in conservation planning, habitat restoration, and district-level resource allocation.
- **Community-based Conservation:** Local stakeholders, including Panchayats and BMCs, are more likely to implement protective measures such as habitat fencing, land-use regulation, and restoration.
- **Integration into PBR and Tourism:** The species' inclusion in People's Biodiversity Registers and promotion through eco-tourism initiatives strengthens both conservation and sustainable livelihood options.

Declaration of *C. malabaricum* as the 'District flower of Kasaragod' serves as a strategic and symbolic conservation action. It not only enhances community engagement and awareness but also reflects the growing importance of localized, participatory approaches in protecting endangered species and ecologically sensitive habitats.

Chapter 6

Molecular Phylogenetic Analysis

Chapter 6

Molecular Phylogenetic Analysis

6.1. Introduction

Molecular markers serve as essential tools in modern biological research, offering precision and reliability in identifying genetic traits and distinguishing between species (Schulman, 2007). Unlike traditional markers influenced by environmental conditions, molecular markers provide objective and reproducible data by analysing variations at the DNA level. These markers have revolutionized various disciplines, including conservation biology, taxonomy, and evolutionary studies, by enabling the precise identification of species and understanding their evolutionary history (Gepts, 1993). In conservation biology, molecular markers play a crucial role in identifying, prioritizing, and conserving species at risk of extinction. Genetic diversity, a cornerstone of species adaptation and survival, can be effectively assessed using these markers, offering insights into intra- and interspecific relationships (Primmer, 2009).

Non-coding chloroplast DNA regions and nuclear ITS sequences are widely used molecular markers in plant phylogenetics, providing accurate taxonomic classification and insights into evolutionary relationships. These markers have proven effective in resolving species boundaries and clarifying evolutionary relationships in many plant families, including Amaryllidaceae (Meerow & Snijman, 2001). Nuclear ITS markers are recognized for their efficiency in elucidating genetic diversity and evolutionary relationships in critically endangered plant species (Atasagun, 2022; Liu *et al.,* 2023).

The *trnL-trnF* region has an optimal sequence length for barcoding and contains highly variable, parsimony-informative sites. This intergenic spacer region enhances the resolution of closely related species and serves as a powerful tool for assessing genetic diversity, making it well-suited for phylogenetic analysis (Yılmaz, 2023; Filiz *et al.,* 2024). These markers are particularly valuable in species-rich and taxonomically complex groups like *Crinum*, where they facilitate the development of informed conservation strategies.

The genus *Crinum* is an ecologically and morphologically diverse group within the family Amaryllidaceae, comprising approximately 115 species distributed across Asia, Africa, the Americas, and Australia. It is the only pantropical genus within the family, with its broad distribution attributed to oceanic seed dispersal (Koshimizu, 1930; Arroyo & Cutler, 1984; Snijman, 2001). Within this global diversity, the Indian subcontinent harbours 15 species, occupying a range of ecological niches from coastal regions to inland plateaus, many of which remain taxonomically unresolved due to a lack of molecular studies (Lekhak *et al.,* 2015; Patel & Patel, 2019). Taxonomically, *C. brachynema*, *C. woodrowii*, and *C. latifolium* belong to the subgenus *Codono Crinum*, while the remaining species, including *C. malabaricum*, fall under *Stenaster*. However, the phylogenetic relationships among these species remain poorly understood due to a scarcity of molecular studies focusing on Indian *Crinum*.

Molecular phylogenetics has emerged as a critical tool in resolving taxonomic ambiguities and elucidating evolutionary relationships within this genus (Kwembeya *et al.,* 2007). Chloroplast and nuclear markers, such as *trnL-F* and nrITS regions, have been particularly effective in reconstructing phylogenies and clarifying species boundaries (Meerow *et al.,* 2003; Kwembeya *et al.,* 2007). However, comprehensive studies integrating molecular data to explore the evolutionary placement of species like *C. malabaricum* within the Indian flora remain sparse. Given its critically endangered status and restricted distribution on laterite plateaus in northern Kerala, a molecular phylogenetic assessment is essential for understanding its evolutionary placement and informing conservation strategies.

This study aims to elucidate the evolutionary relationships among *Crinum* species in India, with a specific focus on *C. malabaricum*, using ITS and *trnL-F* markers. By employing these molecular markers, this research reconstructs phylogenetic relationships within the genus and provides insights into its evolutionary trajectory. The findings contribute to the taxonomic refinement of *Crinum* species and emphasize the role of molecular data in supporting conservation efforts. This work represents a significant step toward understanding the genetic diversity and evolutionary significance of *C. malabaricum*, a critically endangered species, while also enhancing the phylogenetic framework of the genus in the Indian subcontinent.

6.2. Materials and Methods

6.2.1. Plant Specimen Collection

Field exploration trips were conducted across various phytogeographic regions of India between 2021 and 2024. Data including latitude, longitude, altitude, locality, and habitat of the plants were recorded. Photographs of each species were captured in their natural habitats using digital camera (Canon EOS 70D). Leaf samples were preserved under dry conditions using silica gel crystals, while bulbs from each accession were collected and introduced into the germplasm at the KSCSTE-MBGIPS, Kozhikode.

Species identification was confirmed through consultation with protologues, type specimens, and references to national and regional floras, as well as monographic and revisionary works. Herbarium material from the Central National Herbarium, Kolkata (CNH), Madras Herbarium (MH), Royal Botanic Gardens, Kew (K), and Sivaji university Herbarium (SUK), were consulted for species identification. Herbarium specimens for each accession were prepared and deposited in the MBGH herbaria**.** The study includes species of the genus *Crinum* found in India, along with select ornamental species and species with similar habitats to *C. malabaricum*. Below are the details:

Crinum **Species in India:**

1. *C. amoenum* Roxb. ex Ker Gawl., J. Sci. Arts (London) 3: 106 (1817)
2. *C. Asiaticum* L., Sp. Pl. 1: 292 (1753)
3. *C. Brachynema* Herb., Edwards's Bot. Reg. 28(Misc.): 36 (1842).
4. *C. malabaricum* Lekhak & S.R.Yadav, Kew Bull. 67(3): 521 (2012).
5. *C. reddyi* M.Patel & H.Patel, Nordic J. Bot. 37(4)-e02172: 2 (2019).
6. *C. solapurense* S.P.Gaikwad, Garad & Gore*,* Kew Bull. 69(2)-9505:1 (2014).
7. *C. woodrowii* Baker ex W.Watson, Gard. & Forest 10: 324 (1897)
8. *C. lorifolium* Roxb., J. Sci. Arts (London) 3(5): 110 (-111) (1817).
9. *C. Latifolium* L., Sp. Pl. 1: 291 (1753)
10. *C. viviparum* R.Ansari & V.J.Nair, J. Econ. Taxon. Bot. 11: 205 (1988).
11. *C. wattii* Baker, Handb. Amaryll.: 76 (1888)

Ornamental *Crinum* species

12. *C. amabile* Donn ex Ker Gawl., Bot. Mag. 39: t. 1605 (1814)
13. *C. jagus* (J.Thomps.) Dandy, J. Bot. 77: 64 (1939)

Species with similar habit of *C. malabaricum*

14. *C. thaianum* J.Schulze, Pl. Life 27: 127 (1971) – Native to Thailand
15. *C. natans* Baker, Fl. Trop. Afr. 7 : 396 (1898) – Native to W&C Africa
16. *C. calamistratum* Bogner & Heine, Aqua Pl.: 127 (1987) – Native to Cameroon

6.2.2. DNA Isolation

The fresh leaf samples, silica gel dried samples (to ensure rapid drying and minimal DNA degradation), and specimens from herbarium were used for DNA isolation. A modified CTAB DNA isolation protocol (Doyle & Doyle, 1987; Valiya Thodiyil *et al.*, 2024) and Dneasy® Plant Pro Kit (QAIGENE, Germany) were used for DNA isolation. The equipment used in the study (Table 31) and the stock solutions required for genomic DNA extraction (Table 32) are listed.

Equipment	**Make/ Model**
Autoclave	Trueklav, India
Deep freezer (-20^{0}C)	Cellfrost, India
Deep freezer (-80^{0}C)	U101, New Brunswick, Germany
Electronic balance	Sartorius, Germany
Electrophoresis Power Unit	Bio-Rad, USA
Gel Documentation system	Gel doc XR+, Bio-Rad, USA
Hot Air Oven	Beston, India
Micro Centrifuge	Eppendorf, Germany
Micro Wave Oven	LG, India
Micropipettes	Eppendorf, Germany
pH Meter	LAQUA pH-1100, Horiba, Japan
Refrigerated Micro Centrifuge	Eppendorf, Germany
Thermal cycler (PCR)	S1000, Bio-Rad, USA
Nanodrop Spectrophotometer	Multiskansky, Thermo-scientific, USA
Vortex Mixer	Neuation, iswix VT
Water bath	SW 22, Julabo, Germany
Water purification system	Millipore, France

Table 31: List of Equipment used in the study

Solutions Composition

Solutions	**Composition**	**Amount**
Tris Buffer pH 8	Tris 1 M H2O	12.11 gm 100 ml
EDTA pH 8	Na2 EDTA H2O	18.61 gm 100 ml
CTAB Extraction Buffer pH 8.0 Stored at room temperature	CTAB 2% w/v Tris Buffer 100 mM Na2 EDTA 20 mM PVP 1% NaCl 1.4 M H2O	2 gm 10 ml 4 ml 1 gm 8.2 gm 100 ml
High Salt TE pH 8.0 Stored at room temperature	Tris Buffer 10 mM Na2 EDTA 0.1 mM NaCl 1 M H2O	1 ml 20 ul 5.85 gm 100 ml
CTAB Precipitation Solution pH 8.0 Stored at room temperature	CTAB 1% w/v Tris Buffer 50 mM Na2 EDTA 10 mM H2O	1 gm 5 ml 2 ml 100 ml
TE Buffer	Tris Buffer 10 mM Na2 EDTA 1 M H2O	1 ml 0.2 ml 100 ml
Sodium Acetate	Sodium Acetate 3 M H2O	40.8 gm 100 m

Table 32: Stock solutions required for Genomic DNA extraction

Procedure (Modified Protocol).

- Taken 100mg of fresh young leaf sample. The sample frozen in liquid nitrogen and ground to fine powder using mortar and pestle.
- Preheated the extraction buffer. Added Polyvinylpyrrolidone (PVP) was added to the extraction buffer (0.2 g for 0.5 mL) just before use.
- Added 500µl of CTAB Extraction buffer to the pulverized leaf sample. Mixed thoroughly and transferred into 2 mL micro centrifuge tubes.

- Incubated at 65˚C for 45 – 60 min in a water bath with occasional mixing at regular intervals.
- To facilitate phase separation, Added 700µl Chloroform: Isoamyl (24:1) to the mixture. Mixed well to make an emulsion, followed by centrifugation at 12,000 rpm for 15mins at 25˚C.
- Collected the upper aqueous phase, containing the extracted DNA to a fresh micro centrifuge tube. Added equal volume of chloroform and mix well.
- The mixture was centrifuged at 12,000 rpm for 10 mins at 4˚C.
- Upper layer is transferred to a fresh Eppendorf tube. To this added 500 µl 100% chilled ethanol/ isopropanol along the sides of the tubes. The tube was gently inverted to mixed the contents. Kept in -20˚C for 1-2 hour or overnight.
- Centrifuged the samples at 10,000 rpm for 15 mins at 4˚C.
- Collected the Pellet and wash with 70% ethanol. Dried under vacuum.
- Resuspended the pellets in 100-200µl of TE buffer.
- Added 3 – 4 µl Rnase A (Sigma) and incubated at 37 ˚C for 1-2 hours in a water bath.
- Added 500 µl Phenol: Chloroform: Isoamyl alcohol (25:24:1) to the suspension, mixed well and centrifuged at 10,000 rpm for 10 mins at 4˚C.
- Collect the supernatant.
- Add 500 µl Chloroform to this solution, mix gently and centrifuge at 10,000 rpm for 5 mins at 4˚C.
- Collect the supernatant in a fresh 1.5ml micro centrifuge tube. Add double volume of chilled isopropanol and 1/10th (50 µl) volume of 3M solution of sodium acetate. Kept overnight for incubation at 20˚C.

- Collect the pellet by centrifuging the solution at 12,000 rpm for 15 mins at 4˚C.
- Wash the pellet with 70% cold ethanol twice at 10000rpm for 5min.
- Re-suspend the pellets in 50 µl of 1X TE buffer and stored at -20˚C.

Procedure for Dneasy® Plant Pro Kit

- Taken 100 mg of fresh plant tissue and add 500 µl of solution CD1 to a 2 ml tissue disruption tube. Homogenized using a vortex at maximum speed for 10 min.
- Centrifuged the tissue disruption tubes at 12,000 x g for 2 min.
- Transferred the supernatant to a clean 1.5 ml microcentrifuge tube.
- Added 200 µl Solution CD2 and vortex for 5 s.
- Centrifuged at 12,000 x g for 1 min at room temperature. Transferred the supernatant to a clean 1.5 ml microcentrifuge tube
- Added 500 µl of buffer APP and vortex for 5 s.
- Loaded 600 µl lysate onto an MB Spin Column. Centrifuged at 12,000 x g for 1 min.
- Discarded the flow-through and repeated step 8 to ensure that all of the lysate has passed through the MB spin column.
- Placed the MB spin column into a clean 2 ml collection tube.
- Added 650 µl Buffer AW1 to the MB spin column. Centrifuged at 12,000 x g for 1 min.
- Discarded the flow-through and place the MB spin column back into the same 2 ml collection tube.
- Added 650 µl of Buffer AW2 to the MB spin column.

- Centrifuged at 12,000 x g for 1 min. Discarded the flow-through and place the spin column into the same collection tube.
- Centrifuged at up to 16,000 x g for 2 min. Placed the MB spin column into a new 1.5 ml elution tube.
- Added 50–100 µl of Buffer EB to the center of the filter membrane.
- Centrifuged at 12,000 x g for 1 min.
- Discarded the MB spin column. Stored the DNA at -20˚C.

6.2.3. Estimation of DNA

The extracted DNA was assessed for both quality and quantity. Agarose gel electrophoresis was utilized for the qualitative estimation of DNA. The extracted DNA was mixed with a 6X loading buffer and loaded in to the wells on a 0.8% agarose gel containing 0.5 mg/mL ethidium bromide (Table 33). One well was loaded with 1 kb BenchTop DNA ladder (Promega, USA) to estimate the molecular weight of the DNA. The gel was subjected to electrophoresis at 100 V for 20 – 40 minutes using a gel electrophoresis unit (Bio-Rad, USA). Afterward, the gel was visualized under UV light using the GelDoc system (Bio-Rad, USA), and the images were recorded.

Reagents	**Composition**
Loading buffer (6X)	Bromophenol blue- 0.25% Xylene cyanol FF- 0.25% Sucrose- 40%
TBE buffer (10X)	Tris base- 21.6 g Boric acid-11.0 g 0.5 EDTA (pH 8.0)- 8.0 mL Made up to 200 mL with dist. H2O
Ethidium bromide	0.5 mg/mL

Table 33. Stock solutions required for agarose gel electrophoresis

The optical density (O.D.) of the extracted DNA was measured in the range of 230–300 nm using a spectrophotometer (Multiskan Sky, Thermo Scientific, USA). The purity and concentration of the DNA samples were calculated using SpectraManager software (Thermo Scientific). Samples with high purity (A260:A280 ratio of 1.8–2.0) were diluted to a final concentration of 50–100 ng/µL and stored at -20°C for subsequent use in downstream applications.

6.2.4. PCR Amplification of Ribosomal Region

Amplification of the ribosomal DNA ITS1/5.8S/ITS2 region was carried out in a 25 µL reaction mixture using KAPA2G Robust HotStart Ready-mix (Sigma-Aldrich, Germany). The flanking primers used were ITS-AB-101 (5′-ACGAATTCATGGT CCGGTGAAGTGTTCG-3′) and ITS-AB-102 (5′-TAGAATTCCCCGGTT CGCTCGCCGTTAC-3′), targeting the 18S and 26S regions, respectively. Primers were synthesized by Integrated DNA Technologies, USA, following the protocol described by Douzery et al. (1999). The reaction mixture contained 50 ng of genomic DNA, 25 mM dNTP Buffer A (100 mM Tris-HCl, pH 8.0 at 25°C; 15 mM $MgCl_2$; 500 mM KCl;10% Triton X-100), 5 µL of Enhancer, and 1 µL each of 10 µM forward and reverse primers (Table 34).

Amplification was carried out using a thermal cycler (S100, Bio-Rad, USA) with the following PCR conditions: an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 30 seconds at 94°C (denaturation), 30 seconds at 55°C (annealing), and 2 minutes at 72°C (extension). A final extension was carried out at 72°C for 5 minutes (Table 35). The amplified products were resolved on a 2 % agarose gel containing 0.5 µg/mL ethidium bromide. The gel was run on a gel electrophoresis system and analysed for the quality of the PCR products.

6.2.5. PCR Amplification of *trnL-F* Region

Amplifications were performed using the S1000 thermal cycler (Bio-Rad, USA) with Emerald® GT Mastermix (Takara, Japan). Each reaction was carried out in a 25 µL volume containing the following components: 1.0 µL of template DNA (~50–100 ng), 16 µL of nuclease-free water, 12.5 µL of Mastermix (100 mM Tris-HCl, pH 8.0 at 25°C; 15 mM $MgCl_2$; 500 mM KCl; 10% Triton X-100), 0.2 µL of 25 mM dNTP mixture, 0.74 µL of Taq polymerase, and 1 µL each of 10 µM forward and reverse primers (Table 34). The amplification process followed these parameters: an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 45 seconds at 94°C (denaturation), 45 seconds at 56.5°C (annealing), and 3 minutes at 72°C (extension), with a final extension at 72°C for 5 minutes (Table 35). Quality check for the samples was carried out by gel electrophoresis (2% agarose gel).

Locus	Primer	Sequence	Reference
nrITS	ITS- A 101	5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3'	Douzery *et al.,* 1999.
	ITS- A 102	5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3'	
trnF – trnL	trn A50272	5'-ATTTGAACTGGTGACACGAG-3'	Taberlete *et al.,*1991.
	Trn B49317	5'-CGAAATCGGTAGACGCTACG-3'	

Table 34: Primers used for PCR and sequencing.

Stage	KAPA2G Robust HotStart ReadyMix ITS region.	Cycles	Emerald® GT PCR master mix rbcL Region	Cycles
Initial denaturation	95°C/3 min		94^0 C/3 min	
Denaturation	95°C/15 sec	40 Cycles	94^0 C/45 sec	35 Cycles
Annealing	55°C/15 sec		56.5^0 C/45 sec	
Elongation	72°C/60 sec		72^0 C/ 3min	
Final extension	72°C/1 min		72^0 C/5min	

Table 35: Thermal cycling conditions for amplification

6.2.6. Sequencing and Sequence Analysis

Samples were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) and prepared for sequencing with the Big Dye Terminator v3.1 kit on an MiniAmp Plus Thermal Cycler (Applied Biosystems, USA). The primer-extended products were then subjected to capillary electrophoresis and analysis using an 3730xL Genetic Analyzer (Applied Biosystems, USA) with Pop7 polymer, employing the Sanger di-deoxy chain termination method. The quality of the resulting sequences was evaluated using Sequence Scanner Software v1 (Applied Biosystems).

The raw sequences using the sequence alignment editor, BIOEDIT, version 7.2.6 (Hall, 1999). The finalized nucleotide sequences were compared against the NCBI database using the BLAST search tool (Altschul *et al.,* 1990) for taxonomic identification, with species determination based on the highest BLAST score. All newly generated sequences have been deposited in GenBank (Table 36).

6.2.7. Phylogenetic Analysis

For the phylogenetic analysis, in addition to the 58 newly sequenced ITS and *trnL-F* region sequences, 70 ITS and 55 *trnL-F* sequences, including outgroups, were retrieved from GenBank (Benson *et al.,* 2013). The outgroup taxa, *Amaryllis belladonna* L., *Ammocharis angolensis* (Baker) Milne-Redh. & Schweick., *A. coranica* (Ker Gawl.) Herb., *A. longifolia* (L.) Herb., *A. baumii* (Harms) Milne-Redh. & Schweick and *A. nerinoides* (Baker) Lehmiller—were selected based on previous studies on Amaryllidaceae (Meerow *et al.,* 2003) (Table 37).

The forward and reverse sequences were aligned using the MUSCLE algorithm (Edgar, 2004) in the MEGA12 interface (Tamura *et al.,* 2021). The alignment was then automatically refined using the del.colgapsonly function from the phangorn R package (Schliep, 2011) to remove poorly aligned regions and excessive gaps. The refined alignment was used for phylogenetic analysis. Maximum Likelihood (ML) phylogenetic analyses were conducted using IQ-TREE version 2.2.2.6 (Minh *et al.,* 2020) on the IQ-TREE web server. ModelFinder (Kalyaanamoorthy *et al.,* 2017) was used for model selection, identifying TIM2+F+G4 as the best-fitting model for the combined dataset. For individual gene analyses, K2+G+I and TVM+F+I+G4 were selected as the optimal models for ITS and *trnL-F*, respectively. Branch support was assessed using 1,000 ultrafast bootstrap replicates (Hoang *et al.,* 2018) and the SH-like approximate Likelihood Ratio Test (SH-aLRT) with 1,000 replicates. The final consensus tree was visualized using iTOL (Letunic & Bork, 2021). Each DNA region was analysed separately and then combined for phylogenetic reconstruction. Well-supported clades (≥75% bootstrap support and ≥0.95 posterior probability) were compared to detect topological conflicts.

Sl.No.	**Voucher No.**	**Species**	**Location**	**GenBank Acession No.**	
				trnL-F	**ITS**
1	18627	*Crinum* × *amabile* Donn ex Ker Gawl	INDIA: Kerala, Palakad	PV031008	PV037396
2	18609	*Crinum* × *amabile* Donn ex Ker Gawl	INDIA: Kerala, Kozhikode	PV232629	PV037383
3	18632	*Crinum amoenum* Roxb. ex Ker Gawl	INDIA: Assam, Kanchanjuri	PV031013	PV037401
4	18637	*Crinum amoenum* Roxb. ex Ker Gawl	INDIA: West Bengal, Sevoke	PV031017	PV037404
5	18638	*Crinum amoenum* Roxb. ex Ker Gawl	INDIA: West Bengal, Jalpaiguri	PV031018	PV037405
6	18665	*Crinum amoenum* Roxb. ex Ker Gawl	INDIA: West Bengal, Alipurduar	PV031034	PV211241
7	18601	*Crinum asiaticum* L.	INDIA: Kerala, Kozhikode	PV030987	PV037376
8	18613	*Crinum asiaticum* L.	INDIA: Kerala, Alapuzha	PV030997	PV037386
9	18628	*Crinum asiaticum* L.	INDIA: West Bengal, Siliguri	PV031009	PV037397
10	18642	*Crinum asiaticum* L.	INDIA: Maharashtra, Mahabaleshwar	PV031019	PV037406
11	18621	*Crinum brachynema* Herb.	INDIA: Maharashtra, Katespoint	PV031002	PV037390
12	18662	*Crinum brachynema* Herb.	INDIA: Maharashtra, Mahabaleshwar	PV031031	PV037411
13	18646	*Crinum calamistratum* Bogner & Heine	Cameroon	PV031022	PV037409
14	18658	*Crinum calamistratum* Bogner & Heine	Cameroon	PV031027	PV037410
15	18607	*Crinum jagus* (J.Thomps.) Dandy	INDIA: Kerala, Wayanad	PV030992	PV037381

16	18629	*Crinum jagus* (J.Thomps.) Dandy	INDIA: Kerala, Kozhikode	PV031010	PV037398
17	18630	*Crinum jagus* (J.Thomps.) Dandy	INDIA: West Bengal, Jalpaiguri	PV031011	PV037399
18	18643	*Crinum latifolium* L.	INDIA: Maharashtra, Thane	PV232631	PV037407
19	18603	*Crinum latifolium* L.	INDIA: Kerala, Pallom	PV030989	PV037378
20	18606	*Crinum latifolium* L.	INDIA: Kerala, Kalpatta	PV030991	PV037380
21	18610	*Crinum latifolium* L.	INDIA: Madhya Pradesh, Jamni	PV030994	PV037384
22	18612	*Crinum latifolium* L.	INDIA: Andhra Pradesh, Nellor	PV030996	PV035726
23	18616	*Crinum latifolium* L.	INDIA: Kerala, Nelliyampathy	PV030999	PV037387
24	18617	*Crinum latifolium* L.	INDIA: Karanataka, Karkala	PV031000	PV037388
25	18631	*Crinum latifolium* L.	INDIA: Karnataka, Putur	PV031012	PV037400
26	18636	*Crinum latifolium* L.	INDIA: Chhattisgarh	PV031016	PV037403
27	18659	*Crinum latifolium* L.	INDIA: Tamil Nadu, Yercaud	PV031028	PV211238
28	18618	*Crinum lorifolium* Roxb.,	INDIA: Karanataka, Udupi	PV031001	PV037389
29	18626	*Crinum lorifolium* Roxb.,	INDIA: Maharashtra, Dabhole	PV031007	PV037395
30	18624	*Crinum lorifolium* Roxb.,	INDIA: Maharashtra, Devgad	PV031005	PV037393
31	18625	*Crinum lorifolium* Roxb.,	INDIA: Maharashtra, Naringre	PV031006	PV037394
32	18653	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Periye	PV031023	PV035720

33	18654	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Cheemeni	PV031024	PV035721
34	18655	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Aravanchal	PV031025	PV035722
35	18656	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Embate	PV031026	PV035723
36	18666	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Periye	PV031035	PV211242
37	18669	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Cheemeni	PV031038	PV035724
38	18670	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Aravanchal	PV031039	PV035725
39	18657	*Crinum malabaricum* Lekhak & S.R.Yadav	INDIA: Kerala, Embate	PV232633	PV211237
40	18645	*Crinum natans* Baker	Cameroon	PV031021	PV211235
41	18667	*Crinum natans* Baker	Cameroon	PV031036	PV211243
42	18634	*Crinum reddyi* M.Patel & H.Patel	INDIA: Gujrat, Tapi	PV031014	PV035718
43	18635	*Crinum reddyi* M.Patel & H.Patel	INDIA: Gujrat, Ghuntvel	PV031015	PV035719
44	18615	*Crinum solapurense* S.P.Gaikwad, Garad & Gore	INDIA: Maharashtra, Solapur	PV030998	PV035717
45	18661	*Crinum solapurense* S.P.Gaikwad, Garad & Gore	INDIA: Maharashtra, Machnur	PV031030	PV211240
46	18644	*Crinum thaianum* J.Schulze	Thailand:Phang Nga	PV031020	PV037408
47	18668	*Crinum thaianum* J.Schulze	Thailand: Ranong	PV031037	PV037414
48	18602	*Crinum viviparum* R.Ansari & V.J.Nair	INDIA: Kerala, Kozhikode	PV030988	PV037377
49	18604	*Crinum viviparum* R.Ansari & V.J.Nair	INDIA: Kerala, Kumarakom	PV030990	PV037379

50	18608	*Crinum viviparum* R.Ansari & V.J.Nair	INDIA: Karanataka, karakala	PV030993	PV037382
51	18611	*Crinum viviparum* R.Ansari & V.J.Nair	INDIA: Maharashtra, Satara	PV030995	PV037385
52	18660	*Crinum viviparum* R.Ansari & V.J.Nair	INDIA: West Bengal, Jalpaiguri	PV031029	PV211239
53	18648	*Crinum wattii* Baker	INDIA: Manipur	PV232632	PV211236
54	18622	*Crinum woodrowii* Baker ex W.Watson	INDIA: Maharashtra, Lingmala	PV031003	PV037391
55	18623	*Crinum woodrowii* Baker ex W.Watson	INDIA: Maharashtra, Pune	PV031004	PV037392
56	18663	*Crinum woodrowii* Baker ex W.Watson	INDIA: Maharashtra, Satara	PV031032	PV037412
57	18664	*Crinum woodrowii* Baker ex W.Watson	INDIA: Maharashtra	PV031033	PV037413
58	18633	*Crinum woodrowii* Baker ex W.Watson	INDIA: Maharashtra, Tungareshwar	PV232630	PV037402

Table 36: Details of the C*rinum spp.* With voucher number and GenBank accession numbers

Sl.No.	Species	Geographic Location	GenBank Acession No.	
			ITS	***trnLF***
1.	*Amaryllis belladonna* L.	Australia	AF373084	AF104795
2.	*Ammocharis angolensis* (Baker) Milne-Redh. & Schweick.	Tropical Africa	EF111004	EF119727
3.	*Ammocharis coranica* (Ker Gawl.) Herb.	South and South-west Africa	AF373080	AY139152
4.	*Ammocharis longifolia* (L.) Herb.	South Africa	AF373093	AY139169
5.	*Ammocharis nerinoides* (Baker) Lehmiller	South-west Africa	AY139116	MW980621
6.	*Ammocharis baumii* (Harms) Milne-Redh. & Schweick.	South Africa	AY139121	AY139156
7.	*Crinum abyssinicum* Hochst. Ex A.Rich.	East Africa	AY139117	AY139153
8.	*Crinum acaule* Baker	Tropical Africa	AY139118	EU523769
9.	*Crinum americanum* L.	North America	AY139119	AY139154
10.	*Crinum asiaticum* L.	South-East Asia and Pacific	AY139120	DQ388360
11.	*Crinum asiaticum* L.	South-East Asia and Pacific	OL584032	AB113161
12.	*Crinum asiaticum* L.	South-East Asia and Pacific	OL584034	AY139155
13.	*Crinum asiaticum* L.	South-East Asia and Pacific	OL584039	MZ047856
14.	*Crinum asiaticum* var.*pedunculatum* (R.Br.) Fosberg & Sachet	Australia	AY139143	AY139167
15.	*Crinum binghamii* Nordal & Kwembeya	Tropical Africa	EF111008	EF119731
16.	*Crinum bulbispermum* (Burm.f.) Milne-Redh. & Schweick.	South africa	AY139123	EU523772
17.	*Crinum bulbispermum* (Burm.f.) Milne-Redh. & Schweick.	South africa	JX464263	JX464340
18.	*Crinum buphanoides* Welw. ex Baker	Tropical Africa	EF111009	AY139157
19.	*Crinum buphanoides* Welw. ex Baker	Tropical Africa	AY139124	EF119732

20.	*Crinum calamistratum* Bogner & Heine	Tropical Africa	EU836631	EU836621
21.	*Crinum carolo-schmidtii* Dinter	Tropical Africa	AY139125	EU523776
22.	*Crinum campanulatum* Herb.	South Africa	AF373088	AY139158
23.	*Crinum crassicaule* Baker	Tropical Africa	AY139126	EF119737
24.	*Crinum crassicaule* Baker	Tropical Africa	EF111016	EF119736
25.	*Crinum cruentum* Ker Gawl.	Tropical Africa	AY139127	AY139159
26.	*Crinum erubescens* L.f. ex Aiton	South America	AY139130	AY139161
27.	*Crinum firmifolium* Baker	Madagascar	AY139138	AY139165
28.	*Crinum flaccidum* Herb.	Australia	AY139132	AY139162
29.	*Crinum glaucum* A.Chev.	Tropical Africa	EF111007	EF119730
30.	*Crinum jagus* (J.Thomps.) Dandy	Tropical Africa	AY139135	AY139164
31.	*Crinum jagus* (J.Thomps.) Dandy	Tropical Africa	EU836633	EU836623
32.	*Crinum jagus* (J.Thomps.) Dandy	Tropical Africa	OL584035	AJ247487
33.	*Crinum kirkii* Baker	East Africa	AY139136	EF119734
34.	*Crinum kirkii* Baker	East Africa	EF111011	DQ388364
35.	*Crinum lugardiae* N.E.Br.	South Africa	EF111010	EF119733
36.	*Crinum lugardiae* N.E.Br.	South Africa	EF111019	EU523781
37.	*Crinum macowanii* Baker	South Africa	EF111017	EF119738
38.	*Crinum minimum* Milne-Redh.	South Africa	DQ386435	DQ388368
39.	*Crinum minimum* Milne-Redh.	South Africa	EF111014.1	EU523783
40.	*Crinum moorei* Hook.f.	South Africa	JX464265	JX464342
41.	*Crinum moorei* Hook.f.	South Africa	AY139141	EU523784

42.	*Crinum natans* Baker	Tropical Africa	OL584028	EU836626
43.	*Crinum oliganthum* Urb.	Central America	AY139142	AY139166
44.	*Crinum ornatum* (Aiton) Herb.	Tropical Africa	EF111000	EF119723
45.	*Crinum ornatum* (Aiton) Herb.	Tropical Africa	AY139129	AY139160
46.	*Crinum politifolium* R.Wahlstr.	Tropical Africa	AY139144	DQ388366
47.	*Crinum stuhlmannii* Baker	East Africa	EF111006	EF119729
48.	*Crinum stuhlmannii subsp. delagoense* (I.Verd) Kwembeya & Nordal	South Africa	AY139133	AY139163
49.	*Crinum stuhlmannii subsp. delagoense* (I.Verd) Kwembeya & Nordal	South Africa	EF111005.1	EF119728
50.	*Crinum verdoorniae* Lehmiller	South Africa	EF111003	EF119726
51.	*Crinum verdoorniae* Lehmiller	South Africa	EF111002	EF119725
52.	*Crinum verdoorniae* Lehmiller	South Africa	EF111001.1	EF119724
53.	*Crinum walteri* Overkott	Tropical Africa	EF111013	EF119735
54.	*Crinum yemense* Deflers	North Africa	AY139151	AF104784
55.	*Crinum thaianum* J.Schulze	South-East Asia and Pacific	EU836637	EU836627
56.	*Crinum thaianum* J.Schulze	South-East Asia and Pacific	OL584022	NA*
57.	*Crinum thaianum* J.Schulze	South-East Asia and Pacific	OL584023	NA*
58.	*Crinum thaianum* J.Schulze	South-East Asia and Pacific	OL584024	NA*
59.	*Crinum thaianum* J.Schulze	South-East Asia and Pacific	OL584025	NA*
60.	*Crinum thaianum* J.Schulze	South-East Asia and Pacific	OL584026	NA*
61.	*Crinum thaianum* J.Schulze	South-East Asia and Pacific	OL584027	NA*
62.	*Crinum latifolium* L.	India	AY139137	NA*
63.	*Crinum latifolium* L.	India	OL584036	NA*

64.	*Crinum latifolium* L.	India	OL584040	NA*
65.	*Crinum defixum* Ker Gawl.	South Africa	AY139128	NA*
66.	*Crinum amabile* Donn ex Ker Gawl.	South-East Asia and Pacific	OL584030	NA*
67.	*Crinum amoenum* Ker Gawl. Ex Roxb.	South-East Asia and Pacific	OL584029	NA*
68.	*Crinum asiaticum* var. *japonicum* Baker	South-East Asia and Pacific	OL584033	NA*
69.	*Crinum mauritianum* G.Lodd.	Madagascar	AY139139	NA*
70.	*Crinum razafindratsiraea* LehMill.	Madagascar	AY139145	NA*

*NA= *trnl-trF* sequences for this species are not available in GenBank.

Table 37: List of External Sequences: GenBank Accessions of *Crinum* spp. and outgroups (*Amaryllis* spp. & *Ammocharis* spp.)

6.3. Results

6.3.1. Characteristics of the nrITS Region in *Crinum*

The multiple sequence alignment of the Internal Transcribed Spacer (ITS) region, including the 5.8S ribosomal gene, from 128 accessions of *Crinum*, comprised 753 sites, accounting for insertions and deletions. Within this alignment, the ITS1/5.8S/ITS2 region exhibited 484 unique patterns, with 436 parsimony-informative sites, 50 singleton sites, and 267 constant sites. The best fit model for this alignment was determined to be TN+F+I+R2, selected based on the Bayesian Information Criterion (BIC). This model is a variant of the Tamura-Nei (TN93) model, which itself is an extension of the Kimura 2-parameter (K2P) model, accounting for differences in transition and transversion rates (Table 38). The model empirically estimating the base frequencies (+F), incorporates a portion of invariable sites (+I) and Discrete Gamma Rate Categories with two different substitution rates across sites (+R2). During the model optimization, 103 iterations were performed to refine parameters and estimate the phylogenetic tree.

6.3.2. Characteristic of *trnL-F* region in *Crinum*

The *trnL-F* intergenic spacer was analyzed from 113 accessions of *Crinum*, yielding an alignment of 875 nucleotide sites, including insertions and deletions. This dataset exhibited 479 unique patterns, of which 431 were parsimony-informative, 72 were singleton sites, and 372 remained constant. Model selection using the Bayesian Information Criterion (BIC) identified K3Pu+F+R2 as the best-fit substitution model. This model, based on the Kimura 3-parameter framework (K3P), accounts for unequal transition rates and empirical base frequencies (+F) while incorporating two rate categories (+R2) to model site heterogeneity. To achieve parameter optimization and phylogenetic reconstruction, multiple iterations (710) were performed, ensuring a refined evolutionary model (Table 38).

6.3.3. Characteristics of the Combined nrITS + *trnL-F* Tree

Integrating both the ITS and *trnL-F* datasets, the combined alignment consisted of 130 accessions and 2531 nucleotide sites, offering a broader phylogenetic resolution. The dataset displayed 1254 distinct patterns, with 1003 parsimony-informative sites, 214 singleton sites, and 1314 constant sites. The best-fitting evolutionary model, determined by BIC, was TPM3+R3, a transitional model that accounts for differential substitution rates and discrete site variation across three rate categories (+R3).

Compared to the individual datasets, the combined tree exhibited a high log-likelihood score (-4605.378), a longer total tree length (1.684), and stronger bootstrap support (0.998 correlation), emphasizing its greater stability and phylogenetic resolution (Table 37). The increased number of informative sites and improved model fit highlight the advantage of combining nuclear (ITS) and chloroplast (*trnL-F*) markers in reconstructing evolutionary relationships within *Crinum*.

Alignment Information	**ITS (ITS1/5.8s/ITS2)**	***trnL-trnF***	**ITS + *trnLF***
Number of sequences:	128	113	128
Number of characters/columns	753	875	2531
Distinct pattern sites	484	479	1254
Parsimony-informative sites	436	431	1003
Singleton sites	50	72	214
Constant sites	267	372	1314
Best fit model (based on BIC)	TN+F+I+R2	K3Pu+F+R2	TPM3+R3
Total tree length	1.630	0.810	1.684
Log-likelihood of consensus tree	-5393.786	-4474.164	-4605.378

Table 38: Alignment characteristic for the nuclear ribosomal and chloroplast plasmid DNA regions analysed and their combined matrices

The total tree length of the ITS tree (TN+F+I+R2 model) was 1.630 units, while that of the *trnL-F* tree (K3Pu+F+R2 model) was 1.510 units, reflecting the evolutionary relationships captured within these sequences. The ITS tree has a longer total branch length than the *trnL-F* tree, suggesting that the ITS region evolves faster, accumulating more mutations over time. This highlights the genetic variability and evolutionary history of the studied taxa, reinforcing the general observation that ITS is more suitable for resolving recent divergence events, while *trnL-F* is better suited for deeper evolutionary relationships. Similarly, the combined ITS + *trnL-F* tree has a total tree length of 1.684 units, which, being slightly higher than the ITS tree, indicates that the combined dataset effectively captures more evolutionary changes.

The *trnL-F* region contains more constant sites (372 vs. 267 in ITS), making it the more conserved marker. In contrast, the ITS region has more parsimony-informative sites (436 vs. 431 in *trnL-F*), suggesting that ITS provides slightly higher resolution for phylogenetic reconstruction. The log-likelihood score is higher (better fit) in the *trnL-F* tree (-4474 vs. -5393 in ITS), indicating that *trnL-F* offers a more stable phylogenetic reconstruction. These differences align with the expected evolutionary patterns, where ITS is valuable for resolving recent divergence events, while *trnL-F* is better suited for inferring deeper evolutionary relationships due to its higher stability and lower rate of evolutionary saturation.

The bootstrap correlation coefficient serves as a key measure of tree reliability, reflecting the consistency of bootstrap replicates in supporting phylogenetic relationships. Among the three datasets, the ITS tree had a bootstrap correlation coefficient of 0.990, the *trnL-F* tree had 0.967, and the combined ITS + *trnL-F* tree showed the highest value at 0.998. These values suggest that while ITS alone provides strong branch support, the *trnL-F* dataset, being more conserved, has slightly lower correlation due to fewer informative sites. However, the higher correlation in the combined dataset (0.998) indicates superior stability and confidence in phylogenetic inference. The increase in bootstrap correlation in the combined analysis highlights the advantage of integrating nuclear (ITS) and chloroplast (*trnL-F*) markers, as it reduces uncertainty and enhances the overall robustness of the phylogenetic tree.

Overall, these results underscore the comprehensive analysis conducted on the ITS and *trnL-F* regions, emphasizing the diversity of patterns, the abundance of informative sites, and the selection of an optimal evolutionary model for phylogenetic inference.

6.3.4. Phylogenetic Analysis

The optimal Maximum likelihood (ML) analysis of 52 species of genus *Crinum* obtained consensus ML tree (Figure 49). The tree was rooted using the outgroups *Ammocharis spp*. and *Amaryllis sp.* The phylogenetic analysis revealed that the genus *Crinum* is monophyletic with the African, American and Asia-Pacific species forms a separate Clade from outgroups. The divergence of monophyletic *Crinum* clade from their last ancestor is strongly supported with a boot strap value of 100. *C. buphanoides* Welw. ex Baker, *and C. amoenum* Roxb. ex Ker Gawl. are the basal to all the *Crinum* species studied in the present analysis. *C. buphanoides* is a species native to tropical Africa and *C. amoenum* native to South-east Asia.

Ammocharis baumii, formerly *C. baumii* is closely related to the genus Crinum but it clusters with outgrouped *Ammocharis* spp., supports its taxonomic regrouping. This species may be a connecting link between the two genera. Most of the studied species with more than one accession were found to be monophyletic with strong support in the phylogenetic analysis.

Crinum malabaricum forms a well-supported clade (BS100) that includes multiple accessions from different locations in Kerala, indicating its genetic cohesiveness as a distinct taxon. This clade is positioned within the broader *Crinum* genus and is closely related to *C. wattii* suggesting a close evolutionary relationship between these species. Additionally, the *C. malabaricum* clade appears to be closely related to the *C. thaianum* (BS 99) supporting the hypothesis that it shares a more recent common ancestor with these taxa and indicating a biogeographical connection with Southeast Asian species. This relationship of *C. malabaricum* is supported by both ITS and *trnL-F* separate trees (Figure 47 & 48). Similarly, *C. malabaricum* forms a clade with South and Southeast Asian and Pacific species. However, the tree topology suggests that *C. malabaricum* is distinct from African and American *Crinum* lineages, highlighting its unique evolutionary lineage within the genus. The high bootstrap (BS 99) support values in this clade further reinforce the reliability of this relationship, suggesting strong phylogenetic support for *C. malabaricum* as a distinct taxonomic unit within the genus *Crinum*.

The combined phylogenetic tree also supports the ITS tree for the clear separation of Indian *Crinum* species, into distinct clusters (Figure 47 & 49). *C. malabaricum*, along with *C. asiaticum, C. viviparum, C. solapurense, C. reddyi* and *C. watti* are form a cluster. Which shows relationship with Southeast Asian, Pacific and Australian species, confirming its close evolutionary relationship with this clade (BS 96). While the remaining Indian species, such as *C. lorifolium C. latifolium, C. woodrowii,* and *C. brachynema*, form a separate well-supported clade (BS98) and shows close relationship with African species. This inference supports the hypothesis that these Indian species might share a common ancestor with African *Crinum* species, possibly indicating historical biogeographical connections between the Indian subcontinent and Africa.

In contrast, these separation of Indian *Crinum* into two clades are not supported by *trnL-F* tree (Figure 48). It presents a slightly different pattern, where the strict separation between Indian and African *Crinum* species observed in the ITS tree is not as distinct (Figure 47). Despite this, *C. malabaricum* still exhibits a closer relationship with Southeast Asian species, consistent with the ITS tree findings. However, other Indian *Crinum* taxa (*C. latifolium, C. lorifolium, C. woodrowii,* and *C. brachynema*) do not form a strictly separate cluster and appear more dispersed. This suggests that *trnL-F* might have retained more ancestral polymorphism or experienced introgression, leading to an overlap in the genetic relationships between these group. This indicates a more complex evolutionary history, possibly involving historical dispersal events or incomplete lineage sorting.

The phylogenetic tree also shows that American species are monophyletic and are grouped with Tropical and West African species. While Australian species grouped with Soth and Southeast Asia and Pacific species. Similarly, three species from Madagascar forms a monophyletic group and are closely related to Southeast Asia and pacific species.

The combined phylogenetic analysis places other Indian aquatic *Crinum* species viz. *C. viviparum, C. solapurense*, and *C. reddyi*, in sister clade with *C. malabaricum* (BS 96) and *C. thaianum* (BS98). also group within the South and Southeast Asia and Pacific clade. This finding supports the hypothesis that these species share a common biogeographical origin, distinct from other Indian *Crinum* species exhibiting a closer affinity to African species. This suggests that *C. malabaricum* and the other three aquatic *Crinum* species from India may have evolved from an ancestral lineage that was more closely related to Southeast Asian species rather than the African lineages.

However, an interesting taxonomic challenge arises from the clustering of *C. viviparum, C. solapurense*, and *C. reddyi*. These three species appear to form a well-supported group in the combined phylogenetic analysis, indicating a close evolutionary relationship. This raises the question of whether they should be treated as distinct species or if their classification needs reconsideration. Their close genetic relatedness may indicate either recent divergence or potential taxonomic misclassification, warranting further morphological and genetic investigation to clarify their taxonomic status.

Additionally, the current classification of *C. viviparum* as a synonym of the South African species *C. bulbispermum* presents another taxonomic paradox. The phylogenetic trees do not support a close relationship between *C. viviparum* and *C. bulbispermum*, as *C. viviparum* is nested within the South and Southeast Asia and Pacific clade, while *C. bulbispermum* is grouped with other African species. This discrepancy suggests the need for a taxonomic reassessment of *C. viviparum* and challenges its synonymizing with *C. bulbispermum*. The phylogenetic evidence supports the recognition of *C. viviparum* as a distinct species within the South and Southeast Asian and Pacific clade rather than as an African species, which may necessitate a revision of its current taxonomic status. These findings highlight the complexity of *Crinum* phylogeny, emphasizing the importance of integrating molecular data with morphological and biogeographical evidence to resolve taxonomic ambiguities and understand the evolutionary history of the genus.

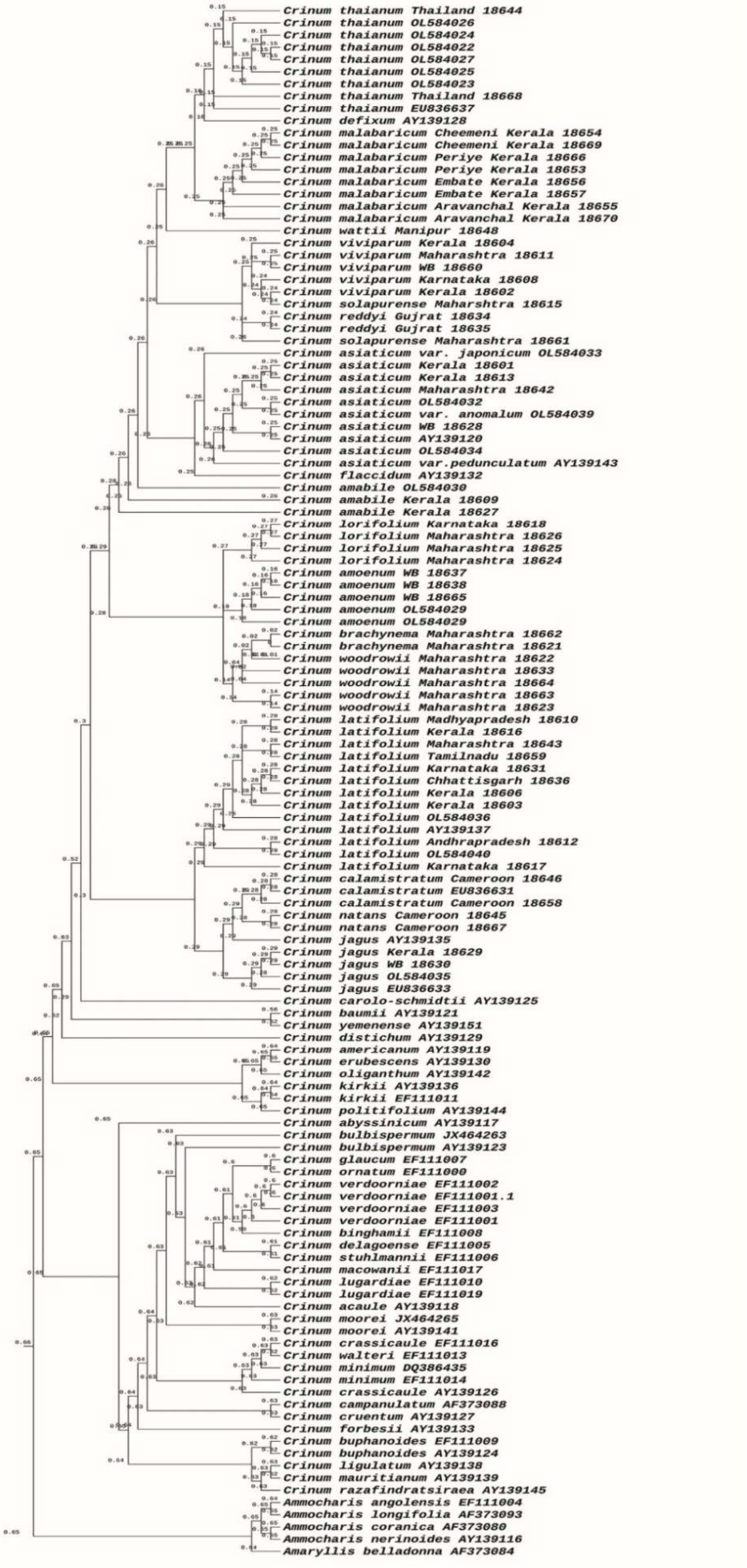


Figure 47: Best ML Tree of the Genus *Crinum* L. based on ITS sequence data

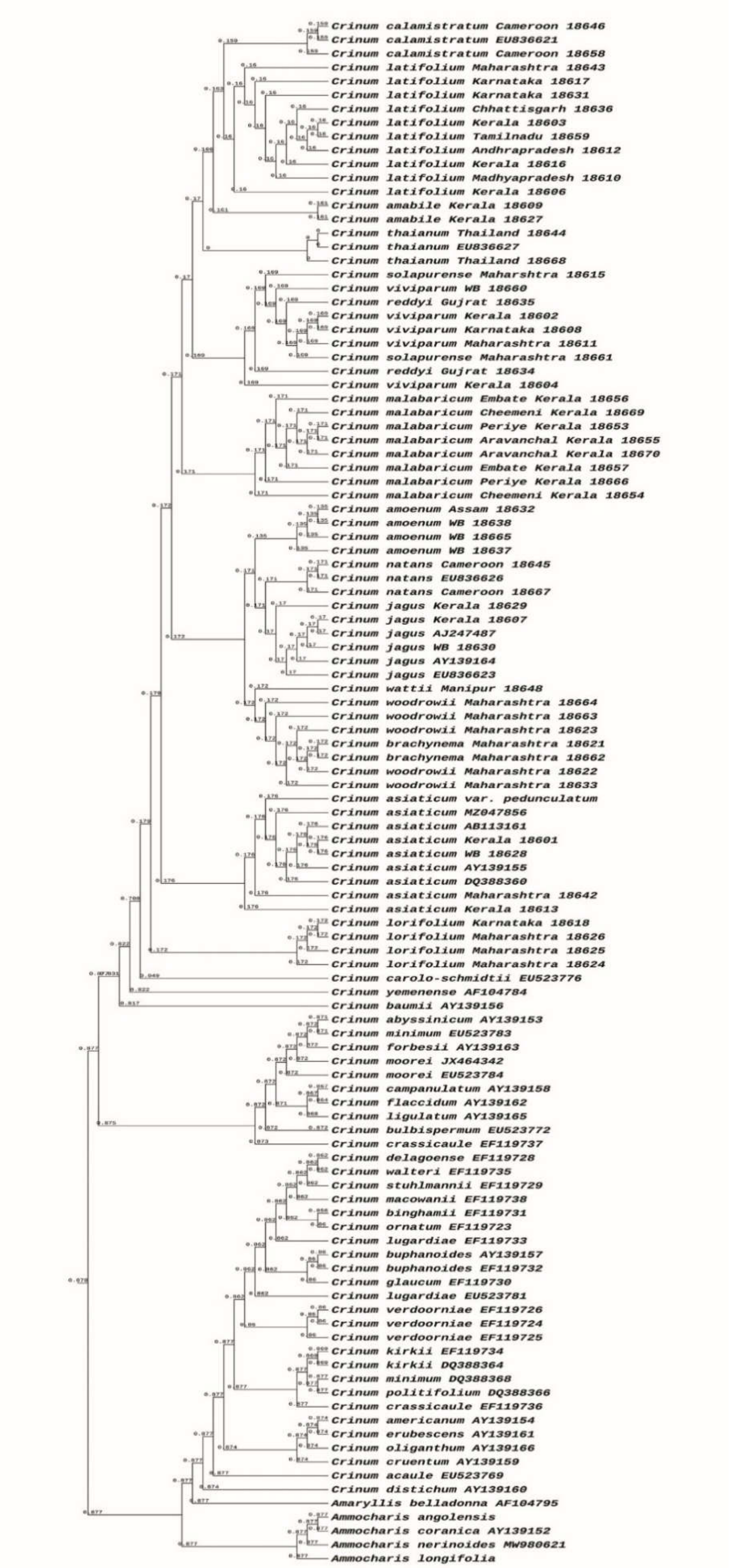


Figure 48: Best ML Tree of the Genus *Crinum* L. based on trnL-F sequence data

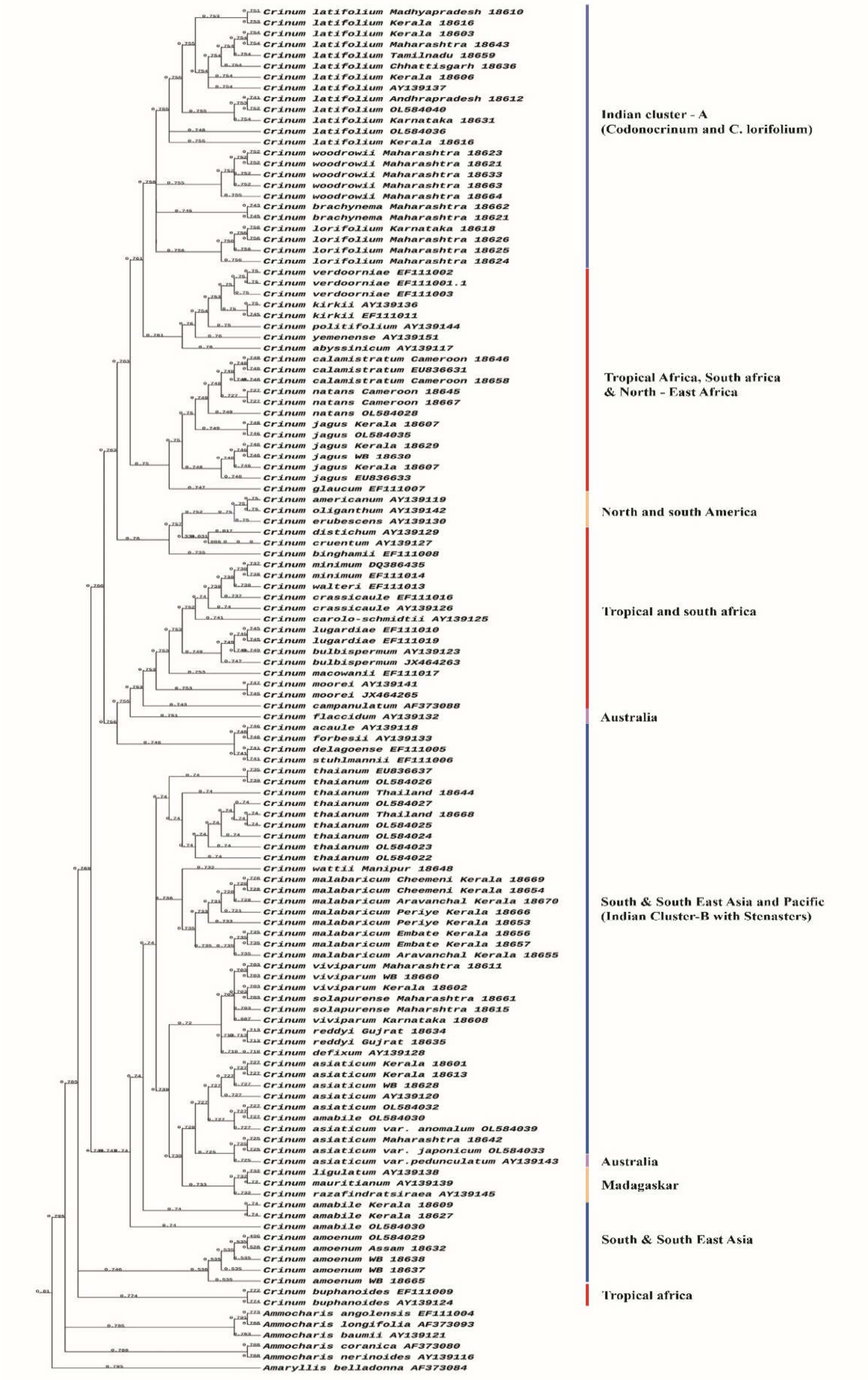


Figure 49: Best ML Tree of the Genus *Crinum* L. based on ITS + trnL-F sequence data

6.4. Discussion

6.4.1. Phylogenetic and Biogeographic Relationship of *C. malabaricum*

Molecular phylogenetic studies play a crucial role in conservation biology by providing deeper insights into the evolutionary relationships and genetic diversity of species (Faith, 1992). For critically endangered plants like *Crinum malabaricum*, understanding their phylogenetic position helps in identifying their closest relatives, tracing their evolutionary history, and making informed conservation decisions. By resolving taxonomic ambiguities and detecting cryptic diversity, molecular phylogenetics aids in prioritizing conservation efforts, ensuring that distinct lineages are adequately protected (Espíndola, 2016). Given the increasing threats posed by habitat destruction, climate change, and other anthropogenic pressures, molecular phylogenetics serves as a crucial tool for guiding conservation actions to prevent the loss of unique and threatened species like *C. malabaricum*.

The molecular phylogenetic analysis of *C. malabaricum* based on nrITS and *trnL-F* markers provides new insights into the evolutionary history and biogeography of the genus *Crinum*. The nrITS-based phylogeny reveals a clear separation between Southeast Asian and Pacific *Crinum* species and those found in the African continent. Previous phylogenetic studies on African *Crinum* using nrITs and *trnL-F* markers (Meerow *et al.,* 2003; Kwebeya *et al.,* 2007) also supports the monophyly of Southeast Asian and Pacific *Crinum* over African species. They also support the relationship of Madagascar species with these clades similar to the present study. Notably, *C. malabaricum*, despite being geographically closer to other Indian species like *C. woodrowii*, *C. brachyaneum*, *C. lorifolium* and *C. latifolium*, shows a distinct phylogenetic lineage. Instead of clustering with these Indian taxa, *C. malabaricum* groups with the Southeast Asian *C. thaianum*, suggesting a possible shared evolutionary history between these regions. The close relationship of *C. malabaricum* with *C. watti* species found in North east India and Myanmar as a sister clade shows the geographical continuity in this evolutionary history. This pattern indicates that *C. malabaricum* might have originated from a Southeast Asian ancestor, differentiating from other Indian *Crinum* species that share closer ancestry with African taxa.

The evolutionary trajectory of *C. malabaricum* is shaped by geographic isolation, habitat specialization, and past climate changes. The collision of the Indian plate with Asia during the early Cenozoic facilitated plant dispersal, leading to the establishment of diverse angiosperms across both regions. As the Indian Plate drifted into perhumid latitudes during the Eocene, mega thermal angiosperms from West Gondwana spread into Southeast Asia (~49 million years ago) (Morley, 2018). Phylogenetic and biogeographic studies support evolutionary links between the Western Ghats and Southeast Asia, particularly through dispersal events (Akashi *et al.,* 2002; Klaus *et al.,* 2010; Biswal *et al.,* 2018). The Myrtaceae family in the Western Ghats, for example, exhibits dispersal patterns from Southeast Asia rather than being solely Gondwanan in origin, indicating a complex biogeographic history (Ray *et al.,* 2020). Many plant lineages in the region are relatively young, arriving via long-distance dispersal during the Eocene and Miocene (Ray *et al.,* 2020). Environmental stability in the Western Ghats has fostered high phylogenetic diversity, shaped by niche conservatism and environmental filtering. The region's ecological diversity reflects plant adaptations to varying climatic conditions over time (Bose *et al.,* 2019).

The present study grouped Indian *Crinum* into two clusters. The first cluster, which includes *C. malabaricum*, is grouped with Southeast Asian and Pacific species. The second cluster, comprising *C. latifolium*, *C. lorifolium*, *C. woodrowii*, and *C. brachynema*, is related to North and East African species. Previous studies also support the grouping of *C. latifolium* with East African species based on phylogenetic studies (Meerow *et al.*, 2003) as well as morphological similarities (Fangan & Nordal, 1993). The grouping of these species is further supported by floral morphology, as most of them belong to Codonocrinum and exhibit zygomorphic floral traits. In contrast, the cluster that includes *C. malabaricum* consists of actinomorphic Stenaster flowers. An exception is *C. lorifolium*, which, despite being in the second cluster, possesses Stenaster-type flowers, making it the only Indian Stenaster in this clade. This suggests that *C. lorifolium* has a distinct evolutionary lineage from other Indian Stenaster species. These findings indicate that subgeneric divisions based on flower morphology have no phylogenetic significance. Floral symmetry in Amaryllidaceae might be under simple genetic control and easily modified (Meerow *et al.,* 1999).

Furthermore, studies suggest that the Australian and Southeast Asian clusters primarily include species with star-shaped flowers from Australia, Asia, Madagascar, and southern Africa, forming what is referred to as the Eastern Stenaster clade. This suggests that star-shaped flowers likely originated once in this region (Kwembeya *et al.,* 2007).

6.4.2. Molecular Marker Performance in Phylogenetic Analysis

Molecular phylogenetic studies rely on different genetic markers, each with its strengths and limitations. In this study, the nuclear ribosomal ITS and plastid *trnL-F* markers were used to infer the evolutionary relationships of *C. malabaricum* and its related taxa. The results indicate differences in phylogenetic resolution between the two markers, leading to variations in tree topologies and support values. The combined phylogeny, integrating both markers, provides a more comprehensive understanding of the evolution of *Crinum*.

The ITS region, a nuclear ribosomal DNA marker, has been widely used in plant phylogenetics due to its rapid mutation rate and high variability at the species level (Baldwin *et al.,* 1995). In this study, ITS provided clear phylogenetic separation between South and Southeast Asia-Pacific *Crinum* species and those from Africa. It also strongly supported the distinct lineage of *C. malabaricum*, which clustered with Southeast Asia-Pacific species rather than other Indian taxa. This suggests that ITS is highly effective in delineating species boundaries and resolving recent divergences within *Crinum*. However, ITS markers are also susceptible to certain evolutionary processes that can affect phylogenetic inference. Concerted evolution, which homogenizes ITS sequences across multiple copies in the genome, can sometimes obscure phylogenetic signals (Soltis & Soltis, 1998; Álvarez & Wendel, 2003). Additionally, the potential for hybridization and incomplete lineage sorting may introduce conflicts between ITS-based phylogenies and other molecular markers (Nieto Feliner & Rosselló, 2007).

In contrast, the *trnL-F* region, a plastid marker, is highly conserved and exhibits lower mutation rates than ITS. While plastid markers are useful for resolving deep evolutionary relationships and biogeographic patterns (Taberlet *et al.,* 1991), their slower rate of evolution makes them less effective for distinguishing closely related species. In this study, *trnL-F* did not show the same strict separation between Indian and African *Crinum* species observed in the ITS tree. Instead, Indian taxa appeared more intermixed, possibly due to retained ancestral polymorphisms or chloroplast capture through past hybridization events (Rieseberg & Soltis, 1991). Despite this, *trnL-F* still confirmed the close relationship of *C. malabaricum* with Southeast Asian species, reinforcing the biogeographic links suggested by ITS data.

The differences in tree topologies between ITS and *trnL-F* can be explained by various evolutionary processes. The nuclear ITS marker undergoes biparental inheritance, which allows for recombination and accumulation of higher sequence divergence, often resulting in better resolution at species-level classification. In contrast, plastid DNA, including *trnL-F*, is maternally inherited in most angiosperms (Birky, 2001; Álvarez & Wendel, 2003). This leads to lower mutation rates and higher conservation, making it less effective in resolving recent evolutionary divergences (Maddison, 1997). The more mixed clustering observed in the *trnL-F* tree suggests that either ancestral polymorphisms have been retained or that there has been chloroplast capture due to past hybridization events among *Crinum* species. This could explain the weaker separation between Indian and African taxa in the *trnL-F* phylogeny.

The integration of ITS and *trnL-F* data into a combined tree generally enhances phylogenetic resolution by incorporating both biparental and uniparental genetic information. In this study, the combined tree maintained the key patterns observed in the ITS phylogeny, particularly the grouping of *C. malabaricum* with Southeast Asian species. At the same time, it retained some of the broader biogeographic relationships suggested by *trnL-F*, such as the connection between Indian and African *Crinum* taxa. The combined analysis demonstrates the importance of using multiple markers in phylogenetic studies to resolve complex evolutionary relationships more effectively. Despite these improvements, some unresolved relationships remain, indicating that additional markers, such as single-copy nuclear genes or genome-wide analyses, may be required for a more robust phylogeny. The use of high-throughput sequencing approaches, such as target enrichment or RAD-seq, could provide deeper insights into the evolutionary history of *Crinum* species (Mandel *et al.,* 2014).

6.4.3. Evolutionary Implications and Biogeographic Insights

The phylogenetic placement of *C. malabaricum* presents a complex evolutionary scenario. While it exhibits a strong affinity with Southeast Asian *Crinum* species, molecular phylogeny reveals that *C. malabaricum* shares evolutionary relationships with the Indian Stenasters (i.e., *C. viviparum, C. wattii, C. solapurense, C. reddyi,* and *C. asiaticum*). However, it remains phylogenetically distant from other Indian *Crinum* species, which suggests that Indian *Crinum* members have followed distinct evolutionary pathways. The tree structure does not strongly support *C. malabaricum* as a basal taxon within *Crinum*, indicating that it is not among the earliest diverging lineages but rather a derived species within the South and Southeast Asia–Pacific clade. This aligns with previous phylogenetic studies that have highlighted the divergence between African and Asian *Crinum* species (Meerow *et al.,* 2003; Kwembeya *et al.,* 2007).

The evolutionary placement of *C. malabaricum* can be attributed to multiple biogeographic and ecological factors. One crucial factor is its adaptation to laterite plateaus in northern Kerala, which present harsh seasonal variations with extreme dry and wet conditions (Pramod & Pradeep, 2020). Such a unique habitat specialization likely drove ecological divergence, isolating *C. malabaricum* from other Indian *Crinum* species that predominantly inhabit riverbanks and wetlands. This pattern is consistent with previous studies showing that habitat specialization can lead to reproductive isolation and speciation in tropical plant groups (Bose *et al.,* 2019; Gopal *et al.,* 2023).

Furthermore, *C. malabaricum* is confined to a few locations in Kerala, particularly in freshwater lateritic rock pools. This limited geographic range and habitat specificity are characteristic features of a narrow endemic (neo-endemic) species. The phylogenetic tree shows *C. malabaricum* forming a well-supported clade with *C. wattii* (from Manipur, Northeast India) and *C. thaianum* (from Thailand). This suggests that *C. malabaricum* is closely related to other Southeast Asian species rather than being an ancient lineage. The relatively short branch length of the *C. malabaricum* clade, compared to more deeply diverged lineages in the tree, supports the idea that it is a neo-endemic species—one that has evolved recently in a restricted geographic region. Its adaptation to lateritic environments might have led to rapid speciation, resulting in its restricted modern distribution.

Unlike paleo-endemic species, which are often relics of ancient, widely distributed lineages that have since become restricted to small areas, *Crinum malabaricum* appears to have close relationships with species from geographically proximate regions. This suggests it is a relatively young species that has evolved within a limited geographic area, rather than a relict species that has persisted from an ancient lineage with a formerly widespread distribution. This observation is consistent with previous studies on other plant groups in the Western Ghats, which show strong links to Southeast Asian taxa due to past dispersal and climatic shifts (Morley, 2018; Ray *et al.,* 2020; Biswal *et al.,* 2018).

6.4.4. Future Directions

While this study provides valuable insights into the phylogenetic placement and evolutionary history of *C. malabaricum*, several key areas remain to be explored for a more comprehensive understanding of its taxonomy, biogeography, and conservation status. One of the primary limitations of this study is the reliance on nrITS and *trnL-F* markers, which, while widely used in plant phylogenetics, have inherent constraints such as incomplete lineage sorting and potential hybridization signals (Mort & Crawford, 2004; Choi *et al.*, 2019; Kolter & Gemeinholzer, 2021, 2021a). Future studies could benefit from genome-wide approaches, such as RADseq (Restriction Site Associated DNA sequencing) or whole plastome sequencing, which can provide higher resolution phylogenies and deeper insights into evolutionary relationships (McKinney, 2016; Stettler *et al.,* 2021).

Additionally, broader geographic sampling is essential to accurately determine the phylogenetic position of *C. malabaricum*. Expanding the sampling to include more Southeast Asian and Indian *Crinum* species, especially poorly studied taxa from Myanmar, Bangladesh, and Sri Lanka, could clarify species boundaries and potential cryptic diversity (Christenhusz & Byng, 2016). Coalescent-based methods, which consider lineage divergence under population-level processes, could further refine species delimitation and evolutionary history (Degnan & Rosenberg, 2009). Morphological and ecological studies are also needed to complement molecular data. Detailed comparisons of reproductive structures, seed dispersal mechanisms, and leaf anatomy across related species could highlight key adaptive traits that contributed to the divergence of *C. malabaricum* (Govaerts *et al.,* 2021). Furthermore, ecological niche modeling (ENM) incorporating climate and soil parameters could provide insights into historical biogeographic processes and potential future threats due to climate change (Svenning *et al.,* 2011).

The phylogenetic and biogeographic analyses of *C. malabaricum* provide crucial insights into its evolutionary history, highlighting its close relationship with the *Stenaster* group within the South and Southeast Asia–Pacific *Crinum* clade. Despite its geographic proximity to other Indian *Crinum* species, *C. malabaricum* follows a distinct evolutionary trajectory, likely shaped by historical dispersal events, habitat specialization, and past climatic shifts. Its adaptation to the seasonally dynamic laterite plateaus of northern Kerala may have played a key role in its divergence, reinforcing its status as a narrow-range neoendemic species.

The molecular marker analysis further emphasizes the need for integrative taxonomic studies incorporating nuclear, plastid, and genomic data to resolve the complexities of *Crinum* phylogeny. Discrepancies between ITS and *trnL-F* topologies highlight potential hybridization, incomplete lineage sorting, or ancestral polymorphism within the genus, necessitating expanded molecular and morphological investigations. Additionally, unresolved taxonomic ambiguities, particularly concerning *C. viviparum*, *C. solapurense*, and *C. reddyi*, underline the importance of future systematic revisions.

Given the restricted distribution and specialized habitat of *C. malabaricum*, conservation strategies should prioritize habitat protection, ex situ conservation efforts, and population genetic studies to assess genetic diversity and resilience. This study underscores the broader relevance of molecular phylogenetics in conservation biology, providing a framework for understanding species evolution, resolving taxonomic uncertainties, and guiding conservation priorities for endangered taxa.

Chapter 7

General Discussions

Chapter 7

General Discussions

This study attempts to understand the current status of *Crinum malabaricum* and its threat and the possible way to sustainably utilise the species and promote the conservation efforts and put forward a conservation strategy. The conservation of the species demands a multifaceted approach that integrates ecological, physiological, and conservation strategies. *C. malabaricum* is not only ecologically significant but also holds potential pharmaceutical value due to its unique phytochemical properties, making its conservation a priority for both biodiversity preservation and medicinal research (Ghane *et al.,* 2018; Priyadarshini *et al.,* 2020; Lekhak *et al.,* 2022). However, *C. malabaricum* faces imminent threats from habitat degradation, climate change, and anthropogenic activities, underscoring the urgency of effective conservation measures (Lansdown & Molur, 2017; Pulparambil & Pradeep; 2023).

This study addresses key challenges to the species' survival by employing ecological niche modeling (ENM) to assess habitat suitability, implementing translocation as a conservation strategy, preserving genetic material through pollen cryopreservation, and evaluating the phytochemical potential of the species. While individual chapters have examined these aspects in detail, a synthesized discussion is essential to assess their collective impact on conservation strategies and provide a holistic understanding of the species' conservation needs. By integrating insights from ecological modeling, *in situ* and *ex situ* conservation efforts, and phytochemical analysis, this discussion strengthens the overall conservation framework for this species. The synthesis of results from diverse methodological approaches enhances decision-making and identifies critical research gaps and future directions, ensuring that conservation strategies are both practical and sustainable.

7.1. Conservation Efforts for *C. malabaricum*

A comprehensive conservation strategy for *C. malabaricum* integrates both *in situ* and *ex situ* approaches. While habitat protection and translocation efforts maintain natural populations, *ex situ* measures like cryopreservation and phytochemical validation serve as safeguards against extinction. The synergy between these strategies highlights the need to combine ecological and genetic tools for long-term species survival.

Ecological niche modeling (ENM) was crucial in predicting the species' habitat suitability under current and future climate scenarios. The analysis confirms its strict ecological specificity, restricted to lateritic plateaus with seasonal water availability. Future projections indicate a significant decline in suitable habitats, underscoring the urgency of conservation interventions. ENM-guided site selection for translocation ensured environmental compatibility, aligning with previous studies that have successfully used predictive models to identify refugia for threatened species (Phillips *et al.*, 2006; Warren *et al.*, 2011).

To mitigate habitat loss, conservation translocation was implemented based on ENM predictions. Experimental translocations across Maharashtra, Karnataka, and Kerala revealed variable survival rates, emphasizing the role of microhabitat factors. Long-term monitoring indicates that hydrological parameters and soil characteristics significantly influence establishment success, reinforcing the necessity of proactive habitat restoration and *ex situ* conservation strategies (Maschinski & Haskins, 2012).

Given the species' small population size and brief flowering window, pollen cryopreservation was explored as a viable *ex situ* conservation method. Pollen collected at peak viability was desiccated to optimal moisture levels and successfully stored in liquid nitrogen. Post-thaw viability assessments confirmed its potential for long-term genetic preservation, ensuring a future resource for propagation and genetic studies. Similar approaches have been employed for other rare and endangered species, demonstrating the importance of cryopreservation in biodiversity conservation (Pence, 2020).

Phytochemical analysis identified a distinct secondary metabolite profile in *C. malabaricum*, with high levels of phenolics, flavonoids, and antioxidants. Notably, bulb extracts exhibited superior antioxidant capacity, suggesting a concentration of bioactive compounds in underground tissues. Comparative analysis with other

Crinum species highlights *C. malabaricum* as a promising source of pharmacologically relevant compounds, including those with neuroprotective potential. These findings strengthen conservation priorities by linking species preservation to medicinal value, fostering interest in sustainable utilization (Atanasov *et al.*, 2015).

7.2. Causes of Endemism in *C. malabaricum*

This study explores the factors driving the species' critically endangered status, restricted distribution, and phylogenetic relationships, offering insights into its endemism and decline. Molecular phylogenetic analysis reveals a close relationship between *C. malabaricum* and *C. thaianum*, an aquatic species endemic to Thailand. This suggests that *C. malabaricum* may have evolved from an ancestral lineage adapted to wetland habitats, supporting its classification as a neoendemic species. Neoendemics are taxa that have evolved relatively recently and are restricted to limited geographic regions, often due to recent speciation events or ecological specialization (Jordan *et al.*, 2016). Conserving the current habitats of *C. malabaricum* is critical, as these ecosystems support the continued evolution and persistence of this narrowly distributed species.

Additionally, *C. malabaricum* exhibits a strong phylogenetic association with Indian stenaster species, including *C. watti*, the *C. asiaticum* clade, the *C. viviparum* - *C. solapurense* - *C. reddyi* clade. These sister clades are closely related to species from Southeast Asia, the Pacific, Australia, and Madagascar. However, *C. malabaricum* shows a distant relationship with Indian codonocrinum - *C. lorifolium* clade, which included species primarily distributed in the Western Ghats. This phylogenetic pattern suggests a complex evolutionary history for *C. malabaricum*. Its connection with Southeast Asian species raises the possibility of historical range shifts between these habitats, potentially driven by climatic or geological events. Furthermore, *C. malabaricum* appears to have evolved independently in the laterite habitats of Kerala, adapting to specific environmental conditions distinct from other endemic *Crinum* species in the region. To better understand its evolutionary trajectory, further genetic studies, including population-level analyses are needed to assess gene flow, genetic diversity, and evolutionary divergence among these related species.

7.3. Relevance to the Broader Scientific Context

This study extends beyond species-specific conservation, contributing to conservation biology, plant ecology, and restoration science. By integrating ecological niche modeling (ENM), translocation, cryopreservation, and phytochemical validation, it provides a scalable model for conserving other threatened species with restricted distributions.

The findings reinforce the importance of a multi-pronged conservation approach that combines *in situ* and *ex situ* strategies. Conservation biology increasingly relies on predictive models like ENM to guide conservation actions under climate change scenarios. By demonstrating the application of ENM in habitat selection and translocation, this study offers a data-driven framework for mitigating biodiversity loss (Hannah *et al.*, 2020). Additionally, incorporating cryopreservation as a genetic safeguarding tool aligns with global efforts to preserve plant genetic resources, particularly for species with limited reproductive windows (Pence, 2020).

Understanding the ecological requirements of *C. malabaricum* through habitat suitability modeling enhances knowledge of plant-environment interactions, a fundamental aspect of plant ecology. This research highlights the conservation significance of laterite plateau ecosystems, which support unique endemic flora but face growing threats from habitat destruction, climate change, and land-use changes. By assessing the species' ecological needs, evaluating habitat suitability, and identifying key threats, this study provides insights into species persistence in these fragile landscapes. Furthermore, it demonstrates the potential of species-specific conservation interventions, *ex situ* conservation through pollen cryopreservation and *in situ* reinforcement via translocation, offering a conservation framework applicable to other endemic and threatened species in lateritic ecosystems.

7.4. Implications for Restoration Strategies

This study underscores the effectiveness of ENM-assisted translocation as a conservation tool. By systematically identifying suitable habitats under current and projected climate scenarios, ENM enhances the likelihood of successful establishment and long-term survival of *C. malabaricum*. Beyond this species, ENM-assisted translocation presents a scalable approach for conserving rare and endangered species across diverse ecosystems. The integration of species occurrence data, environmental parameters, and predictive modeling ensures targeted, evidence-based conservation, minimizing translocation failure risks.

As habitat loss and climate change continue to threaten biodiversity, ENM-based strategies offer a proactive approach to species conservation and habitat management. By integrating ENM predictions with field-based translocation trials, this study exemplifies an evidence-based restoration strategy that can be extended to other species requiring habitat augmentation due to environmental degradation, climate change, or anthropogenic pressures.

7.5. Challenges and Limitations

Despite the comprehensive conservation approach employed for *C. malabaricum*, several methodological and practical challenges were encountered. These limitations highlight the complexities associated with species translocation, genetic studies, and long-term monitoring, emphasizing the need for adaptive management strategies to ensure the species' survival.

7.5.1. Methodological Limitations

The accuracy of Ecological Niche Modeling was constrained by the limited availability of occurrence records and environmental data for *C. malabaricum*. As noted by Elith *et al.* (2006) The reliability of ENM predictions is highly dependent on the quality and quantity of input data, and the scarcity of known populations of *C. malabaricum* posed significant challenges. Similarly, the choice of modeling technique, can influence outcomes. While Maxent is widely used due to its adaptability, it may not always capture complex ecological interactions adequately

(Vasconcelos *et al.,* 2024). Also, ENM results can be sensitive to the selection of environmental variables and their interactions, which may lead to biased predictions if not carefully chosen (Pulparambil & Pradeep, 2023).

Similarly, phytochemical analysis revealed variability influenced by environmental factors, seasonal fluctuations, and plant maturity, making it difficult to establish a definitive chemical profile representative of the species. Research indicates that these elements play a crucial role in determining the quality and quantity of phytoconstituents, which can vary throughout the year and across different growth stages. (Liebelt *et al.,* 2019).

7.5.2. Constraints in Species Translocation

While ENM identified potential translocation sites, field validation revealed discrepancies between predicted site suitability and ground realities. Microhabitat factors such as water availability, soil composition, and biotic interactions significantly influenced transplant success. For instance, some sites exhibited unforeseen challenges like seasonal desiccation or competition from invasive species (Rendall *et al.,* 2021). Translocation survival rates were variable, with stress due to transplant shock, herbivory, and seasonal flooding affecting plant establishment and long-term survival. These findings align with studies on *Euryale ferox*, which also reported high mortality rates in translocated populations (Semwal *et al.,* 2021).

ENM provided a strong predictive framework for identifying suitable translocation sites, field conditions often presented unexpected challenges. Some sites predicted as highly suitable based on environmental variables (temperature, precipitation, soil type) were found to have physical barriers such as altered hydrology, invasive species dominance, or human disturbances that were not accounted for in the model. For example, some locations had seasonal waterlogging or unexpected soil composition variations that affected seedling establishment.

Regarding biotic interactions, pollinator presence and competition from existing vegetation played a significant role in translocation success. In some sites, a lack of native pollinators may have limited reproductive success, while in others, competition from aggressive aquatic or semi-aquatic species hindered establishment. Additionally, mutualistic interactions, such as the presence of specific mycorrhizal fungi or soil microbiota, may have influenced survival rates, though this aspect requires further investigation. Highlighting these real-world ecological factors adds depth to conservation planning by emphasizing the need for integrated field assessments alongside ENM predictions.

7.5.3. Challenges in Long-Term Monitoring

Long-term monitoring of translocated populations and *ex situ* conserved material requires sustained funding, personnel, and institutional support. However, resource and funding constraints often limit the frequency and scope of monitoring efforts. Periodic assessments of survival, reproductive success, and habitat stability are essential but often hindered by financial and logistical challenges (Reilly & Lawton, 2024). Additionally, the laterite plateau ecosystems where *C. malabaricum* thrives are highly vulnerable to climate change and anthropogenic disturbances such as mining, agriculture, and urbanization (Pramod & Pradeep, 2021; Jithin *et al.,* 2023).

7.6. Future Directions

The conservation efforts and research on *C. malabaricum* have provided valuable insights into its ecology, threats, and potential conservation strategies. However, several knowledge gaps remain that warrant further investigation. Addressing these gaps and refining methodologies will not only enhance long-term conservation strategies for *C. malabaricum* but also provide a framework for conserving other endangered species with similar ecological constraints.

7.6.1. Research Questions and Gaps

One critical area for future research is the long-term ecological viability of translocated populations. While short-term survival rates have been assessed, the reproductive success, genetic integrity, and ecological adaptability of these populations over extended periods remain uncertain. Studies on pollination ecology, seed dispersal mechanisms, and population dynamics will be crucial to determine sustainable establishment. Additionally, investigating genetic and epigenetic adaptations in both natural and translocated populations could provide deeper insights into the species' resilience. Whole-genome sequencing and transcriptomic studies, as demonstrated in conservation research on *Cypripedium forrestii*, could help identify genetic bottlenecks and adaptive mechanisms (Lin *et al.,* 2024).

Cryopreservation and germplasm storage optimization also present opportunities for further exploration. While pollen cryopreservation has been successfully implemented, seed-based cryopreservation strategies remain underdeveloped (Benelli, 2021). Furthermore, given the pharmaceutical importance of *C. malabaricum*, future research should assess seasonal and environmental variations in alkaloid production. Standardizing cultivation conditions to maximize bioactive compounds could support conservation through sustainable utilization (Liebelt *et al.,* 2021).

7.6.2. Potential Improvements in Conservation Strategies

Refining ecological niche modeling (ENM) with climate-resilient parameters is essential for predicting habitat suitability under shifting environmental conditions. Incorporating climate change projections into ENM, would help identify long-term refuge sites and inform adaptive conservation strategies (Lemes & Loyola, 2013; Struebig *et al.,* 2015). In parallel, community-led conservation initiatives could play a pivotal role in strengthening conservation outcomes. Engaging local communities in habitat protection, restoration, and sustainable utilization of *C. malabaricum* is critical for ensuring its long-term survival.

A key recommendation is the declaration and management of *C. malabaricum* habitats as Local Biodiversity Heritage Sites (LBHS) under the Biological Diversity Act, 2002 and Kerala Biological Diversity Rules, 2008. LBHS designation would provide a legal and administrative framework for site-specific conservation, empowering Biodiversity Management Committees (BMCs) to oversee habitat protection and community participation. Establishing such community-driven conservation programs within the LBHS framework could promote local stewardship, enhance ecological resilience, and secure the species' future in the face of ongoing environmental threats (Western *et al.*, 1996).

7.6.3. Addressing Policy Implications and Community Involvement

Incorporating policy recommendations and community involvement would strengthen the conservation impact of this study. Engaging with local policymakers to integrate *C. malabaricum* conservation into regional biodiversity action plans could ensure long-term habitat protection. Additionally, working with local communities to raise awareness about the species' ecological importance and potential medicinal applications could foster grassroots conservation efforts. Establishing community-led monitoring programs may also help track population dynamics and habitat changes over time.

7.6.4. *In situ* Seed Banking and Botanical Garden Collaborations

While the study primarily focuses on pollen cryopreservation and translocation, *in situ* seed banking could serve as an additional strategy to enhance genetic conservation. Collaborations with botanical gardens and research institutions could facilitate *ex situ* propagation programs, providing a reservoir of plants for future reintroductions. Establishing living collections in botanical gardens would not only aid in conservation but also provide a controlled environment for studying the species' growth requirements, reproductive biology, and potential threats.

7.6.5. Integrating Genetic Studies for Enhanced Conservation

While this study establishes the phylogenetic placement of *C. malabaricum*, future research should focus on assessing genetic diversity within and between its natural and translocated populations. Genetic monitoring using molecular markers can help evaluate the success of translocation efforts by detecting potential genetic bottlenecks, inbreeding, or adaptation to new environments. Understanding genetic variation is crucial for evaluating population resilience, potential inbreeding effects, and long-term adaptability to changing environmental conditions. Integrating molecular markers such as microsatellites or SNP-based genotyping could provide insights into gene flow, population structure, and genetic bottlenecks.

Additionally, genetic monitoring post-translocation could help determine whether introduced populations maintain sufficient diversity to sustain viable populations.

Establishing a genetic baseline before and after translocation would allow researchers to track adaptive changes, ensuring that conservation efforts support genetically robust populations. Such data could inform future conservation planning, aiding in site selection and optimizing restoration strategies for *C. malabaricum*. Similarly, comparative genomic studies with *C. thaianum and C. lorifolium* may shed light on the historical biogeography and adaptive traits of these species, aiding in the formulation of long-term conservation strategies.

7.6.6. Broader Applications of the Study Framework

The methodologies employed in this study, including ENM, translocation, and cryopreservation, can be adapted for other critically endangered species with restricted distributions, particularly those in fragile laterite ecosystems or aquatic habitats. For example, the conservation framework developed for *C. malabaricum* could be applied endangered aquatic species facing similar threats. Linking conservation strategies with broader habitat restoration efforts can ensure species persistence in the wild. Restoring degraded laterite landscapes, could benefit multiple endemic species beyond *C. malabaricum*.

The phytochemical validation approach used in this study highlights the conservation importance of medicinal plants. Similar frameworks could be applied to other medicinally significant but threatened taxa, aligning biodiversity conservation with pharmaceutical research. Integrating conservation and sustainable utilization of medicinal plants can provide both ecological and economic benefits, fostering a win-win scenario for biodiversity and human well-being (Chen *et al.*, 2016).

This study presents a comprehensive conservation strategy for *Crinum malabaricum*, integrating ecological, genetic, and biotechnological approaches to address its critically endangered status. By employing ecological niche modeling (ENM) for translocation, pollen cryopreservation for long-term *ex situ* conservation, and phytochemical validation to highlight its medicinal value, this research underscores the need for a multi-faceted conservation approach. The findings emphasize the importance of preserving *C. malabaricum* in its natural habitat while securing its genetic material for future restoration, with successful translocation and cryopreservation protocols providing a replicable model for conserving other rare and endemic species, particularly in fragile laterite and aquatic ecosystems. Beyond species-specific conservation, this study contributes to broader fields, including conservation biology, plant ecology, and restoration science, with methodologies that can be adapted to other threatened species, enhancing conservation outcomes at regional and global scales. Additionally, by demonstrating the species' medicinal potential, this work bridges biodiversity conservation with sustainable utilization, reinforcing its ecological and economic significance. In conclusion, this study establishes a strong foundation for future conservation and ecological research, advocating for an integrated approach that combines scientific innovation, habitat protection, and community involvement, with long-term monitoring, adaptive management, and policy interventions being essential to ensuring the survival of *C. malabaricum* and similar endangered taxa.

Chapter 8

Summary and Conclusion

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Over the past decades, global efforts in plant species conservation have yielded significant successes, and India has been progressively contributing to this endeavour. The conservation of critically endangered species requires a multidimensional approach that goes beyond mere population enhancement. A well-balanced integration of *in situ* and *ex situ* conservation strategies, coupled with a deep understanding of species-specific threats and ecological requirements, is essential for ensuring long-term sustainability. Many conservation initiatives lack a comprehensive understanding of the ecological, evolutionary, and utilitarian significance of the species, which is crucial for designing effective conservation strategies.

This study represents a pioneering effort in the conservation of *Crinum malabaricum*, a critically endangered aquatic plant endemic to the laterite plateaus of northern Kerala. The research integrates multiple aspects of conservation biology to develop a holistic approach. The key achievements of this study include:

Understanding Threats and Habitat Assessment

- Detailed ecological and phenological studies revealed that, *C. malabaricum* thrives in seasonal laterite pools, which are highly vulnerable to habitat fragmentation, hydrological alterations, and anthropogenic disturbances.
- The species faces threat not only from anthropogenic activities but also due to its restricted distribution and lack of efficient dispersal mechanisms.
- Phenological events, particularly flowering and fruiting, are directly influenced by rainfall, and changes in monsoon patterns critically affect the species' reproductive cycle.

- Even though new habitats are identified after its assessment in 2016, The species should keep in Critically Endangered category as their AOO is less than 10 sq.km and it is rapidly reducing.

Chemical Profiling for Sustainable Utilization

- Phytochemical analyses identified bioactive compounds with potential medicinal and economic applications. These findings highlight the pharmacological value of the species, encouraging sustainable use while reinforcing the need for its conservation.

- The species was found to be a rich source of alkaloids, exhibiting strong antioxidant activity and acetylcholinesterase inhibition properties, indicating potential pharmacological applications.

Molecular Phylogenetic Analysis

- Using nrITS and *trnL-F* markers, along with a combined phylogenetic approach, the evolutionary history and genetic relationships of *C. malabaricum* were elucidated.

- The study confirmed the close genetic affinity of *C. malabaricum* with *C. thaianum* of Southeast Asia and Indian Stenaster species, while also revealing its distinct evolutionary path from Codonocrinum species of the Western Ghats.

- Phylogenetic evidence provided insights into its restricted distribution and endemism the study suggests that *C. malabaricum* is a neoendemic species that evolved recently due to extreme climatic variations and the geographical uniqueness of the region, tracing its lineage to Southeast Asia - Pacific ancestors.

Ecological Niche Modeling (ENM) for Assisted Translocation:

- Habitat suitability modeling identified optimal reintroduction sites, ensuring that conservation efforts are data-driven and strategically implemented. This approach ensures that conservation actions are data-driven and strategically implemented to enhance survival rates in both natural and semi-natural environments.
- The study initiated the reintroduction of *C. malabaricum* to selected sites within the Malabar Plain, with successful translocation observed in multiple locations across three states. This serves as a pilot project for long-term conservation strategies, offering a model for the conservation programs of similar endangered aquatic plants.

***Ex situ* Conservation Efforts**

- Cryopreservation techniques were developed to safeguard genetic resources, ensuring the long-term preservation of the species' genetic diversity.
- The species was successfully introduced to the 'Aquagene' conservatory of the Malabar Botanical Garden and Institute for Plant Sciences, Kozhikode, Kerala using a unique potting method, ensuring its survival in controlled conditions.

Habitat Restoration and Protection

- Conservation interventions were implemented to restore degraded habitats and mitigate threats from habitat destruction. Measures such as community-based conservation initiatives were employed to enhance the natural environment of the species.
- A proposal was submitted to the Kerala State Biodiversity Board to designate natural habitat for Malabar River lily as a Local Biodiversity Heritage Site (LBHS) under the Biological Diversity Act, 2002, and Kerala Biological Diversity Rules, 2008. Recognition of *C. malabaricum* as the District Flower

of Kasaragod by the district administration further strengthens its conservation initiatives.

This study marks the first comprehensive conservation trial for *Crinum malabaricum*, setting a precedent for future conservation initiatives involving rare and endangered aquatic plants. The reintroduction efforts and site-based conservation strategies developed here provide a valuable model for similar species conservation programs in India and beyond. Furthermore, this work contributes to broader conservation objectives by highlighting the importance of protecting the unique floral and faunal diversity of the laterite plateaus in the Western Ghats.

While this study does not encompass all contemporary aspects of conservation biology, it serves as a significant step toward species targeted conservation. Future research should build on this foundation, integrating advanced biotechnological tools, long-term population monitoring, and community-based conservation initiatives to enhance the effectiveness of conservation strategies. It is hoped that this research will serve as a catalyst for further conservation efforts, ensuring the continued survival of *Crinum malabaricum* and other threatened species in these laterite plateaus.

Chapter 9

Recommendations

Chapter 9

Recommendations

The present study focuses on the conservation of *Crinum malabaricum*, a critically endangered aquatic plant endemic to the seasonal laterite plateaus of northern Kerala. This research integrates ecological niche modeling, molecular phylogenetic analysis, and phenological studies to understand the threats faced by this species and propose conservation strategies. The findings highlight the urgent need for targeted conservation actions, as habitat destruction, anthropogenic pressures, and climate change severely threaten the survival of *C. malabaricum*. The study underscores the importance of preserving such unique habitats and provides a framework for similar conservation initiatives. Based on the outcomes, the following recommendations are proposed for future research and conservation efforts:

1. **Expansion of Research on Endemic Flora:** Future studies should focus on other endemic aquatic plant species inhabiting the laterite plateaus, which are ecologically unique and highly vulnerable to environmental changes. Comprehensive documentation of biodiversity in these habitats is necessary to assess species richness and distribution patterns. Such efforts will enhance understanding of habitat specificity and support targeted conservation strategies.

2. **Continuous Monitoring and Threat Assessment:** Long-term ecological monitoring programs should be established to evaluate population trends and identify emerging threats to *C. malabaricum* and other co-existing species. Regular assessments using field surveys, remote sensing, and GIS-based tools will improve the accuracy of habitat evaluations and conservation planning. Understanding these trends can help predict potential risks and develop timely conservation interventions.

3. **Integration of Biodiversity in Developmental Planning:** Developmental projects in laterite plateaus should incorporate biodiversity assessments to

minimize ecological damage. Infrastructure expansion and land-use changes must be carefully evaluated to ensure that the ecological integrity of these fragile habitats is not compromised. Environmental Impact Assessments (EIA) should mandate the inclusion of rare and endangered species conservation in their reports before approving developmental activities.

4. **Declaration and Management of LBHS:** The natural habitats of *Crinum malabaricum*, particularly the seasonal laterite wetlands of northern Kerala, be declared as Local Biodiversity Heritage Sites (LBHS) to ensure their long-term protection. The management of these sites should be entrusted to the Biodiversity Management Committees (BMCs) of the respective local self-governments, with oversight and coordination support from a dedicated LBHS Management Committee in case of inter-jurisdictional areas. Sustainable management must be community-driven, integrating traditional ecological knowledge, technical support from TSGs, and scientific monitoring. Institutional mechanisms such as the Local Biodiversity Fund (LBF) and allocation of Plan Funds through the 13th Working Group of LSGs should be effectively utilized to support conservation actions. Additionally, livelihood-linked initiatives such as eco-tourism, incentivized conservation schemes, and capacity-building programs should be promoted to ensure active community participation.

5. **Legislation and Policy Implementation:** Strengthening legal frameworks for the protection of laterite plateaus and their endemic species is crucial. Policies must be updated to incorporate the latest scientific findings and conservation strategies. The enforcement of conservation laws, along with the establishment of protected areas or community reserves, should be prioritized to prevent habitat destruction and species loss.

6. **Awareness and Educational Outreach:** Community-based conservation initiatives should be encouraged through awareness programs highlighting the ecological significance of *C. malabaricum* and other vulnerable species. Public participation in conservation efforts can be enhanced through

educational programs in schools, colleges, and research institutions. Collaborative efforts between government agencies, NGOs, and local communities can foster a culture of environmental stewardship.

7. **Reintroduction and Habitat Restoration:** Reintroduction programs should be conducted after thorough ecological impact assessments of the selected sites. Ensuring suitable environmental conditions and addressing potential threats before reintroducing the species is essential for success. Habitat restoration efforts, including water quality management and invasive species control, should be implemented to support species recovery and long-term population sustainability.

8. **Sustainable Utilization and Value Addition:** Investigating the sustainable use of *C. malabaricum* and other aquatic plants for medicinal or ornamental purposes can promote conservation through economic incentives. Value addition studies can provide insights into potential commercial applications while ensuring species survival in natural habitats. Such approaches should align with conservation principles to avoid over-exploitation.

9. **Advanced Phylogenetic and Taxonomic Studies** Future phylogenetic research using high-resolution genomic tools should be conducted to resolve taxonomic uncertainties within the *Crinum* genus. Integrating molecular data with morphological assessments can refine species classification and clarify evolutionary relationships. Comparative studies with other aquatic species can provide broader insights into the phylogenetic history and conservation needs of related taxa.

10. **Extension of Conservation Efforts to Other Threatened Aquatic Species:** The methodologies and conservation strategies developed in this study should be extended to other endangered aquatic plants, particularly those restricted to laterite plateaus. Studying similar species in these unique ecosystems will provide comprehensive insights into their conservation requirements. Collaborative research efforts at regional and national levels can enhance the

scope and impact of conservation initiatives, ensuring the protection of multiple threatened species.

By implementing these recommendations, conservation efforts for *Crinum malabaricum* and other threatened species in laterite plateaus can be strengthened, ensuring the long-term preservation of this unique ecosystem and its biodiversity.

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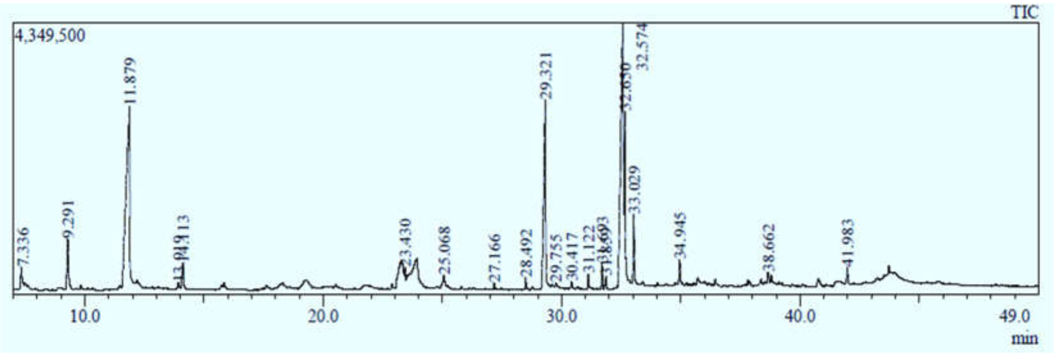
Appendices

Appendix I

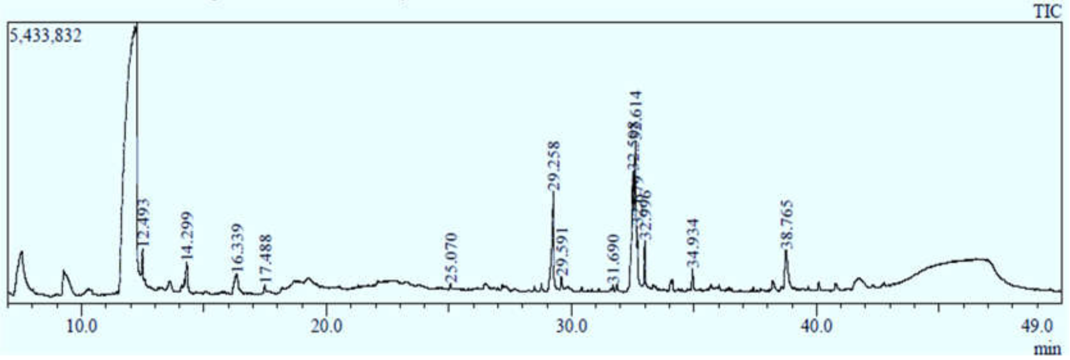
GC-MS Profile of the Methanolic Extract of the Bulb of *Crinum malabaricum* Collected During the Growing Period.



Peak No.	R. time	Area	Area Per.	Height	Height Per.	A/H	Name
1	7.336	1413059	1.36	313280	1.79	4.51	6-Methyl-2-pyrazinylmethanol
2	9.291	3654102	3.5	796173	4.55	4.59	Pyranone
3	11.879	30399300	29.15	2846463	16.25	10.68	5-Hydroxymethylfurfural
4	13.919	401517	0.39	90365	0.52	4.44	3-Heptanol
5	14.113	1933738	1.85	394121	2.25	4.91	1-Amino-4-methylpiperazine
6	23.43	334025	0.32	170255	0.97	1.96	Tridecane-2,11-dione
7	25.068	1132894	1.09	190593	1.09	5.94	Tetradecanoic acid
8	27.166	272222	0.26	107068	0.61	2.54	Pentadecanoic acid
9	28.492	419710	0.4	183421	1.05	2.29	Hexadecanoic acid, methyl ester
10	29.321	18031112	17.29	3011915	17.2	5.99	n-Hexadecanoic acid
11	29.755	190082	0.18	63851	0.36	2.98	9H-Pyrido[3,4-b]indole
12	30.417	311160	0.3	102415	0.58	3.04	2-Phenyl-4,5-methylenedioxybenzaldehyde
13	31.122	609506	0.58	228903	1.31	2.66	Heptadecanoic acid
14	31.693	1038977	1	396800	2.27	2.62	Methyl linoleate
15	31.859	772970	0.74	198509	1.13	3.89	2-Phenyl-4,5-methylenedioxybenzaldehyde
16	32.574	31713992	30.41	4215755	24.07	7.52	10E,12Z-Octadecadienoic acid
17	32.65	6114454	5.86	2339730	13.36	2.61	cis-9-Hexadecenal
18	33.029	2835248	2.72	1066586	6.09	2.66	Octadecanoic acid
19	34.945	1128186	1.08	365694	2.09	3.09	[1,3]Dioxolo[4,5-j] phenanthridine
20	38.662	697255	0.67	166171	0.95	4.2	Crinan-3-ol, (3.alpha.)-
21	41.983	870152	0.83	263990	1.51	3.3	Liriodendromine

Appendix II

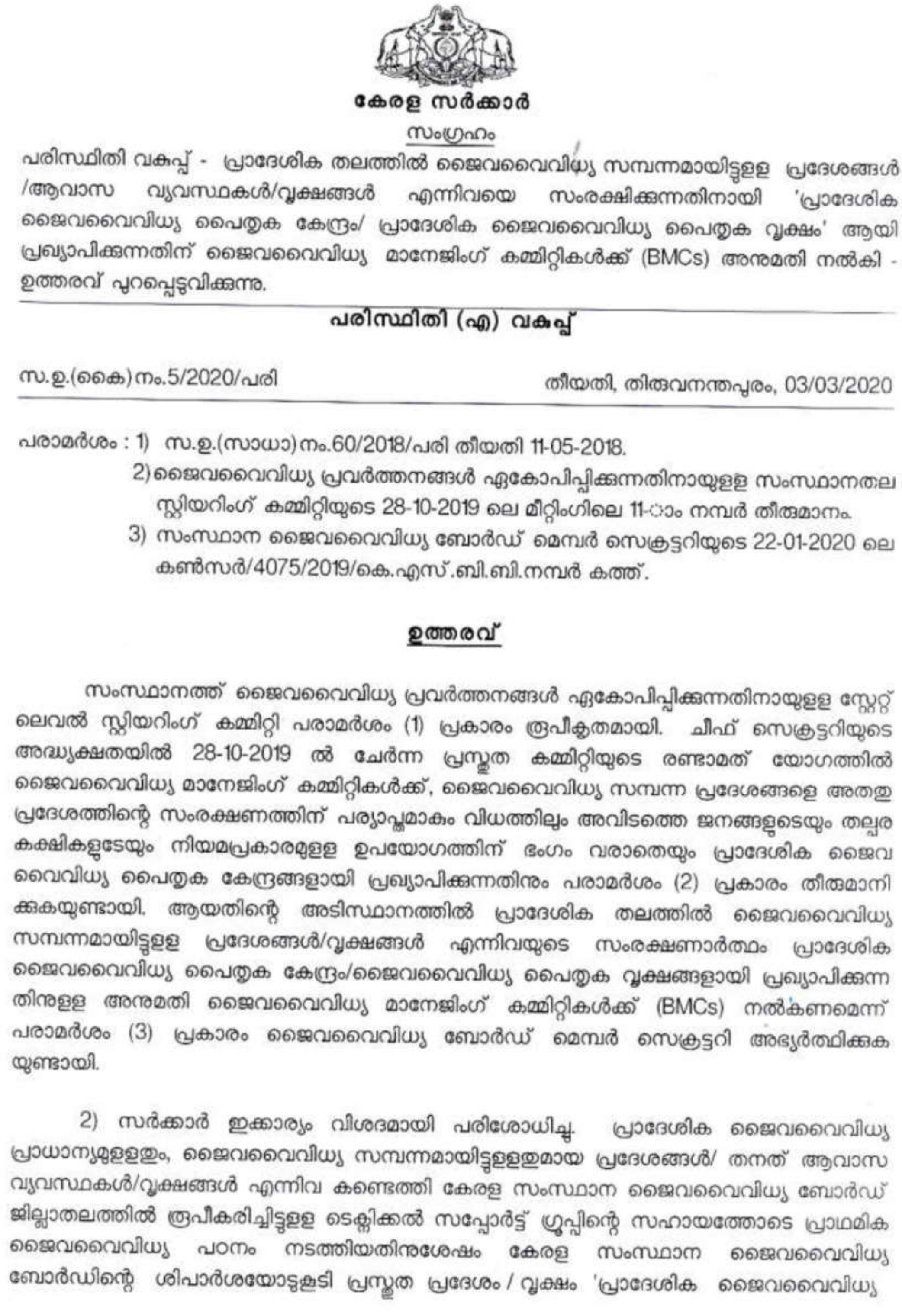
GC-MS Profile of the Methanolic Extract of the Bulb of *Crinum malabaricum* Collected During the Dormancy Period.



Peak No.	R. time	Area	Area Per.	Height	Height Per.	A/H	Name
1	12.493	1385567	3.2	550910	5.46	2.52	1,2,3-Propanetriol, 1-acetate
2	14.299	2909442	6.71	570779	5.65	5.1	N-Formylmorpholine
3	16.339	3319587	7.66	335745	3.33	9.89	Succinic acid, 3-methylbut-2-yl 2-methylbutyl ester
4	17.488	409057	0.94	133859	1.33	3.06	(Z),(Z)-2,5-Dimethyl-2,4-hexadienedioic acid
5	25.07	227560	0.53	105966	1.05	2.15	Tetradecanoic acid
6	29.258	10949369	25.27	1884802	18.67	5.81	n-Hexadecanoic acid
7	29.591	737966	1.7	216606	2.15	3.41	Cirsiumaldehyde
8	31.69	212132	0.49	103931	1.03	2.04	Methyl linoleate
9	32.508	5420381	12.51	1510961	14.96	3.59	Linolic acid
10	32.614	7867133	18.16	1997843	19.79	3.94	Oleic Acid
11	32.679	2258219	5.21	852094	8.44	2.65	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-
12	32.996	2062225	4.76	802640	7.95	2.57	**Octadecanoic acid**
13	34.934	1108536	2.56	376799	3.73	2.94	**[1,3]Dioxolo[4,5-j]phenanthridine**
14	38.765	4465097	10.3	653935	6.48	6.83	**Dihydrocrinine**

Appendix III

Government Order (G.O.) Granting Permission to Biodiversity Management Committees (BMCs) for the Declaration of Local Biodiversity Heritage Sites (LBHS)



കേരള സർക്കാർ

സംഗ്രഹം

പരിസ്ഥിതി വകുപ്പ് - പ്രാദേശിക തലത്തിൽ ജൈവവൈവിധ്യ സമ്പന്നമായിട്ടുള്ള പ്രദേശങ്ങൾ /ആവാസ വ്യവസ്ഥകൾ/വൃക്ഷങ്ങൾ എന്നിവയെ സംരക്ഷിക്കുന്നതിനായി 'പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക കേന്ദ്രം/ പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക വൃക്ഷം' ആയി പ്രഖ്യാപിക്കുന്നതിന് ജൈവവൈവിധ്യ മാനേജിംഗ് കമ്മിറ്റികൾക്ക് (BMCs) അനുമതി നൽകി - ഉത്തരവ് പുറപ്പെടുവിക്കുന്നു.

പരിസ്ഥിതി (എ) വകുപ്പ്

സ.ഉ.(കൈ) നം.5/2020/പരി

തീയതി, തിരുവനന്തപുരം, 03/03/2020

പരാമർശം : 1) സ.ഉ.(സാധാ) നം.60/2018/പരി തീയതി 11-05-2018.

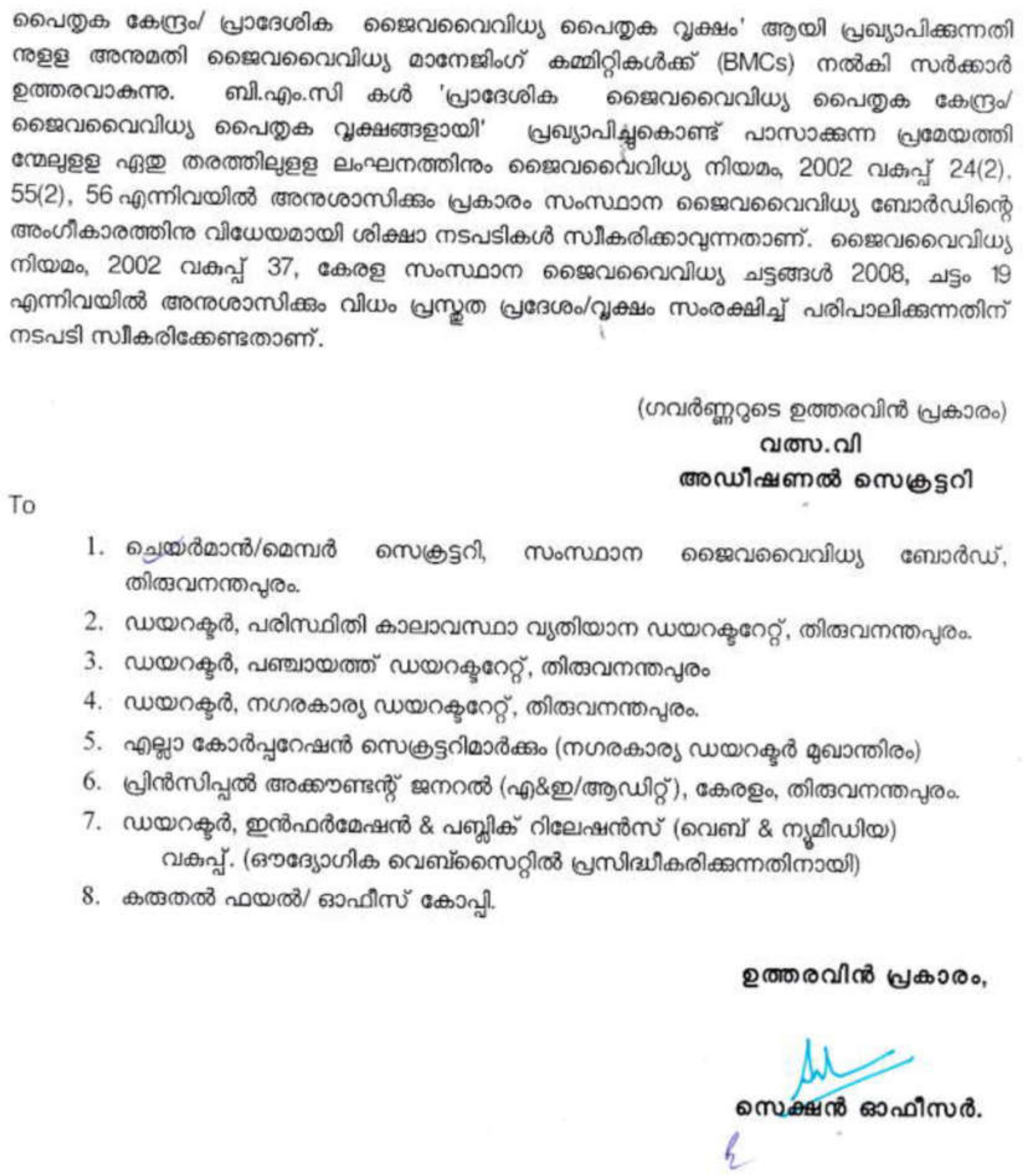
2) ജൈവവൈവിധ്യ പ്രവർത്തനങ്ങൾ ഏകോപിപ്പിക്കുന്നതിനായുള്ള സംസ്ഥാനതല സ്റ്റിയറിംഗ് കമ്മിറ്റിയുടെ 28-10-2019 ലെ മീറ്റിംഗിലെ 11-ാം നമ്പർ തീരുമാനം.

3) സംസ്ഥാന ജൈവവൈവിധ്യ ബോർഡ് മെമ്പർ സെക്രട്ടറിയുടെ 22-01-2020 ലെ കൺസർ/4075/2019/കെ.എസ്.ബി.ബി.നമ്പർ കത്ത്.

ഉത്തരവ്

സംസ്ഥാനത്ത് ജൈവവൈവിധ്യ പ്രവർത്തനങ്ങൾ ഏകോപിപ്പിക്കുന്നതിനായുള്ള സ്റ്റേറ്റ് ലെവൽ സ്റ്റിയറിംഗ് കമ്മിറ്റി പരാമർശം (1) പ്രകാരം രൂപീകൃതമായി. ചീഫ് സെക്രട്ടറിയുടെ അദ്ധ്യക്ഷതയിൽ 28-10-2019 ൽ ചേർന്ന പ്രസ്തുത കമ്മിറ്റിയുടെ രണ്ടാമത് യോഗത്തിൽ ജൈവവൈവിധ്യ മാനേജിംഗ് കമ്മിറ്റികൾക്ക്, ജൈവവൈവിധ്യ സമ്പന്ന പ്രദേശങ്ങളെ അതതു പ്രദേശത്തിന്റെ സംരക്ഷണത്തിന് പര്യാപ്തമാകും വിധത്തിലും അവിടത്തെ ജനങ്ങളുടെയും തല്പര കക്ഷികളുടേയും നിയമപ്രകാരമുള്ള ഉപയോഗത്തിന് ഭംഗം വരാതെയും പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക കേന്ദ്രങ്ങളായി പ്രഖ്യാപിക്കുന്നതിനും പരാമർശം (2) പ്രകാരം തീരുമാനിക്കുകയുണ്ടായി. ആയതിന്റെ അടിസ്ഥാനത്തിൽ പ്രാദേശിക തലത്തിൽ ജൈവവൈവിധ്യ സമ്പന്നമായിട്ടുള്ള പ്രദേശങ്ങൾ/വൃക്ഷങ്ങൾ എന്നിവയുടെ സംരക്ഷണാർത്ഥം പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക കേന്ദ്രം/ജൈവവൈവിധ്യ പൈതൃക വൃക്ഷങ്ങളായി പ്രഖ്യാപിക്കുന്നതിനുള്ള അനുമതി ജൈവവൈവിധ്യ മാനേജിംഗ് കമ്മിറ്റികൾക്ക് (BMCs) നൽകണമെന്ന് പരാമർശം (3) പ്രകാരം ജൈവവൈവിധ്യ ബോർഡ് മെമ്പർ സെക്രട്ടറി അഭ്യർത്ഥിക്കുകയുണ്ടായി.

2) സർക്കാർ ഇക്കാര്യം വിശദമായി പരിശോധിച്ചു. പ്രാദേശിക ജൈവവൈവിധ്യ പ്രാധാന്യമുള്ളതും, ജൈവവൈവിധ്യ സമ്പന്നമായിട്ടുള്ളതുമായ പ്രദേശങ്ങൾ/ തനത് ആവാസ വ്യവസ്ഥകൾ/വൃക്ഷങ്ങൾ എന്നിവ കണ്ടെത്തി കേരള സംസ്ഥാന ജൈവവൈവിധ്യ ബോർഡ് ജില്ലാതലത്തിൽ രൂപീകരിച്ചിട്ടുള്ള ടെക്നിക്കൽ സപ്പോർട്ട് ഗ്രൂപ്പിന്റെ സഹായത്തോടെ പ്രാഥമിക ജൈവവൈവിധ്യ പഠനം നടത്തിയതിനുശേഷം കേരള സംസ്ഥാന ജൈവവൈവിധ്യ ബോർഡിന്റെ ശിപാർശയോടുകൂടി പ്രസ്തുത പ്രദേശം / വൃക്ഷം 'പ്രാദേശിക ജൈവവൈവിധ്യ



പൈതൃക കേന്ദ്രം/ പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക വൃക്ഷം' ആയി പ്രഖ്യാപിക്കുന്നതിനുള്ള അനുമതി ജൈവവൈവിധ്യ മാനേജിംഗ് കമ്മിറ്റികൾക്ക് (BMCs) നൽകി സർക്കാർ ഉത്തരവാകുന്നു. ബി.എം.സി കൾ 'പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക കേന്ദ്രം/ ജൈവവൈവിധ്യ പൈതൃക വൃക്ഷങ്ങളായി' പ്രഖ്യാപിച്ചുകൊണ്ട് പാസാക്കുന്ന പ്രമേയത്തിന്മേലുള്ള ഏതു തരത്തിലുള്ള ലംഘനത്തിനും ജൈവവൈവിധ്യ നിയമം, 2002 വകുപ്പ് 24(2), 55(2), 56 എന്നിവയിൽ അനുശാസിക്കും പ്രകാരം സംസ്ഥാന ജൈവവൈവിധ്യ ബോർഡിന്റെ അംഗീകാരത്തിനു വിധേയമായി ശിക്ഷാ നടപടികൾ സ്വീകരിക്കാവുന്നതാണ്. ജൈവവൈവിധ്യ നിയമം, 2002 വകുപ്പ് 37, കേരള സംസ്ഥാന ജൈവവൈവിധ്യ ചട്ടങ്ങൾ 2008, ചട്ടം 19 എന്നിവയിൽ അനുശാസിക്കും വിധം പ്രസ്തുത പ്രദേശം/വൃക്ഷം സംരക്ഷിച്ച് പരിപാലിക്കുന്നതിന് നടപടി സ്വീകരിക്കേണ്ടതാണ്.

(ഗവർണ്ണറുടെ ഉത്തരവിൻ പ്രകാരം)
വത്സ.വി
അഡീഷണൽ സെക്രട്ടറി

To

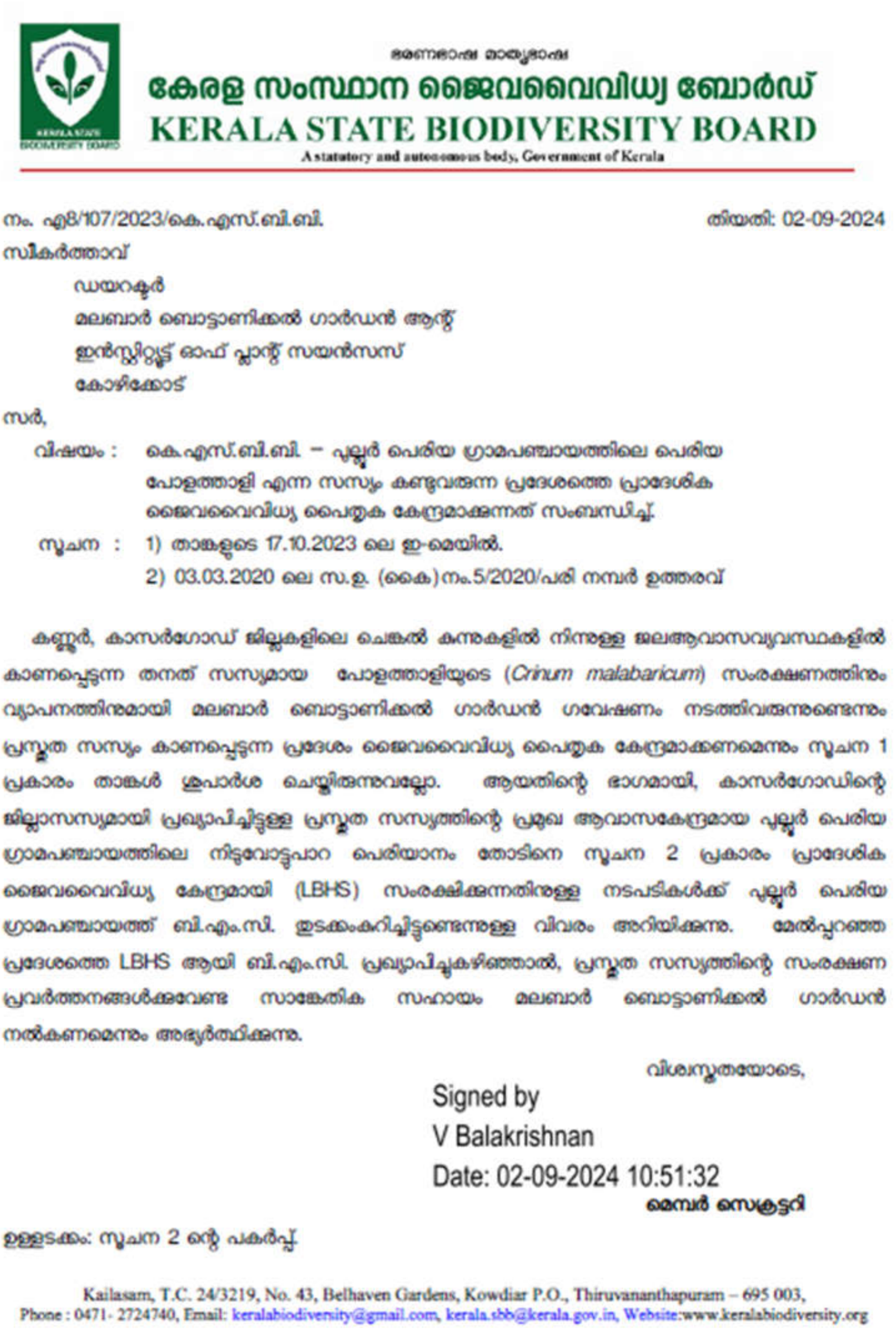
1. ചെയർമാൻ/മെമ്പർ സെക്രട്ടറി, സംസ്ഥാന ജൈവവൈവിധ്യ ബോർഡ്, തിരുവനന്തപുരം.
2. ഡയറക്ടർ, പരിസ്ഥിതി കാലാവസ്ഥാ വ്യതിയാന ഡയറക്ടറേറ്റ്, തിരുവനന്തപുരം.
3. ഡയറക്ടർ, പഞ്ചായത്ത് ഡയറക്ടറേറ്റ്, തിരുവനന്തപുരം
4. ഡയറക്ടർ, നഗരകാര്യ ഡയറക്ടറേറ്റ്, തിരുവനന്തപുരം.
5. എല്ലാ കോർപ്പറേഷൻ സെക്രട്ടറിമാർക്കും (നഗരകാര്യ ഡയറക്ടർ മുഖാന്തിരം)
6. പ്രിൻസിപ്പൽ അക്കൗണ്ടന്റ് ജനറൽ (എ&ഇ/ആഡിറ്റ്), കേരളം, തിരുവനന്തപുരം.
7. ഡയറക്ടർ, ഇൻഫർമേഷൻ & പബ്ലിക് റിലേഷൻസ് (വെബ് & ന്യൂമീഡിയ) വകുപ്പ്. (ഔദ്യോഗിക വെബ്സൈറ്റിൽ പ്രസിദ്ധീകരിക്കുന്നതിനായി)
8. കരുതൽ ഫയൽ/ ഓഫീസ് കോപ്പി.

ഉത്തരവിൻ പ്രകാരം,

സെക്ഷൻ ഓഫീസർ.

Appendix IV

Letter from Kerala State Biodiversity Board (KSBB) Indicating that the Proposal for Declaring the Habitat of *C. malabaricum* as a Local Biodiversity Heritage Site (LBHS) is Under Process



ഭരണഭാഷ മാതൃഭാഷ
കേരള സംസ്ഥാന ജൈവവൈവിധ്യ ബോർഡ്
KERALA STATE BIODIVERSITY BOARD
A statutory and autonomous body, Government of Kerala

നം. എ8/107/2023/കെ.എസ്.ബി.ബി. തീയതി: 02-09-2024

സ്വീകർത്താവ്
ഡയറക്ടർ
മലബാർ ബൊട്ടാണിക്കൽ ഗാർഡൻ ആന്റ്
ഇൻസ്റ്റിറ്റ്യൂട്ട് ഓഫ് പ്ലാന്റ് സയൻസസ്
കോഴിക്കോട്

സർ,

വിഷയം : കെ.എസ്.ബി.ബി. – പുല്ലൂർ പെരിയ ഗ്രാമപഞ്ചായത്തിലെ പെരിയ പോളത്താളി എന്ന സസ്യം കണ്ടുവരുന്ന പ്രദേശത്തെ പ്രാദേശിക ജൈവവൈവിധ്യ പൈതൃക കേന്ദ്രമാക്കുന്നത് സംബന്ധിച്ച്.

സൂചന : 1) താങ്കളുടെ 17.10.2023 ലെ ഇ-മെയിൽ.
2) 03.03.2020 ലെ സ.ഉ. (കൈ)നം.5/2020/പരി നമ്പർ ഉത്തരവ്

കണ്ണൂർ, കാസർഗോഡ് ജില്ലകളിലെ ചെങ്കൽ കുന്നുകളിൽ നിന്നുള്ള ജലആവാസവ്യവസ്ഥകളിൽ കാണപ്പെടുന്ന തനത് സസ്യമായ പോളത്താളിയുടെ (*Crinum malabaricum*) സംരക്ഷണത്തിനും വ്യാപനത്തിനുമായി മലബാർ ബൊട്ടാണിക്കൽ ഗാർഡൻ ഗവേഷണം നടത്തിവരുന്നുണ്ടെന്നും പ്രസ്തുത സസ്യം കാണപ്പെടുന്ന പ്രദേശം ജൈവവൈവിധ്യ പൈതൃക കേന്ദ്രമാക്കണമെന്നും സൂചന 1 പ്രകാരം താങ്കൾ ശുപാർശ ചെയ്തിരുന്നുവല്ലോ. ആയതിന്റെ ഭാഗമായി, കാസർഗോഡിന്റെ ജില്ലാസസ്യമായി പ്രഖ്യാപിച്ചിട്ടുള്ള പ്രസ്തുത സസ്യത്തിന്റെ പ്രമുഖ ആവാസകേന്ദ്രമായ പുല്ലൂർ പെരിയ ഗ്രാമപഞ്ചായത്തിലെ നിട്ടുവോട്ടുപാറ പെരിയാനം തോടിനെ സൂചന 2 പ്രകാരം പ്രാദേശിക ജൈവവൈവിധ്യ കേന്ദ്രമായി (LBHS) സംരക്ഷിക്കുന്നതിനുള്ള നടപടികൾക്ക് പുല്ലൂർ പെരിയ ഗ്രാമപഞ്ചായത്ത് ബി.എം.സി. തുടക്കംകുറിച്ചിട്ടുണ്ടെന്നുള്ള വിവരം അറിയിക്കുന്നു. മേൽപ്പറഞ്ഞ പ്രദേശത്തെ LBHS ആയി ബി.എം.സി. പ്രഖ്യാപിച്ചുകഴിഞ്ഞാൽ, പ്രസ്തുത സസ്യത്തിന്റെ സംരക്ഷണ പ്രവർത്തനങ്ങൾക്കുവേണ്ട സാങ്കേതിക സഹായം മലബാർ ബൊട്ടാണിക്കൽ ഗാർഡൻ നൽകണമെന്നും അഭ്യർത്ഥിക്കുന്നു.

വിശ്വസ്തതയോടെ,

Signed by
V Balakrishnan
Date: 02-09-2024 10:51:32
മെമ്പർ സെക്രട്ടറി

ഉള്ളടക്കം: സൂചന 2 ന്റെ പകർപ്പ്.

Kailasam, T.C. 24/3219, No. 43, Belhaven Gardens, Kowdiar P.O., Thiruvananthapuram – 695 003,
Phone : 0471- 2724740, Email: keralabiodiversity@gmail.com, kerala.sbb@kerala.gov.in, Website:www.keralabiodiversity.org

Publications