Phylogenetic and Commodity Authentication Studies in *Cinnamomum* spp. and *Myristica* spp.

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By

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CERTIFICATE

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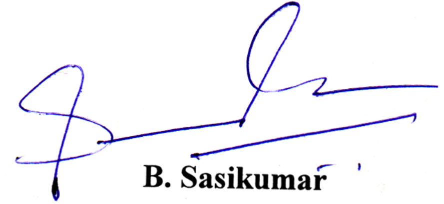

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B. Sasikumar

DECLARATION

I hereby declare that the thesis entitled "Phylogenetic and Commodity Authentication Studies in *Cinnamomum* spp. and *Myristica* spp." submitted for the award of the degree of Doctor of Philosophy in Biotechnology to Calicut University contains the results of bonafide research work done by me at ICAR-Indian Institute of Spices Research, Kozhikode, Kerala under the guidance of Dr. B. Sasikumar, Head and Principal Scientist, Division of Crop Improvement and Biotechnology, ICAR-Indian Institute of Spices Research. This thesis has not been submitted for the award of any other degree or diploma of this or any other University.

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Date :

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I would like to dedicate this thesis to my beloved Dad

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ABBREVIATIONS

%	Percentage
ºC	Degree centigrade
µ	Micro
µg ml^{-1}	Microgram per millilitre
µM	Micromolar
µl	Microlitre
3′	Hydroxyl-terminus of DNA molecule
5′	Phosphate-terminus of DNA molecule
β	Beta
$	Dollars
AP	Arbitrarily Primed
ARMS	Amplification Refractory Mutation System
ASTA	American Spice Trade Association
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
BOLD	Barcode of Life Data Systems
bp	Base pairs
CBOL	Consortium for the Barcode of Life
Cl	Chloride
CITES	Convention on International Trade in Endangered Species
cm	centimetre
CTAB	Cetyl trimethyl ammonium bromide
DAF	DNA Amplification Fingerprinting
DAMD	Directed Amplification of Minisatellite-region DNA
DNA	Deoxy ribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organisation of the United Nations

FSSAI	Food Safety and Standards Authority of India
gm	Gram
g	Relative centrifugal force
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
iBOL	International Barcode of Life
IPTG	Isopropyl- β-D-thiogalactopyranoside
ISO	International Organization for Standardisation
ISSR	Inter Simple Sequence Repeats
ITC	International Trade Centre
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
kb	Kilo base pair
Kg/ha	Kilogram per hectare
K2P	Kimura 2 parameter
LB	Luria-Bertani
M	Molar
m	Metre
mg	Milligram
$MgCl_2$	Magnesium chloride
min	minutes
ml	Millilitre
mm	Millimetre
mM	Millimolar
ML	Maximum Likelihood
MP	Maximum Parsimony
MSA	Multiple Sequence Alignment
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NJ	Neighbour Joining

ng	Nanogram
nm	Nanometre
NMR	Nuclear Magetic Resonance
OTU	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pmole	Picomole
pmol μl^{-1}	Picomole per microlitre
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
RNase	Ribonuclease
rDNA	Ribosomal DNA
SCAR	Sequence Characterised Amplified Region
SDS	Sodium dodecyl sulphate
SIB	Swiss Institute of Bioinformatics
SNP	Single Nucleotide Polymorphism
SRAP	Sequence-Related Amplified Polymorphism
SSR	Simple Sequence Repeat
Taq	*Thermus aquaticus*
T_a	Annealing temperature
TBE	Tris Borate EDTA
TLC	Thin Layer Chromatography
U	Units
UPGMA	Unweighted Pair Group Method
UV	Ultra violet
V	Voltage
WHO	World Health Organisation
WTO	World Trade Organisation

Chapter 1

Introduction

Spices are high value export oriented commodities that occupies a pivotal position in our daily life. Spices are the vegetable products used for seasoning, flavouring and adding aroma to foods (Douglas et al., 2005). They are derived from plant parts like seed, bark, root, rhizome, and flowers having an aromatic and pungent principle and are used as flavorants in the form of whole spices, ground spices, and spice extracts in foods (Sasikumar et al., 2016). Apart from its use as flavorants they also find applications in medicine, cosmetics and perfumery. The essential oils of the spices have therapeutic principles like antioxidant, anti-inflammatory, anti-cancer, antiseptic, antimicrobial, digestive stimulant, carminative and cholesterol lowering that has boosted their application in medical and pharmaceutical fields (Li, 2006).

Spices are traded in the form of whole or dried commodity, powders, pastes, essential oils, oleoresins, and extractives (Sasikumar et al., 2016). The global trade of spices have seen a marked increase in the recent years. According to the trade statistics of the International Trade Centre (ITC) the global export in spices has increased from 2628899 tonnes worth 7978.6 million US $ in 2013 to 2859304 tonnes worth 9836.5 million US $ in 2015. United States of America (USA), Saudi Arabia, United Arab Emirates (UAE) and the European Union are the key players in the global spice import (http://www.intracen.org/itc/market-insider/spices/).

India popularly known as the “Land of Spices” harbors around 52 spices like black pepper, turmeric, ginger, nutmeg, cinnamon, coriander, fennel, cumin, bayleaf, aniseed and allspice that are further classified in to herbs, shrubs, trees, climbers and rhizhomatous herbs based on their growth habitats (Krishnamoorthy et al., 1999).

Cinnamon and nutmeg are two important aromatic tree spices that have wide application in food, medical and cosmetic industries.

Cinnamon (*Cinnamomum verum* Berchthold and Presl., syn. *Cinnamomum zeylanicum* Blume) belongs to *Cinnamomum,* the largest genus of the family Lauraceae, consisting about 250 species of evergreen trees and shrubs that are distributed mainly in the tropical rainforests of Asia, Pacific Islands, Australia, America and Africa at varying altitudes (Joy

and Maridass, 2008; Huang et al., 2016). Asia is the most significant center of *Cinnamomum* species diversity (Lorea – Hernández, 1996) while Sri Lanka is the primary centre of origin (Krishnamoorthy et al., 2007). Forty six species of *Cinnamomum* genus are found in India out of which 26 are recorded from South India and 19 of them are endemic to this region (Remya et al., 2016). Twelve species are reported from Kerala of which 3 are endemic to the state (Kumary et al., 2007). The genus is characterized by economically important species like *Cinnamomum verum* Berchthold and Presl., *Cinnamomum cassia* (L.) Berchthold and Presl. (syn. *Cinnamomum aromaticum* Nees), *Cinnamomum tamala* Nees, *Cinnamomum camphora* (L.) Berchthold and Presl., *Cinnamomum sulphuratum* Nees and *Cinnamomum citriodorum* Thwaites that are valuable as spices, sources of aromatic essential oils and medicine (Chieh et al., 2010; Choudhury et al., 2013).

C. verum, *C. cassia* and *C. camphora* are cultivated commercially to yield cinnamon, cassia and camphor, respectively (Babu et al., 2003). Among the species, *C. verum* also known as true cinnamon, native to Sri Lanka assumes high economical significance (Leela et al., 2012) and is considered to be the most popular spice after black pepper (Dinesh et al., 2015). The dried inner bark of *C. verum* is used as a flavorant in foods, beverages, chewing gum etc. (Chen et al., 2014). The spice is also credited with medicinal properties like antioxidant, anti-inflammatory, antimicrobial, antidiabetic, anticancer, lipid lowering, cardiovascular disease lowering activities and combatting neurological diseases like Parkinson's and Alzheimer's disease (Rao and Gan, 2014). Prolonged treatment with cinnamon extract is also found to induce active cell death and growth inhibition in several cancer lines such as melanoma, breast cancer, colorectal cancer, and hepatoma (Kwon et al., 2009).

Nutmeg (*Myristica fragrans* Houtt.) belongs to the primitive and largest *Myristica* genus of the family Myristicaceae (Sinclair, 1958). *Myristica* has a Malayan centre of origin (Sheeja et al., 2013) and is distributed in the rainforests of South East Asia, India, North Australia and Pacific Islands. There are about 120 species of *Myristica* of which 14 are found in India. The genus is characterised by species like *Myristica fragrans* Houtt., *Myristica malabarica* Lam., *Myristica andamanica* Hook. f., *Myristica fatua* Houtt., *Myristica beddomei* King and *Myristica prainii* King (Krishnamoorthy et al., 2007) of which *M. fragrans* is the most important and commercially cultivated one (Bose et al., 2008a). *M. fragrans* native to Banda Islands in Moluccas is also found in India, Indonesia, Papua New Guinea, Sri Lanka and

Grenada (Sasikumar et al., 2013). In India it is mostly cultivated in Kerala, Karnataka, Tamil Nadu and Maharashtra (Haldankar et al., 2008).

M. fragrans assumes high economical importance due to its aromatic, aphrodisiac and curative properties (Agbogidi and Azagbaekwe, 2013). It is mostly popular for the twin spice it produces – the nutmeg (kernel of the seed) and mace (aril of the seed) that is widely used as a flavorant in baked goods, confectionaries, puddings, meat, sausages and beverages (Asgarpanah and Kazemivash, 2012). Both nutmeg and mace finds application in pharmaceutical preparations due to their carminative, stimulant and astringent properties (Fijida, 2009). Essential oil and nutmeg butter are other derivatives of *M. fragrans* that are of commercial importance (Bose et al., 2008a). Other economically important species of *Myristica* genus are *M. malabarica, M. andamanica* and *M. beddomei* that find application in traditional medicine (Zachariah et al., 2008; Arunachalam and Subhashini, 2011; Manjunatha et al., 2011).

Cinnamon and nutmeg occupies a pivotal position in the international trade. As per the data available from ITC, the global export of cinnamon and cinnamon flowers was 154997 tons worth 482.58 million US $ in the year 2016, with Unites States of America, Asia and European Union purchasing majority of the cinnamon. The global export of nutmeg and mace was 28,123 tons worth 207.32 million US $ in the year 2016 with United States of America as the major importer (http://www.intracen.org/itc/market-info-tools/statistics-export-product-country/).

The economic value coupled with the high cost of cinnamon and nutmeg has resulted in the fraudulent adulteration of these spices. Related species of *C. verum* and *M. fragrans* having very close morphological resemblance are used as adulterants of the commercial produce either by default or design. *C. verum* is found to be adulterated with the barks of related species like *C. cassia* and *C. malabatrum* (Burm.f.) Bl. (Swetha et al., 2014). *C. cassia* barks are rougher, thicker, less aromatic and cheaper with a burning flavour (Thomas and Duethi, 2001) and contain coumarin (benzo-α pyrone), a naturally occurring flavoring substance known to cause kidney and liver damage in rodents (Felter et al., 2006). The bark of *C. cassia* was reported to contain up to 5% coumarin while *C. verum* contains only a trace (0.004%) amount (Lungarini et al., 2008). World Health Organization (WHO) had reported some incidents of hepatotoxicity to humans induced by coumarin intake (WHO, 1995). Studies conducted by the German Federal Institute for Risk assessment showed that 2100 – 4000 mg

of coumarin is present in 1 kg of cassia indicating 5.8 to 12.1 mg of coumarin in one teaspoon of cassia powder, which is a threat to food safety (Dinesh et al., 2015). Bark of *C. malabatrum,* found in homestead gardens of India and Sri Lanka, with no reported toxic effects is also passed off as true cinnamon (Swetha et al., 2014).

M. fragrans is reported to be adulterated with *Myristica argentea* Warb., *M. malabarica* and *Myristica otaba* Humb. and Bonpl. (Krishnamoorthy and Rema, 2001). Mace of *M. malabarica* (Bombay mace), a wild relative of *M. fragrans* is of inferior quality, lacking aroma, yellow in color and often used as a dye source (Swetha et al., 2017). *M. argentea* has an acrid taste (Gokhale, 2009). Both *M. argentea* and *M. otaba* lack the aroma of *M. fragrans* (Krishnamoorthy and Rema, 2001).

Currently physical, chemical and molecular methods are employed for authenticity testing of commodities (Smillie and Khan, 2010). Differentiation of genuine and adulterant entities are possible to an extent using morphological characters but identification becomes tedious on aging, sample drying and processing as they lose their diagnostic features (Swetha et al., 2017). Physical methods used for spice authentication involve the study of macroscopic, microscopic characters, structural evaluation, bulk density etc. while analytical methods involve techniques like chromatography, spectroscopy and capillary electrophoresis that distinguish the samples based on their variation in chemical profile (Sasikumar et al., 2016). The need for skilled personnels and the time consuming procedure often limits the use of physical approaches and analytical approaches are restricted due to the requisite of an expensive standard and non-availability of standards for some botanicals (Shaw et al., 2002; Swetha et al., 2016). Molecular methods involving the amplification of particular regions of genomic DNA are superior over these two approaches due to their effectiveness, accuracy and non-dependance on environmental factors, age, storage and processing conditions (Heubl, 2013; Balachandran et al., 2015). Currently DNA barcoding, a recently evolved molecular marker technique is gaining popularity over other markers like Random Amplified Polymorphic DNA (RAPD), Sequence Characterised Ampilifed Region (SCAR) and Simple Sequence Repeat (SSR) for authentication of food commodities (Swetha et al., 2016).

DNA barcoding is a technique proposed by Paul Hebert, which is based on the sequence variation in short nucleotide regions called barcodes for species identification (Hebert et al., 2003a). An ideal DNA barcode should meet the following criteria (i) universal primers for amplification, (ii) easily amplifiable with a short PCR product of around 1kb, (iii) amenable

to sequencing and sequence annotation, (iv) higher interspecific divergence than intraspecific divergence and (v) recoverable from degraded samples (Kress et al., 2005; Selvaraj et al., 2008; Schoch et al., 2012). A short region of the mitochondrial cytochrome c oxidase (*cox*1) was proposed as the standard barcode for animals (Hebert et al., 2003a). In case of plants, *cox*1 gene has been replaced as a barcode due to its low rate of nucleotide substitutions and high rate of chromosomal rearrangements (Mower et al., 2007; Hollingsworth et al., 2011). The Consortium for the Barcode of Life (CBOL) has proposed chloroplast coding regions like *matK* coding for maturase and *rbcL* coding for the large subunit of RUBISCO as core barcodes and intergenic spacer *psbA-trnH* and nuclear Internal Transcribed Spacer (ITS) as supplementary barcodes in plants (CBOL Plant Working Group, 2009).

DNA barcoding has been used to detect adulteration in different commodities like medicinal plants (Newmaster et al., 2013; Ganie et al., 2015), tea (Stoeckle et al., 2011), olive oil (Kumar et al., 2011), spices like saffron (Gismondi et al., 2013), black pepper (Parvathy et al., 2014), turmeric (Parvathy et al., 2015), star anise (Meizi et al., 2012) and members of family Lamiaceae (de Mattia et al., 2011). It also aids as a taxonomic tool for species identification (Savolainen et al., 2005), taxonomic revision (Lara et al., 2009) and phylogenetic studies (Kress et al., 2010). Barcoding genes have been used in studying the phylogenetic relationship of some species such as *Illicium* (Hao et al., 2001), *Citrus* (Penjor et al., 2013), *Thymus* (Sonboli et al., 2013)*, Dendrobium* (Srikulnath et al., 2015) and *Capsicum* (García et al., 2016).

There are only limited studies on the barcode based phylogenetic relationship of *C. verum* and *M. fragrans* with other species of their respective genus. Morphological characters are given dominance while studying phylogeny. But some of the inherent problems such as phenotypic plasticity and genotypic variability of the characters, interaction of the genotype with the environment, cryptic taxa and difficulty in finding reliable characters due to variation in expression or long maturity periods or incomplete penetrance reduce the reliability of morphological characters in phylogenetic studies (Abeysinghe et al., 2009).
The present work is an attempt to study commodity authentication of cinnamon bark and nutmeg mace. It also focuses on determining the phylogenetic relationship between 12 *Cinnamomum* species (*Cinnamomum aromaticum* Nees, *Cinnamomum alexei* Kosterm., *Cinnamomum camphora* (L.) Berchthold and Presl., *Cinnamomum citriodorum* Thwaites,

Cinnamomum glaucescens (Buch-Ham. ex Nees), *Cinnamomum heyneanum* Nees, *Cinnamomum malabatrum* (Burm.f.) Bl., *Cinnamomum riparium* Gamble, *Cinnamomum sulphuratum* Nees, *Cinnamomum tamala* Nees, *Cinnamomum travancoricum* Gamble and *Cinnamomum verum* Berchthold and Presl.) and six *Myristica* species (*Myristica andamanica* Hook.f., *Myristica amygdalina* Wall. ex Hook. f. and Thomson, *Myristica beddomei* King, *Myristica fatua* Houtt., *Myristica fragrans* Houtt. and *Myristica malabarica* Lam).

The objectives of the study include:

- Tracing out presence of plant based adulterants in traded cinnamon bark and nutmeg mace.
- Generating DNA barcodes for the *Cinnamomum* spp. and *Myristica* spp.
- Studying the evolutionary relationship in *Cinnamomum* spp. and *Myristica* spp.

Chapter 2

Review of Literature

2.1. General Introduction

Spices are high value agricultural commodities that form an integral part of the international commerce. The International Organization for Standardisation (ISO) defines spices and condiments as 'vegetable products or mixtures thereof, free from extraneous matter, used for flavouring, seasoning and imparting aroma to foods' (ISO, 1995). Apart from their usage as flavourants, they assume importance in medicinal and cosmetic applications owing to the antimicrobial, antioxidant, anti-inflammatory, antitoxic, hypolipidermic and other medicinal properties associated with them.

India popularly known as the "Land of Spices" exhibits an amazing diversity of about 52 spices like black pepper, cardamom, ginger, turmeric, garcinia, coriander, cumin, fennel, cinnamon, nutmeg and clove. Based on their growth habit spices are classified as herbs, shrubs, trees, climbers and rhizomatous herbs. Among the spices, tree spices exhibit relatively less variability and also several species are at the risk of endangering. There are 17 tree spices grown in India of which cinnamon and nutmeg are two of the major tree spices as they have great economical significance.

2.2. Genus *Cinnamomum*

The genus *Cinnamomum,* estabilished by Schaeffer (1760), consists of about 200-300 species of evergreen trees and shrubs belonging to family Lauraceae (Rema et al., 2007; Robi et al., 2014). The term *Cinnamomum* is derived from the Greek word *kinnamon* or *kinnamomum* meaning sweet wood that has a semetic origin from the Hebrew word *quinamon* (Ravindran and Babu, 2003). Plants belonging to this genus are distributed mainly in the tropical rainforests of Asia, Pacific Islands, Australia and America at varying altitudes (Ahmed et al., 2000; Rema et al., 2005). Asia is the most significant center of *Cinnamomum* species diversity followed by the neotropical regions (Lorea- Hernández, 1996). Initially *Cinnamomum* was considered to be a purely Asiatic genus occurring in the Asia-Pacific regions. But taxonomists like Nees (1836) and Meissner (1864) recognized the closeness between the species of *Phoebe* and Asiatic *Cinnamomum.* Meissner (1864) also coined the subgenus Persoidea for Asiatic and subgenus Cinnamoidea for American species of *Phoebe*.

Kostermans (1957 and 1961) transferred some *Phoebe* species that exhibited characters similar to *Cinnamomum* to *Cinnamomum* genus. Asiatic *Cinnamomum* are divided into two sections namely section *Camphora* Meissn. and section *Cinnamomum* based on their morphological features like leaf venation and arrangement, presence or absence of perula buds etc. All the native Australian species are placed under section *Cinnamomum* (Hyland, 1989). The neotropical *Cinnamomum* species have the features of both sections of Asiatic *Cinnamomum* species. Thus the genus initially considered purely Asiatic has obtained a pantropical existence, occurring in both hemispheres (Ravindran et al., 2003).

The genus is characterized by features like strong aromatic bark, opposite or occasionally alternate, tripli-nerved or pinnately nerved leaves, bisexual flowers, panicles axillary or sub-terminal inflorescence, 9 perfect stamens, 4- or 2-locular anthers. The fruits are inserted on the slightly expanded or scarcely enlarged cupular or 6-lobed perianth tube, tepals are deciduous from the base or from the middle that differentiate it from other genera of Lauraceae family (Robi et al., 2014).

2.2.1. Taxonomic Classification

Kingdom	Plantae
Division	Angiosperms
Class	Magnolids
Order	Laurales
Family	Lauraceae
Genus	*Cinnamomum*

2.2.2. Origin, distribution and diversity of *Cinnamomum* spp.

Sri Lanka is considered as the primary center of origin of genus *Cinnamomum* and several species of related taxa are also found there (Krishnamoorthy et al., 2007). *Cinnamomum,* a subset of tribe Cinnamomeae is distributed from tropical and subtropical Asia, Australia and Pacific Islands to tropical America and Africa (Huang et al., 2016). The diversity in genus *Cinnamomum* is mainly species diversity and as most of the species occur in the wild, semi-domesticated gene pools of *Cinnamomum* has not been reported (Krishnamoorthy et al., 2007).

Hooker (1886) described the presence of 26 *Cinnamomum* species in India and Kostermans (1983) reported the presence of 12 species in South India. However, now India is reported to contain 46 species of *Cinnamomum* of which 26 species are from South India and 19 species are found endemic to the region (Remya et al., 2016). Twelve species of *Cinnamomum* were reported from Kerala of which 3 are endemic to the state (Kumary et al., 2007). There are two centers of diversity for *Cinnamomum* in India, north east Indian center and south Indian center (Table 1). Some species like *C. sulphuratum* and *C. verum* however, occur in both the regions.

Table 1. Distribution of *Cinnamomum* species in India.

***Cinnamomum* species**	**Place of distribution**
South Indian Species	
Cinnamomum agasthyamalayanum Robi, Sujanapal and Udayan	South Western Ghats, India
Cinnamomum alexei Kosterm.	Ponmudi Hills, India
Cinnamomum caudatum Nees	Western Ghats, Karnataka, India
Cinnamomum chemungianum Mohan and Henry	Western Ghats,India
Cinnamomum citriodorum Thw.	Western Ghats,India
Cinnamomum dubium Nees	Kerala, India
Cinnamomum filipedicellatum Kosterm.	Western Ghats, Nilgiris, Anaimalais, India
Cinnamomum goaense Kosterm	North Western Ghats, India
Cinnamomum gracile Hook. f.	Western Ghats,India
Cinnamomum heyneanum Nees	Western Ghats,India
Cinnamomum keralaense Kosterm.	Western Ghats, India
Cinnamomum litseaefolium Thwaites	South Western Ghats, India
Cinnamomum macrocarpum Hook. f.	Nilgiris, Anaimalais, India

Cinnamomum malabatrum (Burm.f.) Bl.	Western Ghats, India
Cinnamomum nitidum (Roxb). Blume	Western Ghats, Maharashtra, Tamil Nadu, India
Cinnamomum mathewianum Remya Kr., E.S.S. Kumar, Radhamany, Valsalad. and R. Jagad.	Kerala, India
Cinnamomum nicolsonianum Manilal and Shylaja	Western Ghats, India
Cinnamomum nilagiricum Geethakum., Pandur. and Deepu	Tamil Nadu, Kerala, India
Cinnamomum palghatensis Gangop	Western Ghats, Tamil Nadu, India
Cinnamomum perrottetii Meissn	Nilgiris, Western Ghats, India
Cinnamomum riparium Gamble	Nilgiris, Western Ghats, India
Cinnamomum sulphuratum Nees	Western Ghats, India
Cinnamomum travancoricum Gamble	Western Ghats, India
Cinnamomum verum Berchthold and Presl.	Western Ghats, India
Cinnamomum walaiwarense Kostern	Western Ghats, India
Cinnamomum wightii Meissn	Niligiris,India
North Indian Species	
Cinnamomum assamicum S.C. Nath and Baruah	North East India
Cinnamomum bejolghota (Buch-Ham)	Eastern Himalayas, Myanmar
Cinnamomum camphora (L.) Brechthold and Presl.	Sub-Himalayan region
Cinnamomum cassia (L.) Brechthold and Presl.	Mizoram, India
Cinnamomum cecidodaphne Meissner	Assam, Manipur, India
Cinnamomum champokianum Baruah and S.C. Nath	Assam, India
Cinnamomum glaucescens (Buch-Ham. ex Nees)	Eastern Himalayas

Cinnamomum glanduliferum Nees	Himalayas, Khasi Hills, India
Cinnamomum impressinervium Meissner	North East India
Cinnamomum iners Reinw.ex Blume	Assam, India
Cinnamomum parthenoxylon Meisn.	Shillong, Meghalaya, India
Cinnamomum pauciflorum Nees	North East India
Cinnamomum sulphuratum Nees	North Cachar Hills, Assam, India
Cinnamomum tamala (Ham.) Th Nees and Eberm.	North East India, Assam, India
Cinnamomum verum Berchthold and Presl.	North East India

Source: (Modified from Krishnamoorthy et al., 2007).

Holotypes and description of the species studied are enumerated below.

2.2.2.1. *Cinnamomum verum* Berchthold and Presl.

Cinnamomum verum Presl, Prir.Rostl. 2: 36. t. 7. 1825; Manilal & Sivar., Fl. Calicut 250. 1982; Mohanan, Fl. Quilon Dist. 346.1984; Ansari, Fl. Kasaragod Div. 324. 1985; Ramach. & V.J. Nair, Fl. Cannanore Dist. 393. 1988; Antony, Syst. Stud. Fl. Kottayam Dist. 347. 1989; Babu, Fl. Malappuram Dist. 689. 1990; M. Mohanan & Henry, Fl. Thiruvanthapuram 392. 1994; Sivar. & Mathew, Fl. Nilambur 585. 1997; Sasidh., Fl. Periyar Tiger Reserve 345. 1998; Sasidh., Fl. Chinnar WLS 269. 1999; Sunil & Sivadasan, Fl. Alappuzha Dist. 610. 2009; Ratheesh Narayanan, Fl. Stud. Wayanad Dist. 697. 2009.
(http://keralaplants.in/keralaplantsdetails.aspx?id=Cinnamomum_verum).

C.verum has its origin in the central hills of Sri Lanka in places like Kandy, Matale, Belihull, Oya, Haputale and Sinharajan forest range (Ranatunga et al., 2004) while the Western Ghats and its adjascent regions are considered as the secondary center of origin. It was introduced into India from Sri Lanka during the 18th century by British and is cultivated in Kerala, Karnataka, Tamil Nadu and the Western Ghats (Krishnamoorthy et al., 2007). The cinnamon plantation set up by British at Anjarakandi, Kannur, Kerala, though defunct now, is reputed to be the largest and oldest cinnamon estate in the world devoted solely to cinnamon (Lawrence and Farbman, 1984). Among the species in *Cinnamomum* genus, *C. verum* assumes primary importance due to its high economical significance as its bark is widely used as spice in food industry, in pharmaceutical and cosmetic applications.

Description: Trees, upto 20 m high, bark 8-10 mm thick, brown, rough, cracks vertical; blaze creamy pink; bole buttressed; branchlets glabrous. Leaves simple, opposite or subopposite, estipulate; petiole 8-20 mm, stout, glabrous, slightly grooved above; lamina 9.5-14 cm x 3.5-5.5 cm, ovate, elliptic ovate or elliptic-lanceolate, base acute, apex acute to acuminate, margin entire, glabrous, coriaceous, 3-ribbed from base, prominent; lateral nerves 3-6 pairs, obscure, pinnate; intercostae reticulate. Flowers bisexual, in terminal and axillary, pedicel 7 mm long, pale yellow, 5 mm long, 6 mm across; perianth 8 mm, silky, tube campanulate, lobes, 3 mm long, oblong-lanceolate; stamens 9 perfect, those of first and second rows opposite the perianth lobes, introrse and eglandular, those of third row opposite the first row, lateral, bearing 2 large glands at the base; staminodes 3, of the forth row opposite the second row, cordate and stipitate; ovary half inferior, sessile. Fruit a berry, 1-2 cm, ellipsoid to oblong-ovoid, dark purple, surrounded by the enlarged perianth (http://keralaplants.in/ keralaplants details.aspx?id=Cinnamomum_verum).

2.2.2.2. *Cinnamomum cassia* Berchthold and Presl.

Bercht. & Presl, 1825, *Prir. Rostlin* 2: 36, 44-45, 5, 6, 1825; Blume, Bijdr. Fl. Ned. Ind., 11 stuk 570, 1826; Sweet, Hort. Brit. 344, 1827; Th. Ness, Pl. Off. 1: 5.129, 1829; London, Hort. Brit. 190, 1830; Nees & Ebermayer, Handb. Med. Pharm. Bot. 2: 424, 427, 1831; CG Nees in Wallich, Pl. As. Rar. 2: 73 et 74, 1831; Syst. Laur. 42 et 52, 1836; Lindley, Fl. Med. 330, 1838; Miquel, Fl. Ind. Bat. 1(1): 896, 1858: Meissner, In Dc., Prodr. 15(1): 12, 18, 466, 1864; Balfour, Timb. Trees & For. India & Se Asia ed. 3.74, 1870; Kurx. For Fl. Brit. Burma 2: 288, 1877; Gamble, Man. Ind. Timb. 306, 1881 ed 2.560, 1902; Hooker, Fl. Brit. India 5: 130, 1886; Staub, Ge Schichte Genus Cinnamomum 11, 19, 27, 31, 32, 39, 40, 1905; Dunn & Tutcher, inKew Bull., Add. Ser. 10: 223, 1912; Kostermans, in J. Sci. Res. Indon, I: 84, 85, 1952; Commun. For. Res. Inst. Bogor, 57: 24, 1957; in Reinwardtia r: 216, 1956; Chopra et al., Gloss. Ind. Med. Pl. 65, 1956; Wood, In J. Arnold Arb.39: 335, 1958; Kostermans, Bib. Lau., 276, 1964 (Ravindran et al., 2003).

C. cassia has its origin in China and is found in Vietnam, Malayan Archipelago and the north eastern Himalayan region (Ravindran et al., 2003). Its bark is called cassia bark and is used as flavorants in foods, in pharmaceutical preparations and perfumery. As it is often used as a substituent of true cinnamon it is also known as false cinnamon (Krishnamoorthy et al., 1999).

Description: Medium sized trees, 18-20 m, grey brown bark 13-15 mm thick; branchlets yellow brown, more or less tetragonal, longitudinally striated and densely tomentose with grey yellow hairs. Apical buds are small with broad oval scales and densely tomentose with grey hairs. The leaves are simple, alternate/subopposite, oblong oval/narrowly elliptic to sublanceolate, thick, coriaceous, glabrous and shiny green appearance upper, greenish opaque sparsely hairy beneath. Three nerves arise from the base and have a slightly acute apex. The leaf margins are cartilaginous and involute. 1.5-2 cm long petioles; axillary/subterminal panicle; three flowered at branch tips and long peduncle with a length half of its inflorescence. White flowers, 4.5 mm long; 2-6 mm long pedicels; inner and outer side of perianth is densely brown tomentellate and obconical tube, 2 mm long with ovate-oblong lobes. 2-3 mm long stamens, 9 numbers, lateral extorse and 3 staminodes. Glabrous, 1.7 mm long ovary with slender style; ellipsoid fruits, 10x7 mm, pink violet, and about 7 mm broad at the apex (Li and Li, 1998).

2.2.2.3. *Cinnamomum malabatrum* (Burm.f.) Bl.

Cinnamomum malabatrum (Burm. f.) Blume, Bijdr. 568. 1826; Manilal, Fl. Silent Valley 234. 1988; Vajr., Fl. Palghat Dist. 403. 1990; Ansari, Fl. Kasaragod Div. 324.1985; Antony, Syst. Stud. Fl. Kottayam Dist. 346. 1989; M. Mohanan & Henry, Fl. Thiruvanthapuram 392. 1994; Sasidh.et al., Bot. Stud. Med. Pl. Kerala 11,23,31. 1996; Sasidh. & Sivar., Fl. Pl. Thrissur For. 380. 1996; Sasidh., Fl. Shenduruny WLS 267. 1997; Sivar. & Mathew, Fl. Nilambur 583. 1997; Sasidh., Fl. Periyar Tiger Reserve 345. 1998; Sasidh., Fl. Parambikulam WLS 268. 2002; Anil Kumar et al., Fl. Pathanamthitta 423.2005; Sunil & Sivadasan, Fl. Alappuzha Dist. 609.2009; Ratheesh Narayanan, Fl. Stud.Wayanad Dist. 697. 2009 (http://keralaplants.in/ keralaplantsdetails. aspx?id = Cinnamomum_malabatrum).

C. malabatrum is a moderate evergreen tree that is a close relative of *C. verum,* found in the Western Ghats, tropical and subtropical Himalayas, Uttar Pradesh, Eastern Bengal, Myanmar, Khasia and Jaintia Hills in Meghalaya. Both bark and leaves possess condiment properties due to which they are employed as adulterants of *C. verum* barks and *C. tamala* leaves (http://shodhganga.inflibnet.ac.in/bitstream/10603/8765/13/13_chapter%204.pdf).

Description: Trees, about 20 m high, bark 5-10 mm thick, reddish-brown, smooth or slightly, longitudinally cracked; blaze dull-red, aromatic. Apical bud small, densely, subadpresed pilose. Leaves simple, opposite or subopposite, estipulate; petiole 10-20 mm long, stout, glabrous; lamina 10-30 cm x 3-9 cm, elliptic-oblong, oblong or oblong-lanceolate, base

acute, apex acute or acuminate, margin entire, glabrous above, highly aromatic when bruised, coriaceous; 3-ribbed at or a little above the base, the side ribs running almost to the apex, prominent with sparse crisp fine hairs, intercostae scalariform, glabrescent, slender, prominulous. Flowers bisexual, in axillary and pseudo terminal paniculate cymes upto 25 cm long, with densely minute subadpressed pilose hairs; pedicels 3-4 mm long, slender, slightly thickened upwards; perianth tube shallow, 1 mm broad; tepals 6, 3-3.5 mm long, fleshy, ovate, acute, with dense subadpressed pilose hairs; stamens 9 perfect, in 3 rows, 2-2.5 mm long, those of first and second row opposite the perianth lobes, introrse and eglandular, those of the third row opposite the first row, extrorse, bearing large glands on short stipes, the basal part of which adnate to the filaments; filaments pilose, slender, slightly longer than the anthers; anthers oblong to subovate-oblong, 4-celled; staminodes of fourth row 1.5 mm long, hastate, cordate, on as long stipes, opposite the second row. Half inferior ovary, 2 mm, ellipsoid; thick cylindrical style, small peltate stigma. Fruit berry, 8-10 mm, subtended by a cup and tepals are rounded at the base (http://keralaplants.in/ keralaplantsdetails. aspx? id= Cinnamomum_ malabatrum).

2.2.2.4. *Cinnamomum citriodorum* Thw.

C. citriodorum Thw. Enum. Pl. Zey. 253, 1861; Meissn.in.DC Prodr. 15(1): 22, 1864; Miq. Ann. Mus. Bot., Logduno Batavum, 1: 258, 1864; Hook.f.Fl.Br. Ind., 5: 134, 1886; Trimen, Hert.Zey.69, 1888; Trimen, Hanb.Fl.Ceylon 6: 247, 1931; Kostermans, Bib. Laur.284, 1964 (Shylaja et al., 2003).

C. citriodorum is a medium sized tree that is endemic to Sri Lanka and also found in south Western Ghats. Known as ‘Pengiri kurundu’ in Sri Lanka, it is listed as a threatened species in the IUCN Red Data Book published in 2004 by Sri Lanka. It is believed that this species was introduced to India (Rema et al., 2007). Both leaves and barks of *C. citriodorum* are used as spice and medicine by the tribal people (Shylaja et al., 2003).

Description: Medium sized trees with smooth, pale grey/brown bark having lemon grass odour. The branchlets are opposite, slender, smooth and glabrous. Apical bud is silvery and sericeous. The leaves are lanceolate/oval to subovate to lanceolate, gradually tapered, shortly acuminate, coriaceous, glabrous, smooth, glossy, pinnately veined, slender midrib prominent on both surfaces. Four to eight pairs of lateral veins obscurely visible beneath and faintly visible above and two subbasal veins reaching one third of the length of the lamina. 8-10 cm long panicles positioned axially on the peduncle. Greenish white, sericeous flowers, short

tube, narrowly ovate, obtuse, sericeous 3mm long tepals, 2 mm long stamens, anthers of the outer whorls narrowly ovate, sub-acute, small upper cells, anthers of the inner whorl extorse, two-celled, large basal glands and sessile. Small, sagitate, acute staminodes, sub-globose ovary, short style with stigma – small and subpeltate. Ellipsoid/ovate ellipsoid fruit with hemispherical cup. (Dassanayake et al., 1995).

2.2.2.5. *Cinnamomum tamala* (Ham.) Th. Nees and Eberm. (Indian Cassia)

Th. Nees & Ebermayer, Hanb. Med. Pharm. Bot. 2:426 et 428, 1831; CG Nees, in Wallich, Pl. As. Rar. 2:75, 1831; in Flora 15 (2): 591 et 596, 1831; Syst. Laura. 56 et 666, 1836; Th. Nees, Pl. Office, Suppl. 4:22, 1833; Hayne, Getreue Darstell. Arzneijew., 360, 1838; Fl. Ind. Bat.1(1); 892, 1858; Ann. Mus. Bot. Lugd. Bat. 1: 268, 1864; Stendel, Nom. ed. 2, 1: 366, 1840 et 2: 15 et 17, 1841: Meissner, in DC. Prodr. 15 (1): 17, 1864; Bentham, fl. Austral. 5: 303.1870; in Bailey, Queensland. Fl. 4:1309, 1901; Udoy Chand Dutt, Materia Med. Hindus, 224, 1872; rev. ed. 224, 320, 1900; Steward and Brandis, For. Fl. N. W. India, 374, 1874; Gamble, List Trees & Shrubs Darjeeling Dist. 63, 1878; ed. 2:64, 1896; Man. Ind. Timb., 306, 1881; ed. 2, 560, 1902; Hooker f., Fl. Brit. India, 5: 128, 1886; Watt, Dict. Econ. Prod. India 2, 319-323; 1889; Pax, in Engl & Pr. Nat. P.fl. fam; 3(2): 114, 1889; Gage, in Rec. bot. Surv. India 1: 355, 1893; 3(1): 98, 1904; Pharmc. J. 12 May 1894, 941; Kanjilal, For.Fl. School circle, N. W. Pror.275, 1901; For. Fl. Sivalik 326, 1911; Prain, Bengal Pl. 2:899, 1903; in Rec. bot. Survey India 3(2): 270, 1905; Staub, Geschichte gem. Cinnam. 21, 39, 42, T. 17, 1905; Brandis, Ind. Trees, 533, 1906; Lac, List of Trees, shrubs Burma 109, 1912, ed. 2:137, 1922; Duthie, Fl. Upper Gangetic plain 3(1): 57, 1915; Haines, Bot. Bihar & Orissa 797, 1924; Burkill, in Rec. bot, Sur. Ind. 10(2): 351, 1925; Dic. Eco. Prod. Malay Penin, 1: 543 et 556, 1935; Osmanton, For. Fl. Kumaon, 443, 1927; Fischer in Rec. Bot. Sur. India 12(2); 128, 1938; Kanjilal et al.,; Fl. Assam, 4: 56, 1940; Kostermans, Bib. Lau, 354, 1964 (Ravindran et al., 2003).

C. tamala is a small to moderate sized evergreen tree native to India having its origin in Himalayas. It is mainly distributed in India, Indo-China region, Bangladesh and Nepal. It is found in North Western, Eastern and Sikkim Himalayas, Khasi and Jaintia Hills in Meghalaya, North Cachar Hills in Assam, Basantgarh, Rajouri in Jammu and Kashmir, Jaunsar, Tehri Garwal and Kumaon in Uttaranchal and areas of Himachal Pradesh like Drang Forest in Hamirpur, Shimla, Chamba, Mandi, Solan, Nahan etc. Commercial cultivation of *C. tamala* is limited to Khasi and Jaintia Hills, Mikir Hills, Manipur, Arunachal Pradesh,

Nainital and Kangra (Krishnamoorthy et al., 2007). Bark of *C. tamala* known as Indian cassia bark and its leaves popularly known as 'Indian bay leaf ' or tejpat are used as flavorants and in medicinal preparations (Rema et al., 2005).

Description: Evergreen trees, about 7 m high, dark grey, aromatic rough bark that darkens on exposure. Based on their leaf morphology 4 morphotypes were identified by Baruah et al., (2000). The leaves are alternate, sub-opposite/opposite, coriaceous, aromatic, glabrous and young leaves are pink in colour. In morphotype I, leaves are ovate to ovate-lanceolate; in morphotype 2 elliptic-lanceolate; broadly elliptic lanceolate in morphotype 3, small and elliptic to oblong-lanceolate in morphotype 4. The apex is acute to acuminate, it is triplinerved, lateral nerves do not reach the tip. Petiole is about 1.3 cm long and panicle sub-terminal to axillary, equal to the leaves or slightly exceeding them. Flowers 5-7 mm long with 3 mm long pedicel. Perianth 3+3, elliptic-ovate-lanceolate, sub-equal, outer 2.5 mm long, silky pubescent on both surfaces and longitudinally brown ribbed. 9 stamens, 1.5-2 m long, four lobed anther, introrse, extrorse in whorl III, glands of whorl III attached to one third base of the filament, pale yellow anther and glands with pale yellow silky tomentose filaments, pale yellowish green pistil 1.5-2 mm long, filiform style, elliptic-oblong ovary and black drupe fruit supported by thickened peduncle 3-4 mm long (Baruah and Nath, 2003).

2.2.2.6. *Cinnamomum glaucescens* (Wall.ex Nees) Drury

C. glaucescens Drury, Handb. Ind. Fl. 3:55, 1869; St. Lager in Ann. Soc. Bot. Lyon 7: 122; Yamada in Trans. Nat. Hist. Soc. Formosa 17, 440 1927 (Shylaja et al., 2003).

C. glaucescens is an evergreen tree that originated in Western Nepal and is distributed in the Eastern Himalayan tracts. It is distributed mainly in three states of Nepal viz. Dang, Rolpa and Sallyan in Rapti Zone (Rema et al., 2002). Widely known as Sugandha kokila in Nepal it is used as a spice, for flavouring tobacco, in medicinal preparations etc. (Shylaja et al., 2003). The wood of the tree known as Nepal camphor or Nepal sasafras is highly scented and is also used in making furnitures for keeping clothes due to their durability and insect repellant properties (Bose et al., 2008a).

Description: Large trees 25 m tall, highly scented bark. Leaves elliptic or lanceolate, 7-10 cm long, caudate-acuminate, nerves arising on each side of the mid-rib. Short crowded

inflorescence with a densely tomentose panicle and few small yellow pubescent flowers. Fruits are oblong, 3-4 cm long (Bose et al., 2008a).

2.2.2.7. *Cinnamomum sulphuratum* Nees

Cinnamomum sulphuratum Nees in Wall., Pl. Asiat. Rar.2: 74.1831; Hook. f., Fl. Brit. India 5: 132. 1886; Gamble, Fl. Pres. Madras 1225(857).1925; Mohanan, Fl. Quilon Dist. 345.1984; Manilal, Fl. Silent Valley 235. 1988; Vajr., Fl. Palghat Dist. 403. 1990; M. Mohanan & Henry, Fl. Thiruvanthapuram 392. 1994; Sasidh.et al., Bot. Stud. Med. Pl. Kerala 23,31. 1996; Sasidh.&Sivar., Fl. Pl. Thrissur For. 381. 1996; Sasidh., Fl. Shenduruny WLS 267. 1997; Sasidh., Fl. Periyar Tiger Reserve 345. 1998; Ravikumar & Ved, Illustr. Field Guide 100 Red Listed Med. Pl. 91. 2000 Sasidh., Fl. Parambikulam WLS 268. 2002; Mohanan & Sivad., Fl. Agasthyamala 567.2002; Anil Kumar et al., Fl. Pathanamthitta 424.2005; Ratheesh Narayanan, Fl. Stud. Wayanad Dist. 697. 2009 (http://keralaplants.in/keralaplantsdetails.aspx?id=Cinnamomum_sulphuratum).

C. sulphuratum is one of the *Cinnamomum* species endemic to South India (Kostermans, 1983). An evergreen tree endemic to South Western Ghats of India (Rameshkumar and George, 2006), it is found in regions of Nilgiris and Anamalai in Tamil Nadu, Thiruvananthapuram and Wayanad in Kerala, and Coorg, Dakshina Kannada, Hassan, Mysore, Shimoga, Uttara Kannada districts of Karnataka (Shivaprasad, 2015). *C. sulphuratum* was also reported in north east India (Nath and Baruah, 1994). Bark and leaves are used as medicines to cure headache, cough, as a spider poison and mouth refreshner; leaves are also used as substitute for *C. tamala* (Saha et al., 2015).

Description: Trees, 8 m high, bark smooth, odorless, reddish brown; blaze dull red; branchlets slender, angular, densely, minute sub-appressed, yellow, fulvous, pilose. Apical bud small with densely yellow appressed fulvous pilose. Leaves simple, opposite or sub-opposite, estipulate; petiole 8-15 mm long, stout, yellow pilose when young; lamina 4-21 cm x 2-9 cm, elliptic, elliptic-ovate, elliptic-lanceolate or elliptic-oblong, base acute obtuse or shortly cuneate, apex acute or obtusely acuminate or acuminate, margin entire, glabrous and glossy above, glaucous and yellowish pubescent beneath, glabrous afterwards, coriaceous; 3-ribbed from at or a little above the base; side ribs reaching nearly to the apex, prominent beneath; intercostae scalariform, faint. Flowers bisexual, in axillary or terminal lax, yellow fulvous pilose panicles, branchlets few, slender; perianth tube 1-1.5 mm long, densely yellow pilose on both sides; tepals 6, 2-4 mm long, ovate, acute, yellow pilose on

both sides; stamens 9 perfect, those of first and second rows opposite the perianth lobes, introrse and eglandular, those of third row opposite the first row, lateral, bearing 2 large glands at the base; staminodes 3, of the fourth row opposite the second row, narrowly sagitate, stipitate, pilose; ovary half inferior, ellipsoid, as long as the style, stigma minute, peltate. Fruit a berry, 1 x 1.5 cm, cup-shaped; at the rim bears hard end, appressed-pilose, ovate tepals; base of the cup fleshy, obconic, slightly merging into slender pedicel (http://keralaplants.in/keralaplantsdetails.aspx?id=Cinnamomum_sulphuratum).

2.2.2.8. *Cinnamomum camphora* (L.) Brechthold and Presl.

Cinnamomum camphora (L.) Presl, Prir. Rostl. 2: 47. t. 8. 1825; Hook. f., Fl. Brit. India 5: 134. 1886; Gamble, Fl. Pres. Madras 1225(858).1925; Ansari, Fl. Kasaragod Div. 323. 1985; M. Mohanan & Henry, Fl. Thiruvanthapuram 393. 1994 (http://keralaplants.in/ keralaplantsdetails. aspx?id=Cinnamomum_camphora).

C. camphora is an evergreen tree that is distributed in China, Japan, Taiwan, adjoining areas of South East Asia, sub Himalayan regions of India and Australia. In India it is grown in plantations in Dehradun, Saharanpur, Kolkata, Nilgiris and Mysuru. *C. camphora* is an evergreen and aromatic tree. The wood, twigs and bark of the tree on distillation yields a white crystalline substance called camphor that has medicinal, fragrant and insect repellant properties (Babu et al., 2003).

Description: Trees large; branchlets slender, glabrous; terminal buds large, perulate, the outer bud scales glabrous, the inner sericeous. Leaves spirally arranged, glabrous, chartaceous to sub-coriaceous, ovate-elliptic to elliptic to subovate-elliptic, 1-5 cm x 3-10 cm, base acute or tapered-cuneate, both surfaces minutely reticulate or above smooth; main nerves slender. Petiole slender, 2-4 cm long. Panicles axillary, slender, glabrous, many-flowered, up to 10 cm long with few short branches. Pedicels 1-2 mm, obconical.Tepals 2 mm, ovate, acutish, fleshy. Stamens 1.5 mm long; anthers broad; inner anthers truncate, upper cells smaller, latrorse; large gland attached to the basal part of the filament. Staminodes stipitate. Style as long as the ovary with peltate triquetrous stigma. Fruit globose, slightly fleshy, 5-10 mm in diameter, seated on a shallow, thin cup, 3-5 mm in diameter at the apex, 1-2 mm deep, the basal part fleshy, obconical, usually not differentiated from the obconical pedicel, together 5-7 mm long (http://keralaplants.in/ keralaplantsdetails.aspx?id=Cinnamomum_camphora)

2.2.2.9. *Cinnamomum alexei* Kosterm.

Cinnamomum alexei Kosterm., Reinwardtia 7: 454. 1969; Santhosh Kumar et al., Bangladesh J. Plant Taxon.18: 199. 2011. (Kumar et al., 2011).

C. alexei, described by Kostermans (1969) was reported originally from Tjiharum, G Karang, near Tjidadap in Mount Karang, Java, Indonesia. Surprisingly, it was also reported from Ponmudi Hills, Thiruvananthapuram, India (Kumar and Jabbar, 2014). It occured as a member of the third storey evergreen forest in association with *Actinodaphne malabarica, Antidesma menasu, Aporosa acuminata, Cinnamomum malabatrum, Syzygium mundagam* and *Xanthophyllum flavescens* (Kumar et al., 2011). Initially it was placed under *C. verum* but later studies showed remarkable differences between the two and thus it was declared as distinct from *C. verum* (Soh, 2011).

Description: Small trees, 3-5 m high; bark slimy inside, strong smell of betel leaves; terminal bud small with 2 scales, externally densely sericeous; branchlets slender, apically subquadrangular, basally subterete. Leaves opposite or sub-opposite, 4-12 cm × 1.5-5.0 cm, ovate to lanceolate, rounded to cuneate at base, long acuminate to caudate at apex, dark green above, glaucous beneath, entire or undulate at margin, chartaceous, smooth, strong smell of betel leaves; midrib and 2 sub-basal lateral nerves reach below the acumen or rarely almost at blade tip by anastomosing the secondary lateral nerves; secondary nerves closely reticulate, prominent; petioles slender, 0.7-1.5 cm long, concave above. Panicles axillary or extra-axillary, 2.5-4.5 cm long, 5-12 flowered, glabrous, with 3-5 branches; central peduncle slender, 2.0-2.5 cm; pedicels 4.5 mm long, slender, gradually thickened at apex. Flowers 2.5-3.0 mm, dark maroon, glabrous; perianth of 6 tepals in 2 whorls of 3 each, broadly ovate, to 1.5 mm long, acute to obtuse at apex, minutely sericeous at base, dark reddish tomentose within; stamens 9, in 3 whorls of 3 each; whorls I & II with anthers elliptic to ovate, 1.0-1.5 mm long, 2-celled, more or less fleshy, introrse, longer than filaments; filaments sericeous at the very base within; whorls III extrorse; anthers oblong, 2-celled, glands sub-sessile, attached near the basal portion of the filaments; staminodes shorter than anthers, hastate, on 0.5 mm long stipes with 1 or 2 hairs on either sides. Ovary ellipsoid, 1.0-1.5 mm long, glabrous; style as long as the ovary, rather thick with small peltate stigma. Fruit ellipsoid, 15 × 11 mm, cupule cup-shaped, cup shallow, 1.5-2.0 mm × 9 mm, base conical, tepals persistent on fruit and then acute to subacute, indurate (Kumar et al., 2011).

2.2.2.10. *Cinnamomum heyneanum* Nees

Cinnamomum heyneanum Nees in Wall., Pl. Asiat. Rar. 2: 76. 1831 (Kumar et al., 2003).

C. heyneanum described by Nees (1831) is endemic to Peninsular India, distributed in Coorg southwards in Western Ghats. They were found associated with *Myristica malabarica* Lam*., Vateria indica, Humboldtia vahliana* and *Hopea parviflora* (Kumar et al., 2003).

Description: Trees, branchlets slender, minutely grey tomentellous, hairs very thin, wavy. Terminal bud small, Leaves opposite and sub-opposite, 9-13 cm x 1.2-2.5 cm, chartaceous, linear-oblong, attenuate-acuminate at apex, base attenuate-acute, above glabrous, sparsely, minutely tomentellous underneath, obscurely, chartaceous midrib prominent, (starting at lateral nerves reaching two third of the lamina length, and then continuing in large loops to the apex, in this lamina part the loops belong actually to a few, rather patent, faint lateral nerves; secondary nerves faint, parallel, sub-horizontal, minutely, smoothly, reticulate. Petiole very slender, sparsely, minutely tomentellous, 5-8 mm long, flattened above. Cymes axillary or extra axillary, up to 6.5 cm long; main peduncle 4-5 cm long, slender and glabrous. Pedicels glabrous and 1-5 cm long. Flowers 3 per cyme, 4 mm long, densely minutely sericeous; perianth tube 1.5 mm long and cup shaped. Infructescence in the axils of terminal leaves with slender, glabrous, 5-8 cm long peduncle unbranched or with a single, apical, 1 cm long branch. Fruit ellipsoid, smooth, 7 x 12 mm; fruit pedicel obconical, 1 cm long, slender. Persistent tepals, densely and coarsely appressed pilose inside. Single ellipsoid seed (Kumar et al., 2003).

2.2.2.11. *Cinnamomum riparium* Gamble

Cinnamomum riparium Gamble, Bull. Misc. Inform. Kew 1925. 128. 1925 & Gamble, Fl. Pres. Madras 1224 (856). 1925; Ramach. & V.J. Nair, Fl. Cannanore Dist. 393.1988; Sivar. & Mathew, Fl. Nilambur 584. 1997; Ramach.et al., Journ. Econ.Tax. Bot. 1: 94. 1980; Mohanan & Sivad., Fl. Agasthyamala 567. 2002 (http://keralaplants.in/ keralaplantsdetails. aspx? id = Cinnamomum_riparium).

C. riparium was first described by Gamble (1925) based on the specimens collected by Bourdillion from the banks of Periyar river (Geethakumary et al., 2015). It is a native of India found in Kerala, Karnataka and Tamil Nadu, distributed along the coast of Travancore Hills, Coorg and Western Ghats (World Conservation Monitoring Centre, 1998).

Description: Small trees. Leaves opposite to subopposite, 7-11 cm x 1.5-3.5 cm, acuminate at apex, broadly attenuate at base, 3-ribbed, 0.5 cm above from base. Panicles few-flowered, peduncles 3-4 cm long, pedicels slender, 0.8-1 cm long. Flowers 0.3-0.35 cm, perianth lobes 6, 0.1 cm long. Stamens in 3 rows; first and second introrse, third extrorse, glands attached to middle of filaments. Ovary ellipsoid and stigma peltate (http://keralaplants.in/keralaplants details. aspx?id=Cinnamomum_riparium).

2.2.2.12. *Cinnamomum travancoricum* Gamble

Cinnamomum travancoricum Gamble, Bull. Misc. Inform. Kew 1925: 128. 1925 & Gamble, Fl. Pres. Madras 1224(857).1925; Ramach.& V.J. Nair, Fl. Cannanore Dist. 393. 1988; M. Mohanan & Henry, Fl. Thiruvanthapuram 392. 1994; Gopalan & Henry, Endemic Pl. Agasthiyamala 81.2000; Mohanan & Sivad., Fl. Agasthyamala 568. 2002 (http://keralaplants.in/keralaplantsdetails.aspx?id=Cinnamomum_travancoricum).

C. travancoricum is an evergreen tree endemic to Western Ghats first described by Gamble (1925) based on collections of T.F. Bourdillion from Chemunji near Ponmudi in 1895. It is often confused with *C. sulphuratum* (Kumary et al., 2013).

Description: Trees, about 8 m high; branchlets slender, angular, minutely densely subappressed or appressed pilose; branches smooth, glabrous, striate. Leaves simple, opposite, estipulate; petiole 5-15 mm long, slender, appressed pilose; lamina 5-9 cm x 1.5-4 cm, elliptic, elliptic-oblong or elliptic ovate, base acute, apex acute or acuminate, margin entire, appressed pilose when young, glabrous above when mature; glabrescent beneath, coriaceous; 3-ribbed from a little above the base, prominent beneath, side ribs ends 1-2 cm below the leaf tip, connected to the midrib at their ending point by a prominent transverse nerve; intercostae scalariform, faint. Flowers bisexual, in axillary and pseudo terminal, 1-3 cm long, light brown, subappressed pubescent racemes; peduncle slender; pedicel 1-3 mm long, funnel shaped; tepals 6, 4 mm long, oblong, acute, densely subappressed light brown pilose, subequal; stamens 9 perfect, 2.5-3 mm long, those of first and second rows opposite the perianth lobes, introrse and eglandular, those of third row opposite the first row, lateral, bearing 2 large glands at the base; staminodes 3, of the fourth row opposite the second row, cordate and stipitate, staminodes 3, in row 4, 1-1.5 mm long, sagittate, stipe pilose; ovary half inferior, sessile, glabrous, at the bottom of the perianth tube, narrowed into thick style,

stigma peltate (http:// keralaplants.in/ keralaplantsdetails. aspx? id = Cinnamomum_ travancoricum).

2.3. Genus *Myristica*

The genus *Myristica* belongs to the family Myristicaceae that has around 18 genera and 300 species. They are pantropical and are seen in the rainforests of Asia, Africa, Madagascar, South America and Polynesia (Krishnamoorthy et al., 2007). In India four genera of Myristicaceae are found - *Myristica, Horsfieldia, Knema* and *Gymnocranthera* mostly in Andaman and Nicobar Islands as well as in *Myristica* swamps found in valleys of Shendurney, Kulathupuzha and Anchal forest ranges in southern Western Ghats and Uttara Kannada district of central Western Ghats (Krishnamoorthy et al., 2007). *Myristica* is considered to be the most primitive (Sinclair, 1958) and largest genus of Myristicaceae family. Blume and Bentham divided the genus into sections Knema, Irya, Pyrrhosa and Eumyristica according to their androecium modifications (Hooker and Thomson, 1855) while Hooker (1856) classified it into three sections Knema, Myristica and Pyrrhosa. However, Gamble (1957) put forth another classification in which the species of Myristicaceae in India were placed in three genus viz., *Knema*, *Gymnocranthera* and *Myristica* based on their anther column attachment.

Myristica genus is characterized by alternate, entire leaves. Flowers are small, dioecious in cymes, umbels or fasciles from the leaf axis/of the scars of fallen leaves. The peduncle is usually thick bract and persistent brancteoles. Perianth is 3 lobed, androecium stalked and filaments connective connate on a column. Anthers are elongate and 12-30 in number while ovary is ovoid. They have large, ovoid or oblong fruits. Seeds conform to fruit, testa hard, albumen ruminate and cotyledon connate (Gamble 1957).

2.3.1. Taxonomic Classification

Kingdom	Plantae
Division	Angiosperms
Class	Magnolids
Order	Magnoliales
Family	Myristicaceae
Genus	*Myristica*

2.3.2. Origin, distribution and diversity of *Myristica* spp.

Myristica genus has a Malayan centre of origin (Sheeja et al., 2013) and is distributed from South East Asia and India to North Australia and the Pacific Islands (Rema and Krishnamoorthy, 2012). *Myristica* contains about 120 species and are mostly found in New Guinea. In India, 14 *Myristica* species are distributed as depicted in Table 2.

Table 2. Distribution of *Myristica* species in India.

Myristica species	Place of distribution
Myristica amygdalina Wall.	Nagercoil,India.
Myristica andamanica Hook.f.	Andaman Islands,India
Myristica attenuata Wall.	Western Ghats,India
Myristica beddomei King	Western Ghats, India
Myristica dactyloides Gaertn	Western Ghats,India
Myristica fatua Houtt.	Western Ghats, India
Myristica fragrans Houtt.	Western Ghats, Tamil Nadu, Karnataka and Andaman Islands, India
Myristica gibbosa Hook.f. and Thoms.	Khasi Mountains, India
Myristica glabra Blume	Silhet, Tinnevelly and Andaman Islands,India
Myristica glaucescens Hook.f. and Thoms.	Tinnevelly and Andaman Islands,India
Myristica irya Gaertn. Fruct.	Tinnevelly and South Andaman Islands, India
Myristica kingii Hook.f.	Sikkim Himalaya, India
Myristica longifolia Wall.	Sikkim Himalaya, Assam and the Khasi Hills,India.
Myristica magnifica Hood. f.	Western Ghats, parts of Tinnevelly and South Karnataka, India
Myristica malabarica Lam.	Western Ghats, India
Myristica prainii King	Andaman Islands, India

Holotypes and description of the species studied are enumerated below.

2.3.2.1. *Myristica fragrans* Houtt.

Myristica fragrans Houtt., Hist. Nat. 3: 333. 1774; Hook. f., Fl. Brit. India 5: 102. 1886; Gamble, Fl. Pres. Madras 1214 (850). 1925; Manilal & Sivar., Fl. Calicut 249. 1982; Mohanan, Fl. Quilon Dist. 342.1984; Antony, Syst. Stud. Fl. Kottayam Dist. 345. 1989; M. Mohanan & Henry, Fl. Thiruvanthapuram 388. 1994; Subram., Fl. Thenmala Div. 311. 1995; Sivar. & Mathew, Fl. Nilambur 582. 1997; Anil Kumar et al., Fl. Pathanamthitta 421.2005; Sunil & Sivadasan, Fl. Alappuzha Dist. 608.2009; Ratheesh Narayanan, Fl. Stud.Wayanad Dist. 693. 2009. (http://keralaplants.in/keralaplantsdetails. aspx?id= Myristica_ fragrans).

M. fragrans is an aromatic evergreen tree spice native to Banda Islands, Moluccas and successfully grown in other Asian countries such as India, Malaysia, Papua New Guinea, Sri Lanka, and in the Caribbean, namely Grenada. Introduced to India in 18th century by the British, it is now cultivated in parts of Kerala, Tamil Nadu and Karnataka (Sasikumar et al., 2013). Commonly known as nutmeg, it is the only cultivated *Myristica* species. The tree is popular for the twin spice it produces; the mace or aril and the kernel or seed. Both kernel (nutmeg) and mace are used as spice, condiment and in medicines. The nutmeg oil and butter are also used in medicinal preparations (Bose et al., 2008b).

Description: Evergreen dioecious tree, 8 m high; bark black; branches horizontal. Leaves simple, alternate, estipulate; petiole 9-15 mm long, slender, grooved above, glabrous; lamina 9-18 cm x 2.5-6 cm, elliptic, elliptic-lanceolate, elliptic-oblanceolate or elliptic-obovate, base acute or attenuate, margin entire, glabrous, shiny above and glaucous beneath, coriaceous; lateral nerves 10-14 pairs, pinnate, prominent, intercostae reticulate. Flowers unisexual, yellow; male flowers: 3-5 in axillary cymes, urceolate; pedicels drooping; bracts deciduous; bracteoles persistent; perianth 3-lobed, tawny villous; stamens 9-12, connate in to a central stipitate column; female flowers: urceolate, larger than the male, few in axillary cymes; pedicels drooping; bracts deciduous; bracteoles persistent; perianth 3-lobed, tawny villous; ovary superior, ovoid, 1-celled, 1-ovuled; stigmas connate, 2-lobed. Fruit a capsule, yellow to 5 cm long with red aril and lacinate brown seed (http:// keralaplants.in/ kerala plantsdetails.aspx?id=Myristica_fragrans).

2.3.2.2. *Myristica malabarica* Lam.

Myristica malabarica Lam., Acad. Roy. Sci. Mem. Math.Phys. (Paris) 162.1791; Hook. f., Fl. Brit. India 5: 103. 1886; Gamble, Fl. Pres. Madras 1213(850). 1925; Manilal & Sivar., Fl. Calicut 249. 1982; Mohanan, Fl. Quilon Dist. 342.1984; Manilal, Fl. Silent Valley 233. 1988; Babu, Fl. Malappuram Dist. 687. 1990; Vajr., Fl. Palghat Dist. 401. 1990; M. Mohanan & Henry, Fl. Thiruvanthapuram 387. 1994; Subram., Fl. Thenmala Div. 311. 1995; Sasidh.et al., Bot. Stud. Med. Pl. Kerala 24.1996; Sasidh. & Sivar., Fl. Pl. Thrissur For. 378. 1996; Sasidh., Fl. Shenduruny WLS 261. 1997; Sivar. & Mathew, Fl. Nilambur 581. 1997; Sasidh., Fl. Periyar Tiger Reserve 341. 1998; Ravikumar & Ved, Illustr. Field Guide 100 Red Listed Med. Pl. 250. 2000 Mohanan & Sivad., Fl. Agasthyamala 559.2002; Anil Kumar et al., Fl. Pathanamthitta 421.2005; Sunil & Sivadasan, Fl. Alappuzha Dist. 608.2009; Ratheesh Narayanan, Fl. Stud.Wayanad Dist. 692. 2009 (http://keralaplants.in/ keralaplants details. aspx?id=Myristica_malabarica).

M. malabarica is found in the evergreen forests of Western Ghats in Konkan, Coorg and North Malabar regions (Maya et al., 2006).The ripe fruits of *M. malabarica* are largely employed as an adulterant of true nutmeg. The mace of *M. malabarica* is called as Bombay mace and it lacks odour and taste.The seeds are employed for treating ulcers, rheumatism etc. Kino, a wood derived from its bark is used for building purposes, making light furniture, match boxes and splints (Bose et al., 2008b).

Description: Dioecious tree, height 15 m, girth 45 cm; bark 8-10 mm thick, greenish-black, smooth, tuberculate-lenticellate, semi fibrous, blaze reddish, exudation deep red; branchlets glabrous. Leaves simple, alternate, distichous, estipulate; petiole 10-15 mm long, stout, grooved above, glabrous, lamina 7-20 cm x 3-10 cm, elliptic or elliptic-oblong, base acute or attenuate, apex acute, or subacute, margin entire, glabrous and glossy above and glaucous beneath, coriaceous; lateral nerves 8-14 pairs, pinnate, slender, obscure, intercostae obscure. Flowers unisexual, white; male in cymes, axillary or from the old axils below the leaves, generally cymosely branched, rarely simple. Male flowers: more numerous than in female and smaller, umbelled at the apex of branchlets; perianth urceolate, minutely puberulous outside, glabrous inside, 3-4 cleft at apex; staminal column stalked, slightly produces above the anthers, pubescent; anthers 10-15, linear; female flowers: slightly larger than male peduncle generally simple with 3 umbelled pedicels at the apex, rarely once branched and bearing 5-6 flowers; bracteole forming a narrow linear cup round the base of the perianth;

ovary superior, sessile, globose, hairy, stigma 2 clefted, glabrous. Fruit a capsule 5-7.5 cm x 1.8-3.5 cm, oblong, pubescent, seed one, oblong, obtuse, slightly flattened on one side, aril yellow, irregularly lobed, laciniate, extending to the apex of the seed (http://keralaplants.in/ keralaplantsdetails. aspx?id= Myristica_ malabarica).

2.3.2.3. *Myristica andamanica* Hook.f.

M. andamanica is a tree endemic to India, found in the Andaman and Nicobar Islands, used in the folklore medicines (Arunachalam and Subhashini, 2011).

Description: Tree, 15 m high with slender branches, quite glabrous with younger shoots. The bark is blackish green that becomes dark red with blood red juice when cut. The leaves are 3-4 inches in diameter and have a pale brown appearance on both sides on drying. The leaves are pale silvery or coppery beneath with an acute base with spreading nerves. The leaves are glabrous, membraneous, elliptic-oblong, sub-acute and have a strong petiole, 1-1.5 inches long. There are 12-15 pairs of slender nerves. Small, urceolate, globular and white flowers axillary, fasciculate and positioned at the leaf axils. The male flowers are sessile or in peduncled clusters on short pedicelled branches. It has a large bracteole with a three-lobed perianth which is glabrous and smooth. Obtuse and oblong stamina column. Fruit with the shape and size of a hen's egg with a thick and brown pericarp and blood red seed (Parkinson, 1923; Hooker 1973).

2.3.2.4. *Myristica fatua* Houtt.

Myristica fatua Houtt. var. *magnifica* (Bedd.) Sinclair, Gard. Bull. Singapore 23: 282. 1968; M. Mohanan & Henry, Fl. Thiruvanthapuram 387. 1994; Rama Bhat & Kaveriappa, Journ.Econ.Tax. Bot. 20: 213. 1996; Subram., Fl. Thenmala Div. 310. 1995; Sasidh., Fl. Shenduruny WLS 261. 1997 (http://keralaplants.in/ keralaplantsdetails. aspx?id= Myristica_ fatua_var._magnifica).

M. fatua is an evergreen, dioecious tree endemic to the Western Ghats – Aghastyamalai (west) and Central Malanad India. It is also distributed in Kalimantan, Sulawesi, Moluccas in Indonesia, Philippines, New Guinea, Solomon Island and South Pacific Islands. The nut and mace are used in herbal medicines and as substituents of true nutmeg spice (Lim, 2012).

Description: Lofty deciduous trees; 30 m high, bole often buttressed, with large aerial roots and pneumatophores; bark surface purplish-black, smooth; blaze pale chocolate; young parts

clothed with golden brown tomentum. Leaves simple, alternate, bifarious, estipulate; petiole 16-30 mm long, stout, grooved above, clothed with golden tomentum when young, glabrous on maturity, lamina 30-60 cm x 10-15 cm, oblong or elliptic-oblong, base round, apex acute or acuminate, glabrous above, reddish tomentose beneath, coriaceous; lateral nerves 20-26 pairs, prominent, parallel, intercostae obscure. Flowers unisexual, urceolate, rusty tomentose; male flowers 5-6 mm long, 10-20 fascicled on woody tubercles in the axils of leaves; female 6-8 mm long, 2 or 3 in axillary fascicles; perianth ovoid-globose, silky tomentose outside, glabrous inside; lobes 3-4 acute; staminal column much shorter than the perianth, with a short hairy stalk; stalk ovoid-cylindric, bluntly apiculate; anthers about 15, narrow, slightly unequal in length, covering almost the column. Fruit a capsule 8-10 cm x 6-7.5 cm, oblong-ovoid, covered with tomentum; seed one cylindrical, brown; aril orange red deeply cleft into a few broad divisions and covering the apex of the seed (http:// keralaplants.in/ keralaplantsdetails.aspx?id=Myristica_fatua_var._magnifica).

2.3.2.5. *Myristica amygdalina* Wall.

Myristica amygdalina Wall.mFl.Br. Ind., V, 106; King Ann. R. Bot. Gdns Calcutta, 1891, 3, 300 Pl, 128 (Hooker, 1973).

M. amygdalina is a tall glabrous tree native to South East Asia distributed in the dense forests on mountain slopes, ravines and sparse forests of Bangladesh, India, China, Laos, Myanmaar, Thailand and Vietnam (Shen, 2008). In India they are found in Sibsagar, Cachar, North Cachar Hills, Lushai Hills, Goalpara, Garo Hills and foot of Khasi Hills (Sastri, 1962a).

Description: Trees about 65 feet high. The leaves are 38 mm to 76 mm in diameter, coriaceous and pale brown on both surfaces with a 13 mm long petiole. Leaves are 6-8 inches long, elliptic lanceolate and acute at both ends. 8 to 12 pairs of nerves are present on the leaves. The male panicles come from the axils of the fallen leaves and are 3-5 inches long. They branch from the base and are quite glabrous. Slender loosely clustered flowers with pedicels as long as perianth is present. The staminal column is fleshy, concave and globosely trigonous. There are about 8 anthers that are combined. Fruits are shortly peduncled, 1.5 inches long with thin and glabrous pericarp and yellow and lacerate aril (Hooker, 1973).

2.3.2.6. *Myristica beddomei* King

Myristica beddomei King, Ann. Roy. Bot. Gard. (Calcutta) 3: 291, t. 118. f. 1-8, 1891, ssp. beddomei: de Wilde, Blumea 42: 151. 1997; Gamble, Fl. Pres. Madras 1214 (850).1915; Ratheesh Narayanan, Fl. Stud.Wayanad Dist. 692. 2009.(http://keralaplants.in/ kerala plantsdetails. aspx?id=Myristica_beddomei_ssp._beddomei).

M. beddomei is an evergreen tree found in Western Ghats from Konkon southwards and in Anamalai and Nilgiri hills upto an altitude of 1500 m.They are usually planted for providing shade in cardamom plantations. Also its wood is found useful for making tea boxes, match boxes and splints (Sastri, 1962b).

Description: Evergreen dioecious trees, 25 m high, bark 10-14 mm thick, surface blackish-green, smooth, exfoliations small, outer bark 1-2 mm thick, dead, inner bark 10-12 mm thick, fibrous, striate, deep red; exudation watery, red; branchlets glabrous except for terminal bud and inflorescence; leaves simple, alternate, distichous, estipulate; petiole 10-25 mm long, grooved above, glabrous; lamina 12-25 x 4-10 cm, oblong or elliptic-ovate, base acute, round or rarely cuneate, apex acute, margin entire, glabrous, shining above and glaucous beneath, coriaceous; lateral nerves 10-25 pairs, pinnate, prominent, intercostae reticulate, faint. Flowers unisexual, white; male flowers 10-20 together in short axillary dense clusters; peduncle mostly 2-cleft and woody, prominently marked with cicatrices of the bracts; pedicels slender, ferruginous tomentose; perianth thin, fleshy, rusty tomentose, connate into an urceolate tube, constricted above, suddenly expanded, breaking into 3 ovate, spreading acute lobes; staminal column narrow to oblong, ferruginous, produced beyond the anther; anthers 7-15, linear-oblong; female flowers as in male, only few generally 3-4 in the heads; ovary superior, sessile, ovoid-globose, appressed pubescent, 1-celled, ovule 1; stigma oblique, 2-lobed. Fruit a capsule, 5-7.5 cm x 3.7-6 cm, ovoid, apiculate, grooved on one side along the suture, pericarp rufous pubescent when young, thick, succulent; seed one, ovoid; aril orange red, encircling the seed, deeply cut down into many lobes, each of which is more or less lanceolate at the apex into filiform segments (http://keralaplants.in/keralaplants details. aspx?id=Myristica_beddomei_ssp._beddomei).

2.4. Trade of cinnamon and nutmeg

2.4.1. Cinnamon

Cinnamomum is an economically important genus as majority of the species of this genus are recognized as spices, essential oil yielding plants with aromatic potential and applications in pharmaceutical industry as the leaf and bark of these species are reported to have astringent, stimulant, carminative, blood purifier, digestive, anti- fungal, anti-viral, antiseptic properties etc (Choudhury et al., 2013). Among the species, *Cinnamomum verum* is the most important with high economic significance.

Cinnamon, the inner bark of the tree of *Cinnamomum verum* (syn. *C. zeylanicum*), indigenous to Sri Lanka referred to as the "true cinnamon" or "Ceylon cinnamon" is an important spice next to black pepper (Ranasinghe et al., 2013; Dinesh et al., 2015). Bark is the most important commercial product of cinnamon tree traded in the form of quills, featherings, chips and powder (Abeysinghe et al., 2009). Other commercial products of cinnamon are cinnamon bark oil, cinnamon leaf oil and cinnamon extracts (http://www. srilankabusiness.com/ blog/ ceylon- cinnamon- reigns- supreme- in- the-global-cinnamon - market.html).

The use of cinnamon has been reported since antiquity in Bible, ancient Roman and Egyptian histories and medieval Europe as a flavorant, preservant, in medicine and as a perfuming agent for embalming (Singletary, 2008). It is also said that the Dutch, Portughese and British explorer's goals in finding an easy route to the Far East in 15th century was to monopolise the spice trade including cinnamon (Susheela, 2006). Presently cinnamon is an important condiment in food industry due to its aromatic and sweet taste. It is employed in the preparation of chocolates, biscuits, desserts, teas, cocoa, soups, pickles, beverages etc (Maheshwari et al., 2013). It is also employed in chewing gums as a flavorant due to its mouth refreshing and bad breath removing ability (Jakhetia et al., 2010). Cinnamon bark oil finds application in preparation of perfumes, shampoos, cosmetics and soaps (Singletary, 2008). Apart from its use as a spice, cinnamon is gaining popularity as a medicine as it is credited with various therapeutic principles like antioxidant, anti-inflammatory, antimicrobial, antidiabetic, anticancer, lipid lowering and cardiovascular disease lowering activities (Vangalapati et al., 2012). Cinnamon also possesses activities to combat neurological disorders like Parkinson's and Alzheimer's disease (Rao and Gan, 2014). The carminative, antispasmodic, aromatic, stimulative, stomachic, haemostatic and astringent

effects of cinnamon bark has also been described in the Ayurvedic texts (Krishnamoorthy and Rema, 2004).

The major producers of Ceylon cinnamon are Sri Lanka, Madagascar and Seychelles of which Sri Lanka has monopolized 90% of the share of the international trade of true cinnamon. But the trade of true cinnamon is facing stiff competition from Cassia cinnamon, the term used collectively for cinnamon from *C. cassia* (China), *C. burmannii* (Nees) Blume (Indonesia) and *C. loureirii* Nees (Vietnam). A 5% loss has been witnessed in the Ceylon cinnamon market in the last 5 years while an increasing trend has been observed in case of cassia products exported from China, Indonesia and Vietnam (Piyasiri and Wijeratne, 2016).

As per the trade statistics of ITC (International Trade Centre) the global export of cinnamon and cinnamon flowers was 1,54,997 tons worth 482.58 million US $ in the year 2016 with Unites States of America, Asia and European Union purchasing majority of the cinnamon. The global import of cinnamon and cinnamon flowers in 2016 was estimated to be 1,53,213 tonnes worth 470.21 million US $ which were mainly exported from Indonesia (48,900 tonnes worth 94.15 million US $), China (42,417 tonnes worth 91.51 million US $), Vietnam (29,981 tonnes worth 71.75 million US $) and Sri Lanka (16,133 tonnes worth 158.69 million US $). The trade statistics of 2016 also revealed that India imported 27,393 tonnes of cinnamon worth 52.94 million US $ mainly from Vietnam (19,190 tonnes worth US $ 41.04 million) and China (4577 tonnes worth US $ 4.71 million). India imported only 474 tonnes of Ceylon cinnamon worth 2.50 million US $ in 2016 (http://www.intracen.org/ itc/ market-info-tools/statistics-export-product-country/).

2.4.2. Nutmeg

Myristica fragrans known as nutmeg is popular for the twin spice it produces – nutmeg (kernel) and mace (aril covering the seed) which are the most commonly known and widely traded; other products are their essential oils, extracted oleoresins and nutmeg butter. Nutmeg with its pleasant fragrance and warm taste has been used as a flavorant in baked foods, puddings, confections, meats, sausages, beverages etc and as components of curry powder, teas and soft drinks (Asgarpanah and Kazemivash, 2012). Nutmeg is reported to be a nutritional source as it is rich in energy, carbohydrates, proteins, dietary fibres, vitamins, electrolytes, minerals and phytonutrients (Agbogidi and Azagbaekwe, 2013). It has the potential of a therapeutic agent due to its stimulant, narcotic, carminative, astringent, aphrodisiac, antithrombic, antiplatelet aggregatory, antifungal, antidysentric and anti-

inflammatory activities. It is also used to treat stomach ache, rheumatism and vomiting during pregnancy (Muchtaridi et al., 2010). Though mace and nutmeg have a similar flavor, the taste of mace is more refined and it is costlier than nutmeg. Mace is available as whole, broken or powdered in the market.(http://webcache. googleusercontent.com/ search? q =cache: http://www. Doc -developpement-durable.org/ file/ Cultureepices/ Nutmeg% 2520and%2520Mace.pdf&gws_rd=cr&ei=acOyV9m7EM6gjwO9wKmACA).

The major nutmeg producing countries in the world are Gautemala, Indonesia, India, Grenada and Sri Lanka. Twenty eight thousand one hundred and twenty three tonnes of nutmeg and mace worth 207.32 million US $ were exported in the world during 2016 with Gautemala, India and Indonesia as the major exporters. The global import of nutmeg and mace during 2016 was 28,263 tonnes worth 215.37 million US $ (http://www. trademap.org/ tradestat/ Product_ SelProduct_ TS.aspx). According to statistics of international trade centre, 5228 tonnes of nutmeg and mace worth 36.71 million US $ were exported from India during 2016 (http://www.trademap.org/ tradestat/ Product_ SelCountry_TS.aspx? nvpm= 1|699||||0908|||4|1|1|2|2|1|1|1|1).

2.5. Adulterations in spices – cinnamon and nutmeg

Spices are export oriented agricultural commodities having importance in food, pharmaceutical and cosmetic industries (Dhanya and Sasikumar, 2010). Traded forms of spices include dried or fresh whole commodity, powdered forms, pastes, dehydrated material, oils, oleoresin and extractives. Good quality spices are very relevant for the perceived biological efficiency of these commodities, their flavour and aroma. The health conscious public all over the world is increasingly looking for quality spices, be it for health, culinary or cosmetic uses. However, spices and herbs are often adulterated with inferior, similar looking entities leading to erosion of the perceived biological value and public faith in these products (Sasikumar et al., 2016).

Adulteration refers to the admixture or replacement of genuine material with inferior parts of the same or different plant, harmful substances or synthetic chemicals which are not authorized by the official standards (Prakash et al., 2013). Adulteration can be in two ways- direct/intentional adulteration and indirect/unintentional adulteration. Direct/intentional adulteration includes practices of substitution partially or fully with inferior materials owing to their morphological resemblance or chemicals or inert materials in order to attain financial

gain (Preethi et al., 2014). Unintentional adulteration results mainly due to clerical errors (Zhao et al., 2006), absence of a proper evaluation method (Preethi et al., 2014) etc.

Spices and herbs are reported to be adulterated with plant-based and known synthetic adulterants (Singhal et al., 1997; Dhanya and Sasikumar, 2010). Microbial contamination, insect filth contamination and addition of adulterants like dyes are the common quality-related issues of spices (Dallasta, 2013). Adulteration is also related to fraud concerning botanical or geographical origin. Whole spices are usually adulterated with related botanical species of the target species, unrelated species bearing physical resemblance with the target commodity in attributes such as shape, colour, aroma etc, immature dried material and exhausted spices while spice powders are prone to admixture with powders of the above substituents, grain flours, starch etc (Singhal et al., 1997). Deceitful addition of these substituents which may contain undeclared constituents may cause health problems such as allergy in sensitive individuals (Marcus and Grollman, 2002; Asensio et al., 2008; Sasikumar et al., 2016). Value added products such as powder and paste are more vulnerable to adulteration than the entire spice commodity (Sasikumar et al., 2016).

Cinnamon and nutmeg are reported to be substituted by their inferior counterparts. Though morphological identification is possible to an extent in the fresh form for these two spices, it becomes problematic in case of dried or processed forms such as powder, due to the loss of morphological diagnostic features of the spices, increasing the possibility of fraudulent substitutions or adulteration (Swetha et al., 2014, 2016).

C. verum barks are reported to be frequently adulterated with a rougher, thicker, cheaper and less aromatic bark of *C. cassia* (syn. *C. aromaticum* Nees) characterized by a sweet and pungent flavor. *C. cassia* is reported to contain coumarin (a class of compounds that contain 1, 2-benzopyrone structures) that is present in vegetables, spices, fruits and medicinal plants. (Wang et al., 2013). *C. cassia* was found to contain upto 5% coumarin while *C. verum* contained coumarin in trace amounts (0.004%) (Lungarini et al., 2008). The US Food and Drug Administration had imposed a ban on the use of coumarin as a flavouring agent due to its hepatotoxic effect on animals and recommended to withdraw it from markets in United Kingdom (UK) in 1965 (Lake, 1999). The Council of the European Committee (1988) had proposed a limit of 2 mg/kg of coumarin in food and beverages (Council of European Committee, 1988). Some isolated incidents of hepatotoxicity to humans by coumarin intake were also reported by World Health Organization (WHO, 1995). But it was later found that

risk to humans occur mostly in coumarin induced hepatotoxicity in sensitive individuals. Also the carcinogenicity caused was revealed to be non-genotoxic and a tolerable level of daily intake of coumarin was determined as 0.1 mg/kg bodyweight by the European Food Safety Authority (EFSA) based on the no observed adversed effect level in animals (AFC, 2004). The high level of coumarin in foods in the European markets led to the evaluation of the maximum permissible level of coumarin by the European Parliament and formulation of the European regulation (EC) No 1334/2008. According to EC No 1334/2008 the maximum limit of coumarin in food stuffs was decided as follows : 50 mg/kg of coumarin in cinnamon flavoured bakery, 15 mg/kg in other baked items, 20 mg/kg in breakfast cereals and 5 mg/kg in cereals (Regulation (EC). No. 1334/2008). Studies conducted by the German Federal Institute for Risk assessment showed that 2100 – 4000 mg of coumarin is present in 1kg of cassia, which means 5.8 to 12.1 mg of coumarin is present in one teaspoon of cassia powder, which is a threat to food safety (Dinesh et al., 2015).

Traded cinnamon was found adulterated with *C. cassia* barks in markets throughout the world. Out of the cinnamon samples collected from Italian markets, 51% were found to be *C. cassia,* 10% a mix of *C. cassia* and *C. verum* while only 39% were authentic cinnamon (Lungarini et al., 2008). Blahová and Svobodová (2012) reported that the ground cinnamon available in Czech markets was predominantly *C. cassia* and *C. verum* was available only in some specialty shops. Adulteration of traded cinnamon bark samples with *C. cassia* was also reported by Swetha et al., (2014), out of ten bark samples procured from local markets in Kozhikode, seven were found to be substituted with *C. cassia.*

Recently, a ban of Cassia cinnamon by the Food Safety and Standards Authority in India has been reported by the vernacular press in Kerala.The report also states that inorder to curb the trade of *C. cassia* the food safety agencies has proposed that 100 g of traded cinnamon should contain only 0.3% coumarin (Mathrubhoomi, 8.12.2016).

Dried bark of *C. malabatrum,* common in many tropical countries and rarely seen in homestead gardens in India and Sri Lanka, is also passed off as true cinnamon though there is no toxic effects associated with it (Swetha et al., 2014).

The economical value coupled with its high cost has led to the fraudulent adulteration of *M. fragrans* with its closely related species *Myristica argentea* Warb., (Papuan nutmeg) from New Guinea, *Myristica otaba* H, et B. (Otaba nutmeg) and *M. malabarica* (Bombay nutmeg) from south India by unscrupulous dealers (Bose et al., 2008b). Mace of *M. malabarica,* a

common wild relative of the nutmeg, also known as Bombay mace, is yellow in colour, lack aroma and is of inferior quality mainly exploited as a natural dye source while that of *M. argentea* and *M. otaba* has a pungent aroma. Seeds of *M.argentea* and *M. malabarica* are acorn shaped while true nutmeg seeds are egg shaped (http://gernot-katzers-spice-pages.com/engl/Myri_fra.html).

Recent studies have reported the adulteration of traded nutmeg mace with that of *M. malabarica*. Three out of the five mace samples procured from Kozhikode markets were found to be substituted with *M. malabarica* (Swetha et al., 2017).

2.6. Methods to detect plant based adulterations

Detection of adulteration and determination of authenticity and quality of foods and food ingredients, including spices, are major challenges for the agricultural and food industry, challenges that have become increasingly important in recent years (Mafra et al., 2008). Regulatory agencies, food processors and consumers are all interested in detecting adulterants or authenticating raw materials of food products in order to satisfy food quality and safety requirements (Man et al., 2005). With globalisation of trade, the role of standards and conformity assessment are of paramount importance especially in herbs and spices and their value added products because non-tariff agreements such as Sanitary and Phytosanitary (SPS) and Pre shipment Inspection (PSI) agreements insist that the product(s) is safe, free from adulterants and has the desired quality. Though acceptable levels of many of the synthetic adulterants are available, there is a paucity of faster and efficient methods to detect the plant-based adulterants in herbs and spices, especially in value-added items (Sasikumar et al., 2016).

International organisations like the International Organisation for Standardisation (ISO), American Spice Trade Association (ASTA), The Food Safety and Standards Authority, India (FSSAI) etc. impose strict regulations on the quality of spices traded. Globalisation of food trade requires the development of integrated approaches, such as traceability of origin, quality and authenticity to ensure food safety and quality (Barbuto et al., 2010). In the post-WTO era, importing countries as well as the consumers pay more and more attention to food quality, demanding clearer product traceability as well as the use of detailed and accurate product labels.

Numerous techniques have been developed to counter adulteration owing to the increased consumer awareness of food safety and quality control. Adulteration determination is mainly accomplished by comparing measured analytical data with a proper reference set of historical or control data (Wilhelmsen, 2004). Three strategies are employed for demonstrating admixture in agricultural commodities: demonstrating the presence of a foreign substance in the commodity, demonstrating that a component is present at a concentration which deviates significantly from its normal level and checking the chemical profile of the sample, of which the first strategy is considered as the simplest and efficient one (Dhanya and Sasikumar, 2010). Authentication tools utilised vary widely depending on the plant and processes involved ranging from structural evaluation using physical methods, chemical profiling-based analytical methods and the most advanced biotechnological approaches (Smillie and Khan, 2010).

Physical methods involved in the authentication of spices are macroscopic and microscopic structural evaluation and other parameters such as solubility, bulk density, texture etc. (Dhanya and Sasikumar, 2010). Analytical methods used for food authenticity testing involve chromatographic techniques like High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Gas Chromatography (GC), spectroscopic methods like UV spectroscopy, Raman Spectroscopy and its variants, Nuclear Magnetic Resonance spectroscopy (NMR), Mass spectroscopy, capillary electrophoresis and hyphenated techniques that differentiate the samples based on the variation in their chemical profile (Sproll et al., 2008; Mohammad et al., 2015; Cercaci et al., 2003; Zalacain et al., 2015; Reid et al., 2006; Petrakis et al., 2015; Silvis et al., 2015; Cianchino et al., 2007; Reinholds et al., 2015; Sasikumar et al., 2016). Though physical and chemical methods are amenable for food authentication, in certain instances they fail to give exact results (Bansal et al., 2017). The usage of physical methods is often limited due to their time consuming procedure and need for a skilled expertise. The requirement of an expensive standard and the non availability of standards for certain botanicals restrict the use of analytical methods in food authentication (Shaw et al., 2002). Molecular methods can compensate these limitations and are dominant over the physical and chemical approaches due to its accuracy, effectiveness and non dependence on age, environmental factors, storage and processing conditions, especially for the biological adulterants (Heubl, 2013; Balachandran et al., 2015).

Molecular methods involve the amplication of one or more regions of genomic DNA using polymerase chain reaction and have a great potential in food authentication due to its

sensitivity, rapidity, specificity and simplicity (Mafra et al., 2008). The different PCR based methods used for food authentication and traceability are Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990; Mane et al., 2006), Arbitrarily Primed PCR (AP-PCR) (Welsh and McClelland, 1990), DNA Amplification Fingerprinting (DAF) (Anolles et al., 1991), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz et al., 1994), Directed Amplification of Minisatellite-region DNA (DAMD) (Heath et al., 1993), Sequence Characterised Amplified Region (SCAR) (Paran and Michelmore, 1993; Dhanya et al., 2011), Amplification Refractory Mutation System (ARMS) (Newton et al., 1989), Simple Sequence Repeat (SSR) analysis (Litt and Lutty, 1989; Melchiade et al., 2007), species specific PCR (Sasaki et al., 2004), Single Nucleotide Polymorphism (SNP) (Johnston et al., 2013) and real time PCR (Lockley and Bardsley, 2000). Apart from these techniques, DNA barcoding, a recently evolved molecular marker is gaining acceptance as a tool for food authentication and traceability over the other DNA based methods due to its universality and reliability (Galimberti et al., 2013; Heubl, 2013).

2.6.1. DNA Barcoding

The concept of DNA Barcoding, proposed and developed by Dr. Paul Hebert, a Canadian Biologist, in animals for species identification, is based on the sequence variation in short nucleotide stretches called “barcodes” between species (Hebert et al., 2003a). These barcode regions could act as a species recognition tag by comparing it with the sequences present in a reference database containing sequences of the standardized barcode region of almost all the organisms. If an organism fails to match with any sequence in the database, it could be considered as a possible new species (Singh et al., 2012). A short 648 bp region at the 5’ end of mitochondrial *cox1* (*CO1*) gene known as the Folmer region, coding for the cytochrome c oxidase subunit serve as the standard barcode in animal kingdom (Hebert et al., 2003b).

Increasing work in the arena of DNA barcoding paved way for the formation of two International collaborations; The Consortium for the Barcode of Life (CBOL) and International Barcode of Life (iBOL), for the progression of DNA Barcoding. The Consortium for the Barcode of Life is an organization consisting of more than 200 members representing 50 countries estabilished in May 2004 with the support from Alfred P Sloan foundation, USA to promote DNA barcoding as a global tool for species identification by compiling sequences in a reference DNA library. International Barcode of Life, comprising members from 25 nations was set up in 2014 at Guelph, Ontario with an objective to barcode

5 million specimens and 5,00,000 estimated species present on earth by 2015 (Sundari et al., 2015). The huge data generated by these two organizations initiated the development of Barcode of Life Database (BOLD), a workbench for the acquisition, storage, analysis and publication of DNA barcode data maintained by the University of Guelph, Ontario, Canada (Ratnasingham and Hebert, 2007).

DNA barcoding have been successfully used for differentiating species of birds (Hebert et al., 2004a), butterflies (Hebert et al., 2004b), fish (Ward et al., 2005), spiders (Greenstone et al., 2005), amphibians (Smith et al., 2008), insects (Wilson, 2012), etc. based on the sequence variation in *CO1* region. The use of the mitochondrial region in barcoding was dominant over the nuclear genome as it did not exhibit gene duplications common in nuclear genome (Ford et al., 2009).

Attempts at plant barcoding using *CO1* gene was a failure due to the low rate of plant species discrimination, owing to lack of nucleotide substitutions and high rate of chromosomal rearrangements that occurred due to the intramolecular recombination in mitochondrial plant genome (Kress et al., 2005; Mower et al., 2007; Hollingsworth et al., 2011; Bhagwat et al., 2015). Genome - wide horizontal gene transfer, hybridization and homoplasy also restricted the development of barcoding regions for plants (Chase et al., 2005). Inspite of these hindrances, regions of chloroplast and nuclear genome were proposed as possible candidates for barcoding in plants. Among the nuclear genomic regions, only the internal transcribed spacer (ITS) of nuclear ribosomal DNA could be used as a barcode owing to lack of universal primers for amplifying the other single copy genes or introns (Hollingsworth et al., 2011) and the technical issues caused by gene duplication in the nuclear genome (Cowan et al., 2006). So focus was shifted to chloroplast genome for barcoding in plants. Regions of chloroplast genome, analogous to mitochondrial genome, sharing its characteristics like conserved gene arrangement, high copy number and availability of universal primers, were proposed as potential candidates as plant barcodes (Vijayan and Tsou, 2010). The structural stability, uniparental inheritance and haploid nature of chloroplast genome also facilitated its use as a barcode in plants (Abeysinghe et al., 2009).

2.6.1.1. Barcoding loci for plants

An ideal barcode should be easily amplifiable, amenable to sequencing, exhibit higher interspecies variation than intraspecies variation, easily annotated for evaluation of sequence quality and error detection and recoverable from degraded samples (Selvaraj et al., 2013).

Internal transcribed spacer of the nuclear ribosomal cistron, some of the coding and non coding regions of the chloroplast genome that meet the above requirements were proposed as candidate barcodes in plants.

2.6.1.1.1. Internal Transcribed Spacer (ITS)

The major ribosomal genes of plants are arranged in clusters of conserved repeat sequences coding for the 16S, 5.8S and 28S ribosomal subunits. These repeat sequences are separated by Internal transcribed spacers ITS1 (between 16S and 5.8S ribosomal subunits) and ITS2 (between 5.8S and 28S ribosomal subunits) (Ritland et al., 1993). ITS is present as a part of its transcriptional machinery in almost all organisms excluding vertebrates (Calonje et al., 2009). Though they are not incorporated into mature ribosome, ITS1 and ITS2 spacers assume a role in the maturation of nuclear RNAs (Baldwin et al., 1995) and the length of ITS is approximately 565-750 bp in angiosperms (Poczai and Hyovenen, 2010) and 1500-3500 bp in seed plants (Gernandt and Liston, 1999).

The use of ITS locus as a barcode candidate was suggested due to its characteristics like sequence divergence, high copy number, universality and size (Vijayan and Tsou, 2010).

Sequence divergence: The concerted evolution of the spacers resulted in sequence homogeneity within a species, and sequence divergence between closely related species thereby facilitating species discrimination (Vijayan and Tsou, 2010). The sequence variation in ITS is attributed by the polymorphisms or the insertion-deletion events that occur in this region (Baldwin et al., 1995; Alvarez and Wendel, 2003; Poczai and Hyovenen, 2010).

High Copy Number: ITS, present in high copy number in the form of tandem repeats in the nuclear genome makes it widely applicable in molecular systematics (Calonje et al., 2009). Presence of this spacer in multiple copies makes it amenable to easy isolation, amplification, cloning and sequencing (Baldwin et al., 1995; Alvarez and Wendel, 2003).

Universality and size: Presence of conserved regions flanking both the nuclear spacers and availability of universal primers for their amplification is an added advantage for its use as a barcode. The smaller size of ITS in angiosperms renders it favourable for amplification even from herbarium plant material (Baldwin et al., 1995).

Though many workers support its use as barcode, it faced certain opposition mainly due to the following concern. First is the occurrence of the contamination of fungal sequences.

Universal primers available for amplification of ITS in plants may also amplify any fungal DNA as their sequences are similar which may lead to incorrect identifications or confusions (Hollingsworth, 2011). Second is the presence of divergent paralogous copies of the spacer in individuals due to incomplete concerted evolution that generates mixed sequence data, as different variants of the spacer were being sequenced simultaneously. It resulted in the retrieval of different sequences for different individuals within a species depending on the variant that was sequenced (Hollingsworth et al 2011; Ritland et al., 1993). Existance of ITS as pseudogenes also posed a challenge in its application in barcoding (Alvarez and Wendel, 2003). Sequence recovery is another concern for ITS. Watson-Crick base pairing within strands may affect the *Taq* polymerase activity or restrict the mobility of the DNA amplicons in the sequencing gel. Secondary structure formation due to the GC region in the spacer may also present problems during sequencing (Baldwin et al., 1995).

2.6.1.1.2. Chloroplast/Plastid Genome

Chloroplast is an intracellular organelle having an endosymbiotic origin with a genome size of 120-160 kb in angiosperms with genes coding for proteins of the photosynthetic machinery (Sugiura, 1992). It is a circular molecule containing inverted repeat regions (20-28 kb) separated by large and small single copy region (Whitfield and Bottomley, 1983). The genome contains four rRNA genes, 35 tRNA genes and about 100 protein coding genes (Sugiura, 1992).

Based on the information derived from phylogenetic studies and the studies conducted on plant taxa, the chloroplast coding regions like *rbcL*, *matK*, *rpoC1*, *rpoB*, *accD*, *ycf5*, *ndhJ* and non coding intergenic spacers like *psbA-trnH*, *psbK-psbI*, *atpF-atpH* were recommended as potential barcode candidates in plants (Vijayan and Tsou, 2010) of which *rbcL*, *matK* and *psbA-trnH* assumes significance.

2.6.1.1.2.1. *rbcL*

rbcL is a single copy chloroplast gene devoid of introns coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (RUBISCO) that plays an important role in the carbondioxide fixation in Calvin cycle (Enyedi and Pell, 1992). *rbcL* is the first gene sequenced in plants (Vijayan and Tsou, 2010) and one of the most characterized plastid coding region (Newmaster et al., 2007). It is about 1430 bp in length having a fairly

conservative rate of evolution (https://theses.lib.vt.edu/theses/available/etd-11597-103132/unrestricted/ONE.PDF).

High amplification and sequencing success in angiosperms, gymnosperms and cryptogam species are some of the useful attributes that contribute towards the barcode potential of this locus (Li et al., 2011). Numerous reports also stress the use of *rbcL* as a phylogenetic marker (Chase et al., 1993) as it can assign the plants to the correct genus (Zhang et al., 2015). But problem arises as the locus pose difficulties in resolving closely related species due to its low variability (Gielly and Taberlet, 1994). But it is reported to be useful when used in conjunction with other markers (CBOL Plant Working Group, 2009).

2.6.1.1.2.2. *matK*

matK, a rapidly evolving chloroplast region (CBOL Plant Working Group, 2009) was first identified in tobacco by Sugita et al., (1985). It is about 1500 bp long, embedded in the trnK intron and codes for the maturase enzyme that is involved in the group II intron splicing.

matK has been found applicable in plant barcoding and systematic studies owing to its ideal size, high rate of nucleotide substitutions, low transition/transversion ratio and the presence of conserved flanking regions in *trnK* gene (Selvaraj et al., 2008). Its ubiquitous presence and high rate of evolution that resolves intergenic and interspecies relationship in plants have aided in its use as a marker for phylogenetic studies in plants (Johnson and Soltis, 1995; Hilu and Liang, 1997; Vijayan and Tsou, 2010). The locus is popular for its high discriminatory power in angiosperms (Fazekas et al., 2009) that also aided in its use as a barcode. However, low rate of amplification success due to lack of universality (Hollingsworth et al., 2009) and poor species discrimination limits its application in other plant groups (Tang et al., 2016; Christina and Annamalai, 2014).

2.6.1.1.2.3. *psbA-trnH*

Intergenic spacers are informative regions that assume significance in population genetics and plant systematic studies due to the unambiguous haplotype generation and high rate of nucleotide substitutions (Storchova and Oslon, 2007). It is positioned between the *psbA* gene coding for the protein D1 that is a constituent of the photosystem II of photosynthetic machinery and the gene of histidine transfer RNA (*trnH*) (Degtjareva et al., 2012). It is about 340-660 bp long in angiosperms (Li et al., 2011).The *psbA-trnH* intergenic region is constituted by two regions –*psbA* 3’ UTR that plays a role in regulation of gene expression

and *psbA-trnH* spacer that has no function owing to its high sequence variability across angiosperms (Selvaraj et al., 2013).

psbA-trnH is a chloroplast intergenic spacer that is a prospective barcode candidate (Kress et al., 2005). It exhibits amplification success in wide range of plants like angiosperms, gymnosperms, ferns, mosses etc. (Vijayan and Tsou, 2010). Being a highly variable intergenic spacer due to the presence of nucleotide substitutions, it can be employed for successful species discrimination (Li et al., 2011). But the locus exhibits variation in the form of insertions-deletions that result in differences in the sequence length thereby creating problems in sequence alignment even between closely related species (Chase et al., 2007; Ford et al., 2009). The presence of pseudogenes or *rps19* gene within the spacer also gave rise to issues concerning sequence length (Chang et al., 2006). The locus also exists in duplicated copies in certain plant groups that generated complications while working with the locus (Sass et al., 2007; Hollingsworth et al., 2011). Another concern of this locus is its inability to generate bidirectional sequences in some cases due to termination of sequence reads owing to the presence of poly A-T structure (Devey et al., 2009; Zhu et al., 2010). Eventhough there are limitations, the locus is still being used widely in DNA barcoding (Costion et al., 2011).

2.6.1.1.2.4. Multilocus barcode approach

Th.e barcoding regions proposed in plants includes the slowly evolved regions like *rbcL* and the rapidly evolved regions like *matK* or *psbA-trnH.* No single locus could discriminate plant species on its own as the slowly evolved regions lacked nucleotide substitutions while the rapidly evolved regions were problematic due to absence of priming sites, homoplasy etc. (Pettengill and Neel, 2010). To resolve the above issues the multilocus approach was introduced. The term multilocus barcode was coined by Chase and it involves the use of a multigene approach (Chase et al., 2005) where a phylogenetically conserved easily aligned locus (*rbcL*) is combined with more rapidly evolving variable regions like *matK* or *psbA-trnH* (Kress et al., 2009). *rbcL* was used as a first tier due to its universality and success in differentiating congeneric plant species and one among the variable markers viz, *matK, psbA-trnH* and ITS was used as the second tier (Nithaniyal et al., 2014). *rbcL* assisted in assigning the individuals into genera or groups within genera while *matK* and *psbA-trnH* will differentiate species within these higher group. Complementing of these loci will ensure

species discrimination and assignment of plants to the correct genus (Kress and Erickson, 2007).

The Plant Working Group constituted by the scientists of the Royal Botanic Garden of The Consortium of Barcode for Life (CBOL), an international initiative for development of DNA barcodes, analysed the possible combinations of candidate genes and recommended *rbcL*+ *matK* as core barcodes along with *psbA-trnH* and ITS as supplementary barcodes in plants. But Kress et al., (2005) suggested the combination of *rbcL* + *psbA-trnH* better than *rbcL* + *matK* for barcoding.

2.6.1.1.2.5. Application of DNA barcoding

DNA barcoding finds application as a taxonomic tool to aid identification of species that cannot be identified based on morphological characters (Newmaster and Raghupathy, 2009), taxonomic revision of species (Lara et al., 2009), identification of invasive species in ecosystem (Armstrong and Ball, 2005), studying spatial pattern of underground plant diversity (Kesanakurthi et al., 2011), plant-animal interaction studies (Jurado-Rivera et al., 2009) etc. It is also important in wild life forensic studies where it is employed to differentiate non threatened species from endangered species listed by the Convention on International Trade in Endangered Species (CITES) (Aubriot et al., 2013). Floristic studies also utilized the potential of DNA barcoding for conducting floristic analyses without assistance from skilled personnel and also helped in estimating the species richness in an unknown flora (Costion et al., 2011; Hartvig et al., 2015). It also helped in conservation and prevention of illegal trade of endangered species (Eaton et al., 2010) and logged timber (Lowe and Cross, 2011). Apart from the above uses it is widely employed in authentication of medicinal plants and traded food commodities and phylogenetic studies which is discussed in detail in sections 2.6.1.1.2.6 and 2.7.2

2.6.1.1.2.6. DNA barcoding as an authentication tool

DNA barcoding is an efficient marker technique with an important role in certifying food origin, quality of food, safeguarding public health and minimizing food piracy (Barcaccia et al., 2016; Galimberti et al., 2013). It is based on the analysis of the polymorphic sites in the sequences generated, for the barcoding loci of the organisms, constituting the raw materials employed and the food products derived from them (Hebert et al., 2003a; Ferri et al., 2015). These sequences can be compared with the standard sequences deposited in the easily

accessible reference databases like GenBank and BOLD to ensure food authenticity and protect consumers from food fraud (Ferri et al., 2015). Increased sensitivity, target DNA sequence diversity, amplification of minute amounts of DNA as in processed products makes DNA barcoding amenable as an authentication tool. DNA barcoding is widely used for the authentication of medicinal plants, agricultural commodities like spices, seafood, meat etc (Galimberti et al., 2013).

2.6.1.1.2.6.1. Authentication of medicinal plants

Song et al., (2009) assessed the potential of eight barcoding regions (ITS2*, rbcL, psbA-trnH, ndhJ, rpoC1*, *accD, rpoB* and *YCF5*) for authentication of medicinal plants belonging to family Polygonaceae. The ten species of plants important in Chinese Pharmacopoeia taken in the study could be recognized and differentiated from its adulterants using *psbA-trnH*. Distinguishing the genuine plant from its closely related species and adulterants was also possible through a multilocus approach involving *rbcL*, *rpoC1* and ITS2.

Roots of *Astragalus membranaceus* and *Astragalus membranaceus var. mongholica* refered to as *Radix astragali* significant in the Chinese Pharmacopenia is adulterated with other species of *Astragalus, Oxytropis, Hedysarum* and *Glacyrrhiza* in the traded markets and herbal industry. DNA barcoding was employed to authenticate *Radix astragali* samples (Guo et al., 2010). ITS and *matK* loci exhibited sequence variation to distinguish them from its adulterants. ITS depicted 59 variations at the species level while *matK* locus had 47 variations at interspecies level and 117 variations at genus level thereby facilitating its identification.

Srirama et al., (2010) demonstrated the detection of species admixture in traded samples of *Phyllanthus amarus,* a hepatoprotective plant using *psbA-trnH* based barcoding. A reference library was constructed using 16 species of *Phyllanthus*. *psbA-trnH* could clearly discriminate these species in to separate clusters in the Neighbour Joining (NJ) tree. The market samples clustered along with the respective reference species contained in them. Out of the 25 traded samples collected from different places in South India, 76% contained *Phyllanthus amarus* and the remaining 24% were found to be admixtures of species like *Phyllanthus debilis, Phyllanthus urinaria, Phyllanthus maderaspatensis* and *Phyllanthus kozhikodianus*.

Roots of *Scutellaria baicalensis* significant in the traditional Chinese medicine owing to their pharmacological activities are often adulterated with roots of related *Scutellaria* spp. viz. *S. amoena, S. rehderiana and S. viscidula.* DNA barcoding was proposed as a tool for the quality control of *Scutellaria baicalensis* (Guo et al., 2011*). matK, psbA-trnH* and *rbcL* loci were used in the study. A tiered approach of *psbA-trnH* and *rbcL* could discriminate *S. baicalensis* from its adulterants. *psbA-trnH* was the ideal loci to authenticate the commercial samples due to its shorter amplicon size, high number of variable sites and failure of the other loci to amplify the traded samples.

Sui et al., (2011) used DNA barcoding to distinguish the ethnomedicinal plant *Sabia parviflora* from its adulterants like *S. fasciculata, S. latifolia, S. dielsii, S. yunnanensis, S. swinhoei, Hedera nepalensis* var. *sinensis, Euonymus fortunei* var. *radcans, Euonymus fortunei, Paederia yunnanensis, P. scandens var. tomentosa, Senomenium acutum* and *Cocculus trilobus*. *matK, rbcL* and *psbA-trnH* sequence alignment revealed the presence of polymorphic sites differentiating *Sabia parviflora* from its adulterants.

Gentianopsis paludosa is an important herb of the Tibetan traditional medicine that is usually adulterated with nine species viz. *Gentianopsis barbata*, *G. contorta, G. grandis, Halenia elliptica, Lomatogonium macranthum, L. rotatum, Swertia angustifolia, S. bifolia* and *S. erythrosticta*. ITS locus with its higher interspecies divergence and high degree of polymorphism effectively discriminated *G. paludosa* from its adulterant species. Polymorphisms were exhibited in the 19-30 and 58-73 positions of the locus between *Gentianopsis* and its adulterant species respectively (Xue and Li, 2011).

Paris polyphylla and *P. polyphylla* Smith *var. chinensis* employed as medicinal Paris is often adulterated with the more abundant and cheaper, *Valeriana jatamansi.* The identification of *Valeriana jatamansi* in traded *Paris* samples was possible using *psbA-trnH* based barcoding. Length polymorphism was observed in the *psbA-trnH* amplicons of the genuine and adulterant samples. The size of the *psbA-trnH* amplicons was in the range of 1077-1084 bp and 263-267 bp in *P.polyphylla* and *V. jatamansi,* respectively. Thus discrimination was possible at the band level itself. The sensitivity of this method was 1: 1000 (*V. jatamansi* : *P. polyphylla*) (Yang et al., 2011).

Baker et al., (2012) proposed the use of DNA barcoding in identification of Black Cohosh dietary supplements, a target for adulteration. Sequence analysis revealed the presence of 2 single nucleotide polymorphic (SNP) sites in *matK* locus that distinguished Black Cohosh

from related *Actaea* species. Out of the 36 samples tested, 25 samples were found to be authentic Black Cohosh while 9 were substituted with *A. cimicifuga*, *A. dahurica*, and *A. simplex*.

The commercial medicinal plants of Southern Morocco were identified using DNA barcoding. *rpoC1, psbA-trnH, matK* and ITS loci were tested for 111 medicinal root samples. BLAST clust analysis and phylogram construction based on the multi locus approach involving *rpoC1, psbA-trnH,* and ITS identified most of the samples to their corresponding genus. It could also detect adulterations in eight traded samples belonging to six herbal products like substitution of *Colchicum autumnale* L. (bukbuka) with *Bunium* species (Kool et al., 2012).

Baiying (Herba Solani Lyrati), obtained from *Solanum lyratum* was differentiated from its toxic substituent Xungufeng (Herba Aristolochiae Mollissimae) obtained from *Aristolochia mollissima* using DNA barcoding. Five barcodes ITS*, matK, rbcL, psbA-trnH and trnL-trnF* were used to authenticate five commercial samples each of Baiying and Xungufeng commodities. Comparison of sequences of the five barcodes depicted that three samples of Baiying was substituted with *Aristolochia mollissima* (Li et al., 2012).

DNA barcoding of ITS2 region was used to authenticate the important herbs of family Asparagaceae and Ascelepiadaceae. Attempt to distinguish between *Asparagus racemosus* belonging to Asparagaceae from its close relative and substituent *Asparagus gonoclados* and *Hemidesmus indicus*, a member of family Ascelepiadaceae from its adulterant species *Decalepish hamiltonii* was done by Rai et al., (2012). Though ITS2 was able to distinguish these species from their adulterant entities; ITS2 locus could be applied only for authentication of *H. indicus*. The higher intraspecific divergence value obtained for the sequences of *A. racemosus* restricted the use of ITS2 locus in authentication.

Authentication of natural herbal products was accomplished using DNA barcoding. Among the ginseng products screened, a case of mislabeling was detected based on the four polymorphic sites between American and Korean ginseng in the ITS locus. Fifty percent of the samples labelled as Korean ginseng were found to be American ginseng. Two non-ginseng barcodes were also generated from the ginseng products. In the non-ginseng medicinal products tested two were found to be adulterated. *rbcL* barcode generated from a sample commercially labeled as Echinacea (*Echinacea purpurea*), was similar to the

sequence from walnut and also a sample traded as "Black Cohosh" contained *Acetea asiatica* (Wallace et al., 2012).

DNA barcoding was applied as a forensic tool to detect the presence of tobacco in moassel samples seized by government agencies at Canada Border Security Agency (CBSA) laboratory (Carrier et al., 2013). *rbcL* and *matK* loci identified the presence of tobacco in more than 60 moassel samples.

Identification of *Hoodia* (slimming cactus) listed in the CITES Appendix II was accomplished through DNA barcoding, inorder to prevent its illegal trade, as identification is not possible based on morphological and chemical characters. *psbA-trnH* and ITS sequences could successfully discriminate it from other species in Ceropegieae and help in its authentication (Gathier et al., 2013).

Gao et al., (2013) reported that *psbA-trnH* locus can be employed to authenticate the medicinal plant species of family Fabaceae. BLAST 1, nearest distance method and taxon gap method based on *psbA-trnH* sequences effectively discriminated Fabaceae species. Differentiation of *Sophora tonkinensis*, and *Radix astragali*, medicinal plants of Fabaceae from its adulterants was accomplished using *psbA-trnH* region.

Saw palmetto (*Serenoa repens*), a herbal dietary supplement, was authenticated using *rbcL* and *matK* based barcoding (Little and Jeanson, 2013). Sequence analysis of *matK* revealed the presence of two polymorphic sites at positions 802 and 818 that distinguished saw palmetto from *Brahea aculeata, B. dulcis* and plants of the sub tribe Livistoninae, and Rhapidinae. The two polymorphic sites at 292 and 398 in the *rbcL* alignment differentiated it from *Acoelorrhaphe wrightii.* Based on these diagnostic sites, *rbcL* and *matK* mini barcode primers were developed. Out of the 37 samples analysed, 92% samples gave amplifiable DNA with the *rbcL* and *matK* mini barcode primers. Eighty five percent supplements were found to contain *S. repens*, 6% had adulterant species, and 9% of the supplements could not be determined conclusively.

The efficiency of DNA barcodes in identification of the processed medicinal plant materials like roots, stem, etc. used in the traditional medicine of South Africa was shown by Mankga et al., (2013). *matK* and *rbcL* were used as ideal barcodes in their discrimination. Eighteen market samples were analysed in this study out of which only 16 gave successful

amplication. BLAST analysis proved that 11 samples were genuine while 5 were mislabeled by the sellers.

Murphy and Bola (2013) employed barcoding technique to authenticate *Salvia divinorum*, source of a recreational drug salvinorin A, famous among adolescents. *rbcL* locus differentiated it from other sources of drugs like *Nicotiana tabaccum* and *Cannabis sativa* while *trnL-F* distinguished it from other *Salvia* species like *S. greggi, S.chionophylla, S. microphylla, S. dolomitica, S. clevelandii* and *S. cedrosensis.*

Newmaster et al., (2013) used a multilocus approach of *rbcL* + ITS to authenticate 44 herbal products that represented 30 plant species and 12 brands. It was found that 59% of the samples generated barcodes for plant species that were not listed in the label. Thirty three out of the 44 products tested revealed substitutions and only two out of the 12 companies sold authentic products. Barcodes of filler species like rice, wheat etc. unlisted on labels were also detected in the traded products.

Herbal materials important in Chinese medicine were successfully authenticated using ITS2 based DNA barcoding (Pang et al., 2013). The study showed that ITS2 locus could identify 61 herbs representing 69 species, discriminate 48 herbs from their closely related species and distinguish 34 herbs from their adulterants using BLAST 1, NJ tree method and secondary structure determination.

Gentiana manshurica, G. scabra, G. triflora (traded as Guanlongdan) and *G. rigescens* (traded as Jianlongdan) are four important medicinal plants of Chinese Pharmacopeia that are often substituted with *G. rhodantha* and *Podophyllum hexandrum.* Differentiation of the authentic *Gentiana* species was done by DNA barcoding. The study revealed the potential of *rbcL, matK, psbA-trnH,* ITS*, trnL-trnF,* 5SrRNA and *rpL36-rps8* regions to differentiate Guanlongdan from Jianlongdan and their respective adulterant species in to separate clades with high bootstrap support on NJ analysis (Wong et al., 2013).

DNA barcoding was applied to discriminate *Isatis indigotica*, a medicinal plant from its closely related adulterant species, *Isatis minima* and *Isatis oblongata* using three loci ITS2*, trnL-F* and *rbcL* (Chen et al., 2014). Though there was no sequence variation between the two adulterant species, ITS2, *trnL-F* and *rbcL* exhibited 4, 3 and 1 single nucleotide polymorphisms (SNPs), respectively differentiating *I. indigotica* from its adulterant species.

Among the three loci, ITS2 proved to be the best with a higher sequence variation and clustering of the *I. indigotica* sequences into a clade separate from its adulterant species.

Herrmann and Wink (2014) assessed the potential of *rbcL* locus in authentication of 37 herbal drug samples representing 28 species. Seventy five percent of the drugs were assigned to their species level and 25% to their genus level by BLAST analysis. One drug sample of *Fraxinus rhynchophylla* was found to be switched with *Arctium lappa.* aa

Little (2014) has reported the potential of *matK* barcode in authentication of herbal supplements of *Gingko biloba.* Out of the 37 herbal samples analysed, 9 samples produced amplicons similar to fillers like *Oryza sativa* and an unidentifiable species.

Authentication of threatened and commercial timber trees in the tropical dry evergreen forests of India was done using DNA barcoding. A reference library was created for 143 timber species using *rbcL* and *matK* sequences. This libraray was used to check the wood samples used in commercial timber industries and could identify 21 samples to species level and 4 to the genus level (Nithaniyal et al., 2014).

Authentication of 20 *Cassia* species used in Indian traditional medicine was carried out using DNA barcoding loci *rbcL, matK. psbA-trnH* and ITS2 (Purushothaman et al., 2014). Single locus approach was not successful in species identification but a tiered approach of *rbcL* + *psbA-trnH* provided 100% species identification.

Molecular identification of medicinal plants sold commercially in Manila, Philippines was done by DNA barcoding. *matK, psbA-trnH, rbcL* and ITS loci were used for authenticating the plants. Contamination of ITS sequences by fungal ITS sequences led to its exclusion from further analysis. BLAST analysis of *matK* and *psbA-trnH* successfully authenticated the medicinal plants as *Euphorbia hirta, Senna alata, Annona muricata, Gliricidia sepium, Blumea balsamifera, Psidium guajava, Mangifera indica, Lagerstroemia speciosa* and *Citrus maxima.* Substitution of *Premna odorata* with its related species *P. microphylla* could also be identified (Raterta et al., 2014).

Zhang et al., (2014a) utilized DNA barcoding for the identification of 111 medicinal materials collected from the herbal market of the “three month fair” of Dali region. The potential of ITS2 and *psbA-trnH* was tested and found that ITS2 had better utility at species level while *psbA-trnH* assigned species to the correct genus. Out of the samples tested, 14 adulterants and 12 substituents were identified.

The adulteration of "Bala" (*Sida cordifolia* and *S. rhombifolia*) herbal products traded in South India was checked by Kumar et al., (2015) using DNA barcoding.The study based on *psbA-trnH* and ITS2 barcodes conducted on 10 traded samples revealed that only two belonged to the Bala group while the remaining eight were admixtures.

Vassou et al., (2015) conducted a study for the authentication of *S. cordifolia,* a herbal drug using DNA barcoding. They analysed 25 market samples of *S. cordifolia* traded as roots, stem, whole plant, leaves, seed and powder using *psbA-trnH,* ITS2*, rbcL* and *matK. psbA-trnH* and ITS2 were found to be the best loci for their authentication with a higher interspecies divergence. None of the market samples were reported to be authentic. The traded samples contained 36% *Sida acuta,* 20% *S. spinosa,* 12% *S. alnifolia,* 4% *S. scabrida,* 4% *S. ravii,* 8% *Abutillon* sp., 4% each of *Ixonanthes* sp., *Terminalia* sp., *Fagonia* sp., and *Tephrosia* sp.

Two hundred and fifty seven samples from 8 species namely dried leaves, flowers and roots sold in Brazilian markets were analysed using barcoding loci *rbcL, matK* and ITS. It was found that the substitution exist as high as 71%. *Hamamelis virginiana* was found to be substituted with samples of genus *Lantana* and *Solanum* while *Valeriana officinalis* was substituted with plants of genus *Ageratum* and *Cissampelos* due to their easy availability in Brazil. *Sorocea bonplandii* known as *Maytenus ilicifolia* in Brazil was used as a substituent of *M. ilicifolia* due to its similar morphology and common name. The study also revealed the use of *Vernonia condensata* as an adulterant of *Peumus boldus*. Admixture of *H.virginiana* with *Tilia samples* and *Passiflora incarnata* with *Senna alexandrina* were also identified (Palhares et al., 2015).

Cassia, Senna and *Chamaecrista*, three important medicinal species used in Indian traditional medicine Ayurveda, are susceptible to admixture in the herbal market. The use of DNA barcoding as a screening method was proposed for the quality control of these herbal medicines. Seethapathy et al., (2015) conducted a study to assess the extent of adulteration of these species in South Indian market using barcoding loci ITS2*, matK, rbcL* and *psbA-trnH.* Market samples were collected from different places in India. With a higher interspecific divergence than the other loci, ITS2 successfully discriminated the plant species. It was revealed that *Cassia fistula* species sold in market was authentic. *Senna auriculata* was sold as *S. alexandrina* in a shop in Trichy and Erode. In Coimbatore, *S.*

auriculata was sold as *S. tora*, while in Mysoru and Tuticorin, *S. occidentalis* was mislabeled as *S. tora*.

Aristolochic acid containing herbal materials reported to be toxic, are being sold in the Chinese herbal markets. Inorder to safeguard consumers, DNA barcoding was used as a method to authenticate these materials from their putative, safe, non-aristolochic substituents. BLAST1, nearest distance and NJ method based on ITS2 and *psbA-trnH* sequences clearly differentiated between aristolochiaceous and non- aristolochiaceous plant materials. A real time PCR assay was developed based on the ITS2 sequences for rapid identification of aristolochiaceous plants (Wu et al., 2015).

Xin et al., (2015) proposed the application of DNA barcoding as an efficient tool for supervising the trade of Rhodiola Crenulata Radix et Rhizoma (*Rhodiola crenulata*).The barcoding potential of *matK, rbcL, psbA-trnH* and ITS2 in species identification was assessed for 10 species of *Rhodiola*. *psbA-trnH* and ITS2 effectively discriminated all the species of which ITS2 was selected as the best barcode locus due to its higher interspecific divergence. ITS2 based barcoding of 100 traded samples collected from hospitals and drug stores showed that only 36 samples were authentic *R. crenulata.* Based on BLAST analysis, 35 samples were found to be *R. serrata,* 9 samples were *R. rosea,* 7 samples were *R. gelida,* 2 were *R. quadrifida* and 1 was *R. fastigiata.*

ITS2 based DNA barcoding was able to distinguish the commercially important Chinese medicine Glehniae Radix, root of *Glehnia littoralis* from its herbal adulterants. The NJ tree constructed using ITS2 sequences clustered genuine samples monophyletically and the adulterant species clustered in to a separate clade (Zhu et al., 2015).

Arisaematis Rhizoma and Pinelliae Tuber are two important traditional medicine components used in East Asian Countries that are targets for adulteration. Authentication of these two medicinal plants was done using DNA barcoding (Moon et al., 2016). *matK* sequence alignment showed 45 polymorphic sites while *rbcL* depicted 28 nucleotide sites that distinguished Arisaematis Rhizoma and Pinelliae Tuber from their adulterant entities.

Urumarudappa et al., (2016) assessed the species adulteration in the trade of the raw drug of *Saraca asoca* using DNA barcoding. *rbcL* and *psbA-trnH* were the barcode regions employed in this study. BLAST analysis of these two regions revealed that out of the market samples procured from 25 shops, 80% were found to be adulterated with plants from 7

different families viz. Fabaceae, Combreatace, Caricaceae, Moringaceae, Meliaceae, Rhamnaceae and Putranjivaceae.

2.6.1.1.2.6.2. Authentication of spices

de Mattia et al., (2011) conducted a study for the identification and traceability of different spice groups like mint, origanum, sage and basil using loci *rbcL, rpoB, matK* and *psbA-trnH. matK* and *psbA-trnH* could distinguish *Mentha aquatica* from *M. piperita* and *M. spicata.* In case of origanum samples, barcoding was not found fruitful. *Ocimum basilicum* could be clearly differentiated from *Ocimum gratissimum* and *Ocimum tenuiflorum* by the sequence variations in *psbA-trnH* and *matK* regions, thereby enabling the traceability of basil samples in market. *Salvia officinalis*, the commercial sage could also be distinguished from other related species using *psbA-trnH.*

psbA-trnH locus was used for the authentication of *Ilicium verum* from its closely related adulterant species like *I. micranthum, I. simonsii, I. modestum, I. jgadifengpi, I. henryi,* and *I. dunnianum* var. *latifolium* (Meizi et al., 2012). *psbA-trnH* was found to be best over *matK* and *rbcL* loci due to its PCR and sequencing successes, higher interspecific divergence and 100% species identification efficiency by BLAST analysis and nearest distance method. The indels and variable sites observed in *psbA-trnH* locus helped to clearly discriminate *I. verum* from its congeneric adulterants. Two market samples purchased from market were found to be genuine *I. verum.*

Gismondi et al., (2013) has proposed the use of DNA barcoding as a traceability tool for commercial saffron samples. A study was conducted using different *Crocus* species using loci *rbcl, matK, psbA-trnH* and ITS. ITS locus was found to be ideal for discriminating *C. sativus* (true saffron sample) from other species. This method could also differentiate between saffron samples of European and Italian origin.

Chilli adulteration in traded black pepper powder was first detected by Parvathy et al., (2014) using DNA barcoding. Three loci viz. *rbcL, rpoC1* and *psbA-trnH* were studied out of which *psbA-trnH* was found to be ideal in adulterant detection. Amplicons with a size of 350 bp were produced for genuine black pepper while adulterated samples yielded amplicons of 350 bp and 650 bp. BLAST analysis revealed that the sequence of the 650 bp amplicons showed 100% similiarity to *Capsicum annuum.* This technique was able to detect chillli adulterations upto 0.5%.

DNA barcoding was used to detect the adulteration of traded true cinnamon barks with *C. cassia*. *rbcL* was identified as the ideal barcode over *matK,* ITS and *psbA-trnH* owing to its amplification success and higher interspecific divergence. Sequence alignment of *rbcL* locus also revealed the presence of three polymorphic sites that differentiated *C. verum* from *C. cassia.* Out of the 10 bark samples procured from the market, seven exhibited the SNPs specific to *C. cassia* thereby confirming the adulteration (Swetha et al., 2014).

Parvathy et al., (2015) has applied DNA barcoding for tracing out adulterants in traded turmeric powder. Three loci *rbcL, matK* and ITS were used in this study. ITS was found to be the ideal locus with a higher discriminatory power. Sequence analysis of ITS revealed the presence of variable sites that could distinguish *Curcuma longa* from its closely related adulterant species, *Curcuma zedoaria*. Out of the 10 market samples tested, one showed *C. zedoaria* adulteration. BLAST analysis brought to light the presence of filler species like cassava starch, rye, wheat and barley in two traded samples.

Adulterations of traded saffron with *Buddleja officinalis, Gardenia jasminoides, Curcuma longa, Carthamus tinctorius* and *Calendula officinalis* could be detected using *matK* and *rbcL* locus (Soffritti et al., 2016). Sequence variation in *matK* was higher than *rbcL* locus. Species specific primers developed based on the polymorphic sites helped to identify these adulterants in traded saffron. This method could detect adulteration as low as 0.5%. Authentication of *M. fragrans* from its adulterant *M. malabarica* was accomplished by *psbA-trnH* based barcoding. Sixty polymorphic sites and 9 indel regions in *psbA-trnH* sequence alignment discriminated *M. fragrans* from *M. malabarica.* Three out of the five mace samples collected from the market exhibited the polymorphic regions specific to *M. malabarica* thereby confirming its adulteration with *M. malabarica* (Swetha et al., 2017).

2.6.1.1.2.6.3. Authentication of other commodities

Bruni et al., (2010) assessed the potential of different barcoding loci like *rpoB, matK, psbA-trnH* and nuclear regions like *At103* and *sqd1* in distinguishing the poisonous plants from edible ones. Based on the amplification, sequencing success and resolution power of the tested loci, *matK* and *At103* were proved to be ideal for their discrimination. *psbA-trnH* was excluded from analysis due to the difficulty in aligning sequences.

Canola and saffron oil contamination in olive oil could be detected using DNA barcodes (Kumar et al., 2011). *psbA-trnH* and *matK* primers specific for canola, saffron and olive oil

were designed and successfully amplified. These primers were able to detect 5% and above adulteration in olive oil samples. Further confirmation was done by sequencing the amplicons produced and comparing it with the barcodes deposited in reference databases.

A study on barcoding of herbal teas using barcoding loci *rbcL* and *matK* revealed that 35% of the samples generated barcodes for unlisted ingredients on the brand label. *Matricaria recutita* was the most common unlisted ingredient found in seven herbal tea products. Four herbal teas yielded *Camellia sinensis* barcodes although it was not listed on the label. Barcode from a herbal tea sample was similar to *Poa annua* and four products generated barcodes similar to plants of parsley family (Stoeckle et al., 2011).

Li et al., (2012) employed barcoding to authenticate the plants used in the preparation of Chinese "cooling beverage", prepared from single or mixture of plants. *rbcL, matK, psbA-trnH* and ITS loci were used in the differentiation of Kudidan (*Elephantopus scaber*) from its substituent *Elephantopus tomentosus*; Ludougen (*Pandanus tectorius*) from its adulterant (*Pandanus austrosinensis*); Shepaole (*Rubus reflexus*) from *Rubus parvifolius* and Xiangsizi (*Abrus precatorius*) from *Abrus cantoniensis*. *psbA-trnH,* ITS*, matK* and *rbcL* alignment depicted 12-46, 9-70, 1-7 and 1-5 polymorphic sites respectively, that served as markers to distinguish between these genuine and adulterant species. Though *rbcL* had the least number of polymorphic sites, only it could be successfully amplified in all the 4 traded samples of the cooling beverage tested. Sequence analysis of *rbcL* revealed that traded samples of Kudidan, Ludougen and Xiangsizi was genuine material and Shepaole was substituted.

The increasing demand for non-Camellia teas has paved way for its substitution with adulterants resulting in allergic reactions. BLASTN and phylogenetic analysis based on barcoding loci viz. *rbcL, matK, psbA-trnH* and ITS2 could identify non-Camellia teas from the samples screened thus providing a way to effectively label the commercially available samples and ensure safety to consumers (Long et al., 2014).

DNA barcoding was used to solve a dispute in the international trade of roasted barley tea. Roasted barley tea consignment imported from China was rejected due to substitutions. Barcoding of the rejected barley tea samples using *rbcL, matK, psbA-trnH* and ITS2 loci revealed that out of the 13 batches of samples tested, 1 batch was substituted with *Morus* species. Out of the remaining 12 batches, 2 batches had only *Hordeum vulgare* while 10 had *Hordeum vulgare* admixed with *Morus* sp., *Triticum* spp., *Avena sterilis* and *A. fatua* (Jian et al., 2014).

Bruni et al., (2015) adopted barcoding technique to identify the plant composition in processed honey using *rbcL* and *psbA-trnH* loci. BLAST analysis showed that the four honey samples contained 39 plant species of genus *Castanea*, *Quercus*, *Fagus* and other herbal taxa. One out of the four samples studied showed traces of genomic DNA from *Atropa belladonna,* a toxic plant, thereby promoting the applicability of barcoding in evaluating food safety.

Apart from the plant based products, barcoding has applications in authentication of food derived from animals. Efficiency of DNA barcoding in sea food traceability has resulted in its adoption as a method for authentication of fish based commercial products by the US Food and Drug Administration (Yancy et al., 2008). Barcode based seafood authentication has been successful due to the availability of more than 70,000 reference sequences in the database, Fish Barcode of Life Initiative (FISH-BOl) (Galimberti et al., 2013). Many authors have reported mislabeling and adulteration of seafood, processed seafood and their byproducts like fish fillets using *cox1* gene based barcoding (Nicolè et al., 2012; Maralit et al., 2013; Carvalho et al., 2015; Khaksar et al., 2015; Chang et al., 2016). Meat adulteration in US markets could also be traced using *cox1* based barcoding (Kane and Hellberg, 2016; Quinto et al., 2016).

2.7. Phylogenetic Analysis

The study of evolutionary relationships between species of organisms ranging from bacteria to human is referred to as phylogenetic analysis (Nei, 1996) and the relationship between two species is called phylogeny (Rizzo and Rouchka, 2007). The term phylogenetics is derived from the Greek words phyle and phylon meaning “tribe” and “race” and the term genetikos, from genesis meaning “birth” (Roy et al., 2014). Phylogenetic studies involve the construction and evaluation of the hypothesis regarding the historical patterns of descent in the form of phylogenetic trees (Gregory, 2008) by comparison of the specific characters of a species under the assumption that similar species (species with similar characters) are genetically close (Shamir, 2001). The evolutionary studies are conducted based on classical or modern approach. Classical approach involves the use of morphological characters like size, colour etc. while the modern phylogenetic approach relies on comparison of DNA, RNA or protein sequences in homologous organisms. Differences in these sequences indicate genetic divergence among organisms that arise due to molecular evolution in course of time. Molecular markers are dominant for phylogenetic analysis as morphological

characters may be limited in number and insufficient to differentiate two organisms at phyla, order, family or genus level (Patwardhan et al., 2014). Phylogenetic analysis based on DNA sequences shed light on the relationship between two closely related species while aminoacid sequence based analyses provide insight regarding the more distant realationships (Michu, 2007).

2.7.1. Phylogenetic trees

The evolutionary relationships deduced from phylogenetic analysis is represented as branching tree like diagrams that give information regarding the estimated pedigree of the organisms and these representations are known as phylogenetic trees (Brinkman and Leipe, 2001). In other words, a phylogenetic tree is a diagram that depicts the lines of evolutionary descent of different species, organisms or genes from a common ancestor (Baum, 2008).The first sketch of phylogenetic tree was drawn by Charles Darwin as early as in 1837 while understanding the concept of evolution (Gregory, 2008).

An evolutionary tree consists of different components like node, internode and branch. The tip of the tree is called node or leaf or operational taxonomic units (OTU) that represents the different types of comparable taxa like individuals from same or different species that are defined as the phylogenetic units of the tree (Mount, 2001; Vandamme, 2009). These nodes are interconnected by branches that denote the relationship between two species at points called internodes or hypothetical taxonomic units (Gregory, 2008). Internodes are the hypothetical progenitors or the last common ancestor (LCA) of OTUs that arise from a node and denote the speciation event that gives rise to sister groups (Gregory, 2008; Vandamme, 2009). The two descendants that are derived from the same node are called sister groups which have close relation with each other and share a common ancestor.

The pattern of branching of a tree is called topology, (Figure 1) that depicts the evolutionary relatedness (Gregory, 2008) and the length of branches indicates the time estimate between the species (Rizzo and Rouchka, 2007). The branches may be either scaled (with different lengths based on their evolutionary distance) or unscaled (with same length) (http://www.ncbi.nlm.nih.gov/ Class/NAWBIS/ Modules/ Phylogenetics/ phylo8. html). Based on branching of trees, they may be classified to bifurcating and multifurcating trees. Bifurcating trees are those in which a node is descended by two lineages while multifurcating trees are descended by three or more lineages (Reddy, 2011). The branches may be grouped as monophyletic, paraphyletic and polyphyletic. A group of species that are derived from a

single common ancestor, includes all the species descending from the common ancestor and shares a set of unique characters is called a monophyletic group (Reddy, 2011; Dowell, 2008). Paraphyletic groups include a group of species arising from a common ancestor but do not include all descendants of the common ancestor. Polyphyletic groups include species from more than one ancestor and exclude species that might link them (Kolekar et al., 2011).

There are two types of phylogenetic trees viz. rooted phylogenetic trees and unrooted phylogenetic trees. Rooted phylogenetic trees contain a node known as 'root' from which all other OTUs arise. It is the oldest point in the tree and is the common ancestor of all taxa involved in the analysis (Dowell, 2008). Trees that do not originate from any clear node are called unrooted trees (Rizzo and Rouchka, 2007). Rooted trees represent the divergence of a group of related species from their last common ancestor by successive branching over the time period while unrooted trees show interspecies relationship excluding the identification of the last common ancestor. In a rooted tree, the path from root to node depict the evolutionary time while in unrooted tree the path between the nodes does not signify the evolutionary time (http://bip.weizmann.ac.il/ education/ course/ introbioinfo/ 03/ lect12/phylogenetics.pdf).

Rooted phylogenetic trees are constructed using unrelated species or genes used in the phylogenetic construction. The distantly related or relatively less related taxa known as outgroups are used for rooting of trees (Reddy, 2011). Single or group of species that are not part of a study are likely to be used as an outgroup (Michu, 2007). Trees may also be grouped as species tree and gene trees. A tree that depicts the evolution of a gene is known as gene tree while one that shows the evolution of a species is called species tree (Reddy, 2011).

The trees are represented in three forms namely dendrogram, phylogram and cladogram (Roy et al., 2014) (Figure 2). A dendrogram is any diagrammatic representation of a phylogenetic tree while phylogram is a representation of a phylogenetic tree with branches that have variable lengths proportional to evolutionary changes. Cladograms are phylogenetic trees that have uniform branch length depicting the relationship between the taxa without any significance to evolutionary divergence (Kolekar et al., 2011).

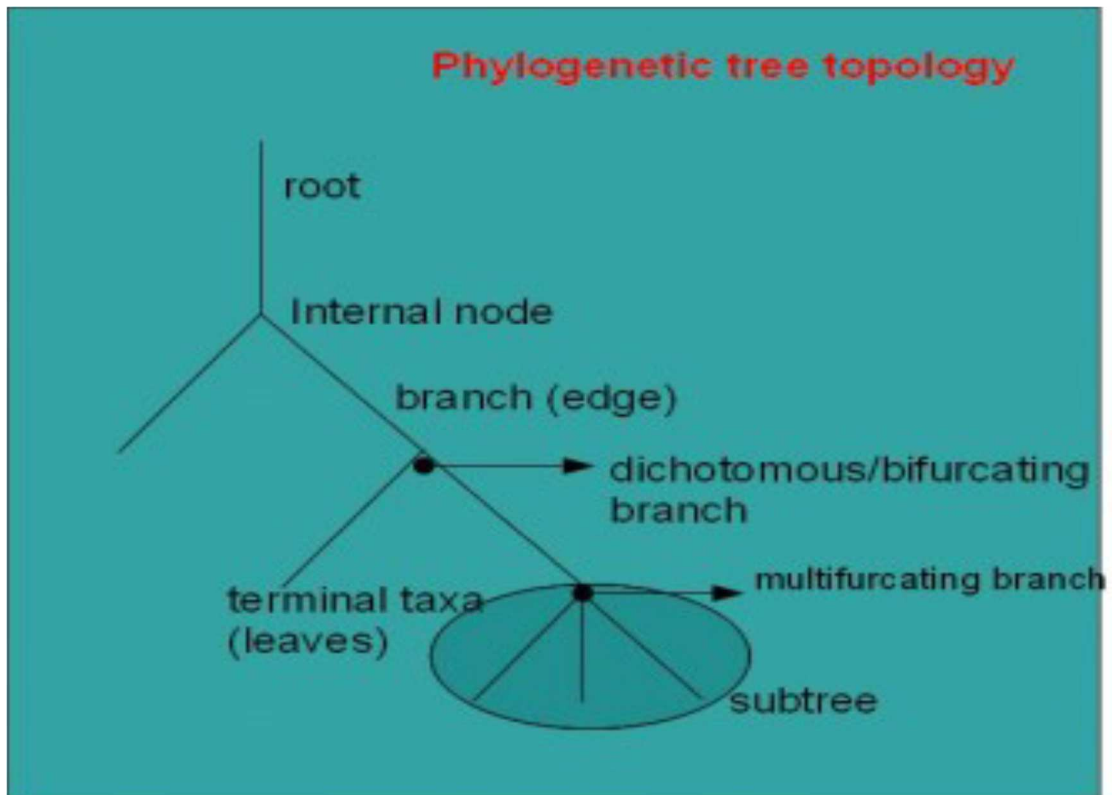


Figure 1. Topology of Phylogenetic tree. (Source: Reddy, 2011).

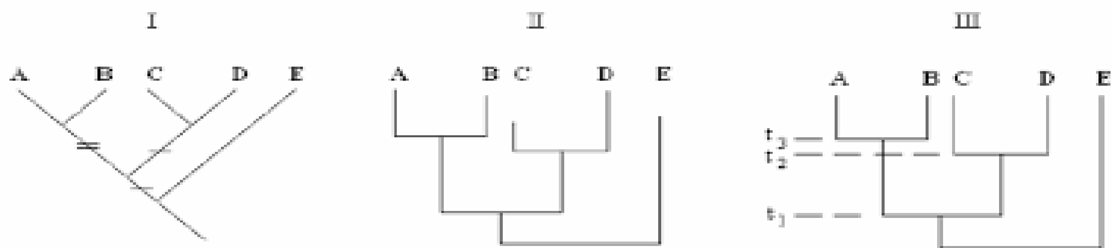


Figure 2. Different forms of phylogenetic trees (I – cladogram, II – phylogram, III – dendrogram) (Source : Kazachkova, 2007)

2.7.2. Steps in Phylogenetic Analysis

The construction of phylogenetic trees involves five steps:

1. Selection of an appropriate marker
2. Multiple sequence alignment
3. Selection of an evolutionary model
4. Phylogenetic construction
5. Evaluation of the phylogenetic tree

2.7.2.1. Selection of an appropriate marker

The biological information that can be employed to estimate the evolutionary relationships among species is called a phylogenetic information marker (Reddy, 2011). The criteria for a marker are as given below:

a) It should be a single copy gene
b) Availability of primers for easy amplification
c) Easy alignment of the sequenced amplicons
d) Optimum substitution rates to present enough informative polymorphic sites
e) Not too much variation in the bases among taxa, as it may not reveal the true ancestry
f) Use of multiple genes may provide better resolution to the tree

The molecular markers that are employed for phylogenetic analysis are the nuclear ribosomal genes, mitochondrial genes and chloroplast genes of which chloroplast markers are primarily used in plant phylogenetic studies (Patwardhan et al., 2014). The chloroplast markers used in phylogenetic studies are the barcoding regions viz. *rbcL, matK, psbA-trnH,* ITS*, rpoB, rpoC,* and *atpB.*

2.7.2.2. Multiple Sequence Alignment (MSA)

MSA, the second step in the phylogenetic analysis involves arrangement of sequences to identify the regions of homology between them. MSA is of two types viz. pairwise alignment and multiple alignment. Alignment of two sequences is called pairwise alignment while that of three or more sequences is called multiple sequence alignment. Pairwise alignment is done either locally or globally to identify the homologous regions. Global alignment involves the comparison of sequences from one end to the other irrespective of their sequence length while local alignment checks for the small homologous regions locally (Reddy, 2011). Sequence alignment would be easy if the sequences are of equal length. But when they are dissimilar and differ in their length, alignment becomes tedious as it requires the insertion of gaps to map the nucleotides of the corresponding sequences (Morrison, 2006). The gaps represent the insertion – deletions that may have occurred in the sequences over the course of time (Dowell, 2008). MSA is carried out by automatic tools or manual alignment. Though manual alignments correctly identify conserved regions its laborious nature paved the way to resort to automatic alignments (Michu, 2007). Automatic tools include softwares like CLUSTAL W (Thompson et al., 1994), T-Coffee (Notredame et al., 2000), MUSCLE

(Edgar, 2004) etc. The calculation of sequence diversities by MSA helps to infer the evolutionary relationship between taxa (Michu, 2007).

2.7.2.3. Selection of an evolutionary model

Evolutionary models are sets of assumptions about nucleotide or aminoacid substitutions that describe the different probabilities of a nucleotide or aminoacid substitution to correct the unseen changes along the phylogeny (Posada, 2003). The need for evolutionary models in phylogeny arises to counter the effects due to homoplasy that may hinder generation of a genuine tree (Patwardhan et al., 2014). Evolutionary models are mathematical models that are built empirically using properties derived from comparison of the DNA/aminoacid sequences or parametrically employing chemical and biological properties of the same. These models calculate the genetic distance (represented as the branch lengths of the tree) between homologous sequences by measuring the nucleotide substitutions per site that has occurred in the evolutionary lineage between them and their recent common ancestor (Liò and Goldman, 1998). There are mainly two types of nucleotide substitution evolution models viz. Jukes Cantor model and Kimura two parameter model.

Jukes Cantor model (Jukes and Cantor, 1969): In 1969, Jukes and Cantor proposed a stochastic nucleotide substitution model which assumes that all nucleotide substitutions occur at an equal rate and the substituted ones are replaced by other nucleotides (Liò and Goldman, 1998). According to this model, each base is considered to have the same frequency (0.25) in a DNA sequence (Mount, 2001). But it can analyse only closely related sequences (Patwardhan et al., 2014).

Kimura two parameter model (Kimura, 1980): According to this model, substitutions are of two types, transitions (purines substituted by purines) and transversions (purines substituted by pyramidines) and transitions occur at a faster rate than transversions. Kimura model also assumes the frequency of base in a sequence to be 0.25 (Patwardhan et al., 2014).

2.7.2.4. Construction of phylogenetic trees

Phylogenetic trees are constructed using distance based methods or character based methods.

2.7.2.4.1. Distance based methods

Distance based methods are also called clustering methods or algorithmic methods. In this method, the pairwise distances are calculated for all the species under study using an evolutionary model of nucleotide substitution like Jukes Cantor or K2P to derive a distance

matrix that is subsequently used to construct a tree by clustering method (Michu, 2007). Once the distance matrix is generated the actual data is discarded and trees are constructed based on the distances (Brinkman and Leipe, 2001). Based on the distance matrix generated, two closely related taxa are placed under a common internode, a third taxa is then added considering the internode as a group followed by progressive addition of other taxa (Reddy, 2011). Distance based methods are computationally less intensive, operate on simpler algorithms, and provide outputs in polynomial time (Brinkman and Leipe, 2001; Dowell, 2008; Uncu et al., 2015). The most commonly employed distance based methods are Unweighted Pair Group Method with Arithmetic averages (UPGMA) and Neighbour Joining Method (NJ).

2.7.2.4.1.1. Unweighted Pair Group Method with Arithmetic averages (UPGMA)

UPGMA, the simplest and oldest distance based method to develop phylogenetic trees was developed by Sokal and Michener (1958) that assumes the rate of substitutions to be constant along a lineage (Uncu et al., 2015). It is a clustering algorithm that generates true topology of a tree if the divergence follows the molecular clock hypothesis that mutational rate is identical in all species (Brinkman and Leipe, 2001; Shamir, 2001). In this method, the pairwise distances between sequences are calculated and the clustering algorithm initially identifies the two most similiar OTUs based on their sequence similiarity and shorter evolutionary distance and cluster them as a single composite OTU. The average pairwise distance of the newly formed cluster or OTU is then calculated followed by the identification of the closely related pair between the newly formed OTU and the remaining sequence. This process is iterated until an evolutionary tree is constructed and a root is identified for the tree (Mount, 2001; Dowell, 2008). However UPGMA has a tendency to provide incorrect phylogeny when the evolutionary rate differs along the branches (Posada, 2003).

2.7.2.4.1.2. Neighbour Joining (NJ) Method

NJ is a rapid reliable method used for phylogentic construction developed by Saitou and Nei (1987) that assumes different branch lengths indicating the evolutionary divergence along the branch (Michu, 2007). The tree is created using mininum evolution criteria to minimize the tree and is more suited for sites with variable rates of evolution (Kolekar et al., 2011). Here tree construction is done by a decomposition process. Initially the algorithm forms a star shaped tree with all the available OTUs descending from a single node. It then searches among the OTUs as neighbours to find the one that share the smallest S value (sum of branch

length) and cluster it as a single composite OTU. This single composite taxon is then treated as a neighbor with the other remaining taxa to find the smallest S value. The process is continued until all taxa are clustered to form a tree (Uncu et al., 2015). An unrooted phylogenetic tree is the ouput of NJ method which can be rooted by introducing an outgroup. Though this method is widely used in phylogenetic analysis, it faces certain problems as the algorithm is based on reduction of sequence information while conversion to distance matrix. Also incorporation of indels may further complicate the procedure (Michu, 2007).

2.7.2.4.2. Character based methods

Character based methods utilize the aligned nucleotide and aminoacid characters than pairwise distances for tree inference (Shamir, 2001). Each position in an alignment is called character and the nucleotide located at a position is called state of that character. All these characters are analysed independent of each other (Kolekar et al., 2011). They scrutinize each column of the multiple sequence alignment and create a tree that suits the informational characters in the alignment. As they depend on the nucleotide and aminoacid characters, it is possible to trace out the evolution of specific regions in an alignment like catalytic regions (Dowell, 2008). But these methods are slower than distance based methods (Kolekar et al., 2011).

There are mainly three types of character based methods that are used in phylogenetic analysis viz. maximum parsimony, maximum likelihood and Bayesian analysis.

2.7.2.4.2.1. Maximum Parsimony (MP) Methods

Maximum Parsimony methods developed initially for morphological characters was used with aminoacid sequence data by Eck and Dayhoff (1966) and nucleotide sequence by Fitsch (1971) and Hartigan (1973). It is used for small number of sequences that are quite similar (Mount, 2001). MP methods involve the calculation of total number of nucleotide or amino acid substitutions and choose the tree topology with the smallest number of changes (Uncu et al., 2015). All sites in the sequence are not useful for constructing phylogeny. The constant sites that have the same nucleotides at all position and the singleton sites in which the nucleotides differ at a single site in a species cannot be used for this analysis. The sites at which atleast two distinct characters are present atleast twice in the sequences are suitable for this analysis and are called parsimony informative sites (Yang et al., 2012). Maximum parsimony methods employ Fitsch's algorithm for phylogeny inference (Kolekar et al., 2011). MP method also allows the incorporation of gaps as an additional alignable character

(Brinkman and Leipe, 2001). There are two types of maximum parsimony methods – unweighted and weighted. Unweighted methods treat transition and transversion substitutions equally while unweighted methods give different weights to transitions and transversions during phylogeny reconstruction (Uncu et al., 2015).

In this analysis all possible unrooted trees are considered and the sequence variations at each site are placed at the tip of the tree. The trees that require the smallest number of change to produce the variation is then determined. After repetition of the above analysis, an overall tree that produce the minimum evolutionary change is finally decided (Mount, 2001).

The simplicity, amenability to mathematical analysis and accesibity are the strong points of this method (Yang et al., 2012). But it also suffers from certain shortcomings like long term attraction (a tendency to group trees with long branch in to a group) and inability to incorporate parameters that alter sequence evolution in the tree (Uncu et al., 2015).

2.7.2.4.2.2. Maximum Likelihood (ML) Methods

The concept of maximum likelihood was developed by R.A. Fisher in 1920 as a statistical tool to estimate unknown parameters in a model (Yang et al., 2012). The potential of ML methods in phylogentic analysis was first stressed by Cavalli S-Forza and Edwards (1967). Felsenstein (1981) modified it to suit the analysis of nucleotide sequence data and Kishino et al., (1990) utilized ML for aminoacid data. The substitutions in each column of the sequence alignment are examined for their fit to a tree that explains the phylogenetic relationship between sequences (Mount, 2001). Two steps are involved in this method: optimizing branch length to calculate the tree score for each tree and search for the maximum likelihood tree. ML method calculates branch length by considering every possible nucleotide at the internal node to maximize the likelihood of the tree. The likelihood of a tree topology is calculated as the sum of the probabilities calculated for each observed site and is designated as log likelihood (Hall, 2008) and the tree with the highest log likelihood is selected as the maximum likelihood tree (Uncu et al., 2015).

Advantage of ML methods are the availability of a number of evolutionary models for analysis, evaluation of trees with variations in mutation rate along different lineages and studying the realationship between diverse sequences (Mount, 2001). But this method is computationally demanding and also the probability of deriving an incorrect topological tree is high when the rate of substitution varies among the lineages.

2.7.2.4.2.3. Bayesian Analysis

Bayesian analysis is a statistical approach that was first used in phylogenetic studies in 1900. It provides an intuitive measure of support for trees and helps to estimate the phylogenetic relationship of large complex data of different genes. Bayesian method is based on the calculation of posterior probabilities of tree from the prior information using Monte Carlo Markov Chain models (Michu, 2007). It starts with the generation of a random tree proceeded by modifications to the subsequent generations followed by calculation of the posterior probability ratio that is the deciding factor for a tree acceptance or rejection (Uncu et al., 2015). Bayesian analysis is a consistent and computationally efficient method. But it also has certain limitations like long branch effect. Another issue is the use of prior information to deduce the posterior probabilities as specification of the prior is always a burden for the user. Also at times use of simplistic models may lead to very high posterior probability values (Yang et al., 2012) that are not advisable.

2.7.2.5. Evaluation of the constructed phylogenetic trees

The validity of the trees are evaluated using statistical approaches of boot strapping and jackknifing. Bootstrap method was proposed by Efron (1979). In bootstrapping method, trees are again constructed equal to the number of pseudoreplicates. Pseudoreplicates are the complete data set with equal number of columns or information sites by removing one column of information site that is replaced by complete column site from the existing data (Reddy et al., 2011). The number of times each of the nodes in the initial tree is repeated in bootstrap trees is given as percentages at the tree nodes and are called bootstrapped values or percentages (Felsenstein and Felsenstein, 2004). Bootstrap represents the number of times in which the sequences are grouped together (Michu, 2007). The trees with more than 70% bootstrap values are considered to be reliable (Reddy et al., 2011). A low boot strap value signifies long branch attraction (Michu, 2007).

Jackknifing is a method proposed by M.H. Quenouille in 1949 which is similar to bootstrapping method. Jacknifing is a resampling method without replacement of columns or information sites (McIntosh, 2016).

2.7.3. Phylogenetic studies in plants using DNA barcoding

Kaufmann and Wink (1994) made a systematic study of the sub family of Lamiaceae and Nepetoideae based on *rbcL* sequences using maximum parsimony and NJ methods. Both the trees were congruent to each other and clustered the 41 taxa belonging to 20 genera in to 12

clades viz. I - *Collinsonia,* II - *Lavandula,* III - *Agastache, Glechoma,* IV - *Satureja, Hyssopus, Dracocephalum,* V - *Nepeta,* VI - *Hormium,* VII - *Prunella,* VIII - *Melissa, Ocimum,* IX - *Monarda, Mentha,* X - *Origanum, Thymus,* XI - *Salvia,* XII - *Rosmarinus,* and XIII – *Perovskia.* These clades represented 3 tribes Elsholtzieae (I), Lavanduleae (II), and Mentheae (III — XIII). Mentheae was further divided in to 3 groups III-VII, VIII and IX-XII. The study also placed *Marjorana hortensis* as a separate genus as reported by other pioneers.

Cros et al., (1998) has carried out the phylogenetic analysis of coffee trees belonging to genus *Coffea* and *Psilanthus* using the maximum parsimony method on sequences of the chloroplast *trnL-trnF* intergenic spacer. Phylogenetic analysis revealed that the species were grouped in to five clades that gave a geographical correspondence. It was found that the genus *Coffea* had a radial mode of speciation and a recent origin in Africa.

The phylogenetic relationships between 15 species of *Illicium* were examined using ITS sequences with neighbor joining and maximum parsimony methods of tree construction. Phylograms showed a dichotomy between *I. floridanum* and *I. parviflorum,* two North American species and the remaining East Asian species. Congruence was seen between phylogeny and palynology as the three species that formed trizonocolpate pollen consistently formed a single clade. The close association between the East Asian species and the low sequence divergence of ITS denoted a recent sequence divergence or a slowdown of mutations (Hao et al., 2000).

Hao et al., (2001) conducted a phylogenetic study on Schisandraceae using morphological and ITS sequence data. A dichotomy was observed between two clades representing *Kadsura* and *Schisandra* genus in the most parsimonious tree drawn from the morphological data. ITS phylogram grouped *S. propinqua* with *Kadsura* while the position of *S. glabra* was uncertain. A tree based on combined data was polytomous due to the variable positions of *K. scandens, K. coccinea* and *S. propinqua.*

The phylogenetic study of the Litsea complex, Lauraceae was conducted using *matK* and ITS sequences. *matK* phylogram depicted 5 resolved clades *Neolitsea, Laurus, Parasassafras, Litsea* and *Lindera clades* in polytomy with unresolved sections of Lindera and Umbellularia. A more resolved phylogram was generated from the combined data of *matK* and ITS that showed four lineages viz. *Laurus*, *Litsea*, *Lindera* and *Actinodaphne* II that were polyphyletic (Li et al., 2004).

Phylogenetic analysis of the genus *Piper* was done using individual *petA-psbJ*, ITS sequences and their combination. *petA-psbJ* failed to highlight any relation between the species in study while the multilocus approach of these loci grouped *Piper* into 10 major clades. New clades and subclades like *Peltobryon*, *Schilleria*, *Isophyllon*, *P. cinereum/P. sanctum* were also identified from the study (Jaramillo et al., 2008).

The phylogenetic analysis of genus Zingiberaceae was attempted by Selvaraj et al., (2008) using NJ and UPGMA methods based on *matK* sequences. The results depicted the polyphylogenetic nature of the Zingiberaceae genera *Afromum, Alpinia, Globba, Curcuma* and *Zingiber.* The species exhibited a variation of 24% between the species and a transition-transversion ratio of 1.54. The *matK* phylogram clearly portrayed the intra and interrelationships between the genus.

A phylogenetic analysis of five *Dendrobium* species was carried out using *rbcL* and *matK* sequences for species identification. Tree construction was done using NJ method and the phylogram clearly differentiated the *Dendrobium* species viz. *D. fimbriatum*, *D. moniliforme, D. nobile, D. pulchellum* and *D. tosaense* from each other. *matK* was efficient over *rbcL* in determining the genetic relationship between the species as the latter exhibited less sequence variation (Asahina et al., 2010).

Oskoueiyan et al., (2010) studied the phylogentic status of *Vavilovia formosa* using chloroplast *trnL-F*, *trnS-G* and nuclear ITS regions. Phylogeny was studied using Maximum parsimony and Bayesian analysis. Both single and multilocus approach showed that *Vavilovia* is closely related to *Pisum* and forms a sister group with *Lathyrus*. They proposed that *Vavilovia* should be included under genus *Pisum* because of the similar morphological, biochemical and molecular characters shared with *Pisum formosa.*

The phylogeny of the genus *Neocinnamomum* and its relation with related genera *Caryodaphnosis* and *Cassyntha* was done using the barcoding regions *psbA-trnH*, *trnK* and ITS2. Maximum parsimony and Bayesian analysis was conducted to decipher their evolutionary relationship. Only the multilocus approach could throw light on its phylogenetic status and revealed the monophyletic nature of the genus *Neocinnamomum.* The sister relationship of *Neocinnamomum* with *Caryodaphnosis* was observed in the Bayesian method that was ascertained by the similiarity in morphology, bark and wood anatomy. A close relationship was seen between *Neocinnamomum* and *Cassyntha* due to long branch effect (Wang et al., 2010).

Techaprasan et al., (2010) evaluated the phylogeny of genus *Kaempferia* using chloroplast sequences *psbA-trnH* and *petA-psbJ*. Maximum parsimony analysis revealed the polyphyletic nature of the genus with *K. candida* clustering separately from other species thereby denoting its distant relationship. *Kaempferia* species were grouped in to four clades.

A phylogenetic study was conducted by Liu et al., (2013) on *Cissus* genus belonging to the grape family Vitaceae using five plastid genes *rps16, trnL-F, atpB-rbcL, psbA-trnH* and *trnC-petN*. Maximum parsimony, maximum likelihood and Bayesian inference showed the polyphyletic nature of *Cissus* genus with three clades – *Cissus*, Cissus striata complex and *Cissus anatartica* - *C. trianae.*

Penjor et al., (2013) conducted a phylogenetic study on 93 accessions of citrus and its relatives using NJ and maximum likelihood analysis of chloroplast *matK* sequences. *Citrus* species was divided into three clusters – citron, pummelo and mandarin and the phylogram showed that *Feroniella oblate* is closely related to *Feronia limonia* and not nested within citrus species. *Murraya paniculata* was found similar to *Merrillia caloxylon* and dissimiliar to *M. koenigii.*

The relationship of *Senecio asirensis* with other *Senecio* species (sequences downloaded from GenBank) was studied using nuclear ITS markers. Maximum parsimony, maximum likelihood and NJ methods produced phylograms that clustered *S. asirensis* in to a separate clade suggesting its endemism to Saudi Arabia (Khan et al., 2013).

Sonboli et al., (2013) assessed the phylogenetic relationship of *Thymus persicus* with other *Thymus* species and plants from other genera like *Origanum, Thymbra and Satureja* using ITS sequences with maximum parsimony and Bayesian analysis. The relationship of *T. persicus* with other *Thymus* species remained unsolved but it was found that *T. marandensis* shared sister relationship with *T. caramanicus, T. migricus, T. pubescens, T. trautvetteri* and *T. daenensis*. Inclusion of the monotypic genus *Saccocalyx* showed the paraphyletic nature of genus *Thymus* and its sister relationship with *Origanum.*

Phylogenetic status of African plant genus *Aneilema* was studied based on chloroplast sequences *matK, psbA-trnH, trnL-trnF* and *rps16*. Parsimony analysis was conducted to infer its phylogeny and it was revealed that *Aneilema* was not a monophyletic genus and that the species did not descend from a common ancestor. The consensus tree showed that *A. brasiliense* was closely related to *Polyspatha* and *Pollia* species than *Aneilema* (Kelly and Evans, 2014).

Vinitha et al., (2014) assessed the phylogenetic potential of *matK, rbcL* and ITS regions in Zingiberaceae using different tree based algorithms like NJ, UPGMA, ML, MP and Bayesian inference. *matK* and *rbcL* was found to be the best loci for species resolution and NJ the best method for tree construction. Out of the 20 species analysed *rbcL* and *matK* resolved 15 species into monophyletic groups and five species into two paraphyletic groups. ITS could only resolve 12 species while the remaining was clustered in to three paraphyletic groups.

Feng et al., (2015) constructed the phylogeny of genus *Dendrobium* using ITS2 sequences. The clustering done using NJ method gave results similar to previous morphological and molecular analysis. NJ method clustered the 21 species under study belonging to 12 sections in to four clusters of which two had high bootstrap values.

ITS and *matK* regions were used to reconstruct the phylogeny of 27 species of *Dendrobium* using maximum likelihood and Bayesian inference. *matK* phylogram grouped the species in to 6 clades but some species did not group together with species of their section. ITS phylogram grouped the species in to two clades thereby indicating the more powerful resolution of ITS in the evolutionary study of *Dendrobium.* A multi locus approach (ITS + *matK*) also gave the same clustering as of the ITS phylogram indicating the dominance of ITS over *matK* in delineating the evolutionary relationship of *Dendrobium* species (Srikulnath et al., 2015).

Phylogenetic status of *Capsicum* was assessed by sampling nearly all taxa of the genus using chloroplast regions *matK, psbA-trnH* and the waxy intron of the nuclear gene. The maximum parsimony and Bayesian analysis revealed the monophyletic nature of *Capsicum* sharing a sister relationship with *Lycianthes*. Eleven well supported clades were obtained out of which four were monophyletic (García et al., 2016).

Yao et al., (2016) studied the phylogenetic relationship of genus *Pogostemon* using ITS region and five chloroplast regions *matK, rbcL, rps16, psbA-trnH* and *trnL-F.* Maximum parsimony and Bayesian inference were carried out to resolve the phylogeny of the genus. Phylogenetic relationships were poorly resolved when the regions were used individually. However, a combined data set of the loci confirmed the monophyletic status of *Pogostemon* and its sister relation with *Anisomeles.* It was clustered in to two major clades, clade A containing *Pogostemon* + *P. amaranthoides* and clade B containing *Allopogostemon* + *Dysophyllus.*

2.7.3.1. Phylogenetic analysis in *Cinnamomum* spp.

Clarification in the genetic diversity of Sri Lankan cinnamon species was done on the basis of the sequence analysis of the chloroplast genomic regions *trnL-trnF, psbA-trnH* and ITS. Although sequence variation in the chloroplast regions were less, the cluster analysis based on ITS sequences employing NJ method could clearly indicate that the *C. litseafolium*, *C. rivulorum* and *C. sinharajaense* were divergent from *C. verum* accessions (Abeysinghe et al., 2009).

Chieh et al., (2010) performed a phylogenetic analysis of seven geographical strains of *Cinnamomum osmophloeum* Kaneh of China on the basis of the partial non-coding ITS2 region of ribosomal genes. UPGMA clustering method of the entities revealed a sequence homology in the tested samples. The phylogenetic trees constructed grouped the samples in to one cluster even though they belonged to different chemotypes.

In a phylogenetic study involving some Chinese *Cinnamomum* species using the locus ITS2, phylogenetic affinity between *C. osmophloeum* and *C. burmanii* was observed and both were found to be more closely related to *C. cassia* (Lee et al., 2010).

Phylogenetic analysis were done using nuclear genes viz. ITS, *LEAFY* and RPB2 on 94 *Cinnamomum* and 13 out group samples. Maximum parsimony and Bayesian phylograms constructed from the combined data set of the loci revealed the monophyletic nature of *Cinnamomum* group with three clades. The Asian section *Camphora* samples formed clade 1, the American *Cinnamomum* species with *Mocinnodaphne cinnamomoidea* and three *Aiouea* species formed clade II and the remaining ingroup samples – three *Camphora,* all Asian *Cinnamomum,* six Australian and one *Ocotea ikonyokpe* formed the third clade (Huang et al., 2016).

2.7.3.2. Phylogenetic Analysis in *Myristica* spp.

Limited literature is available for phylogenetic studies in *Myristica* species. Sheeja et al., (2014) conducted a phylogenetic study on *Myristica* species using a consensus tree of maximum parsimony, maximum likelihood and Bayesian analysis generated from 18S rDNA sequences. Three clusters were obtained for the *Myristica group - M. fragrans* and *M. malabarica* in one cluster, *M. andamanica, M. beddomei, M. amygdalina* and *M. prainii* in another cluster and the third cluster consisted of *Gymnocranthera canarica* and a *Myristica sp.* And *Knema andamanica,* which formed a distinct group separate from the others, pertaining to the sister relation with *Myristica* genus.

Tallei and Kolondam (2015) reported that *matK* locus was not suitable for differentiating the species in *Myristica* genus and that it could be used only to distinguish the different genus in Myristicaceae family.

Chapter 3

Materials and Methods

The present work was carried out at the Division of Crop Improvement and Biotechnology, Indian Council of Agricultural Research (ICAR) - Indian Institute of Spices Research (IISR), Kozhikode, Kerala, India during March 2012-2016.

3.1. Materials

3.1.1. Group I – Cinnamon

3.1.1.1. Authentication of traded cinnamon bark

3.1.1.1.1. Reference database

A reference database was created using the leaves of *C. verum* and its adulterant species *C. aromaticum* and *C. malabatrum* (Table 3) (Figure 3) for authentication of traded cinnamon samples.

Table 3. Details of *Cinnamomum* reference samples

Sl.No	Species	No. of Accessions	Place of collection
1.	*C. verum* Berchthold and Presl.	3	IISR Experimental Farm, Peruvannamuzhi, Chelavoor, Kozhikode, Kerala, India.
		2	IISR Experimental farm, Chelavoor Kozhikode, Kerala, India.
2.	*C. aromaticum* Nees.	5	IISR Experimental farm, Chelavoor Kozhikode, Kerala, India.
3.	*C. malabatrum* (Burm. F.) J. Presl.	4	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Tropical Botanical Garden and Research Institute, Thiruvananthapuram, Kerala, India.
		1	Nagercoil, Tamil Nadu, India.

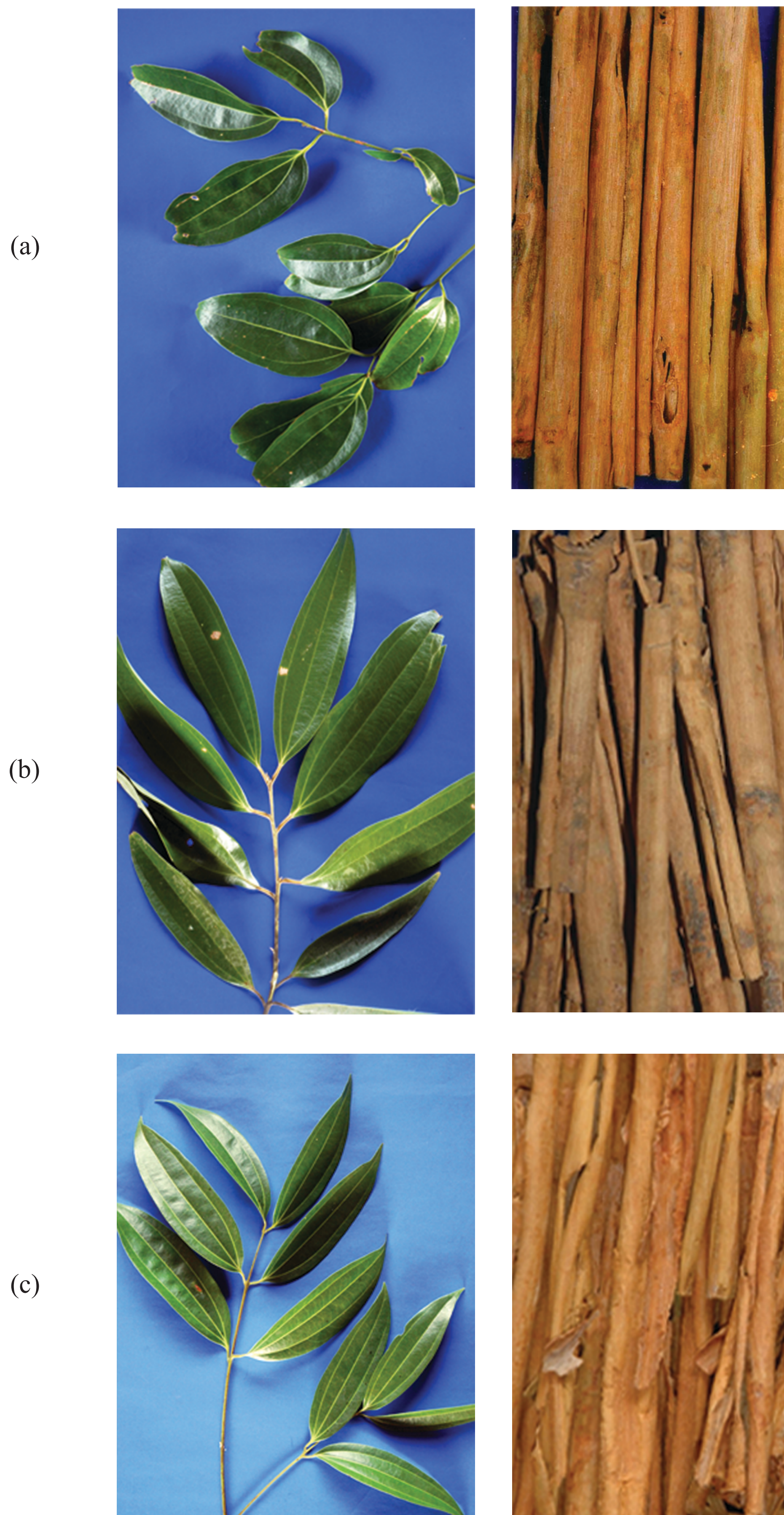


Figure 3. Representative pictures of *Cinnamomum* reference species.(a) branch and bark of *Cinnamomum verum* (b) branch and bark of *Cinnamomum aromaticum* (c) branch and bark of *Cinnamomum malabatrum*.

3.1.1.1.2. Market samples of traded cinnamon

Ten samples of traded cinnamon bark were procured from different shops in Kozhikode, Kerala, India (Figure 4).

Figure 4. Representative pictures of the traded cinnamon bark samples. MS1-MS10 Market samples of cinnamon bark

3.1.1.2. Phylogenetic studies

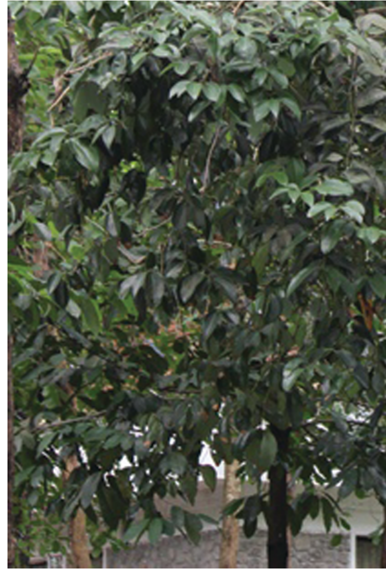
Fresh leaves were collected from 28 accessions representing 12 species of genus *Cinnamomum* from different locations in India (Table 4) (Figure 5)

Table 4. Details of *Cinnamomum* spp. used in phylogenetic studies

Sl.No	Species	No. of Accession	Place of collection
1.	*C. verum* Berchthold and Presl.	5	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
2.	*C. aromaticum* Nees.	5	IISR Experimental farm, Chelavoor, Kozhikode, Kerala, India.
3.	*C. malabatrum* (Burm. F.) J. Presl.	4 1 1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India. Tropical Botanical Garden and Research Institute, Thiruvananthapuram, Kerala, India. Nagercoil, Tamil Nadu, India.
4.	*C. citriodorum* Thwait.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
5.	*C. tamala* (Buch.-Ham) Nees & Eberm.	2 1	IISR Experimental Farm, Chelavoor, Kozhikode, Kerala, India. Nagaland, India.
6.	*C. glaucescens* Nees.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
7.	*C. sulphuratum* Nees.	1	Tropical Botanical Garden and Research Institute, Thiruvananthapuram, Kerala, India.
8.	*C. camphora* Berchthold and Presl.	1 1	IISR Experimental farm, Chelavoor Kozhikode, Kerala, India. Tropical Botanical Garden and Research Institute, Thiruvananthapuram, Kerala, India.
9.	*C. alexei* Kosterm.	1	Tropical Botanical Garden and Research Institute, Thiruvananthapuram, Kerala, India.

10.	*C. heyneanum* Nees.	1	Tropical Botanical Garden and Research Institute, Thiruvananthapuram, Kerala, India.
11.	*C. riparium* Gamble	1	Kozhikkanam, Idukki, Kerala, India.
12.	*C. travancoricum* Gamble	1	Kozhikkanam, Idukki, Kerala, India.

(a)


(b)

(c)

(a)

(b)

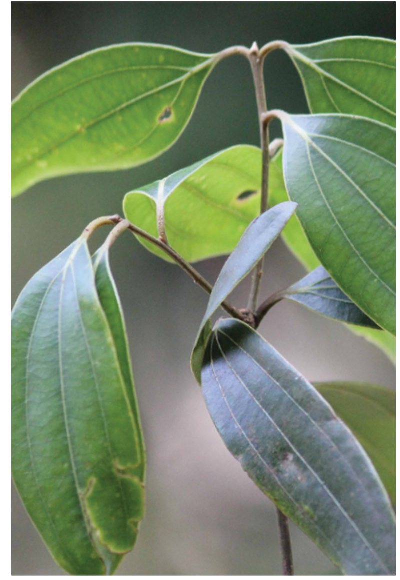
(c)

Figure 5A. *Cinnamomum verum*. (a) habitat (b) close up of tree (c) branch

Figure 5B. *Cinnamomum tamala*. (a) habitat (b) close up of tree (c) branch

(a)

(b)



(c)

(a)

(b)

(c)

Figure 5C. *Cinnamomum sulphuratum*. (a) habitat (b) close up of tree (c) branch

Figure 5D. *Cinnamomum malabatrum* (a) habitat (b) close up of tree (c) branch

(a)

(b)

(c)

(a)

(b)

(c)

Figure 5E. *Cinnamomum citriodorum*. (a) habitat (b) close up of tree (c) branch

Figure 5F. *Cinnamomum aromaticum* (a) habitat (b) close up of tree (c) branch

(a)

(b)

(c)

(a)

(b)

(c)

Figure 5G. *Cinnamomum camphora*. (a) habitat (b) close up of tree (c) branch.

Figure 5H. *Cinnamomum alexei*. (a) habitat (b) close up of tree (c) branch.

(a)

(b)

(c)

(a)

(b)

(c)

(a)

(b)

(c)

(a)

(b)

Figure 5K. *Cinnamomum glaucescens*. (a) habitat (b) close up of tree (c) branch
Source a and b : Wikipedia
Figure 5L. *Cinnamomum travancoricum*. (a) close up of tree (b) branch

3.1.2. Group II – Nutmeg.

3.1.2.1. Authentication of traded nutmeg mace

3.1.2.1.1. Reference data base

A reference database was created using the leaves of *M. fragrans* and its adulterant species *M. malabarica* listed in Table 5 (Figure 6) to authenticate traded nutmeg mace.

Table 5. Details of *Myristica* reference samples

Sl.No	Species	No. of Accession	Place of collection
1.	*M. fragrans* Houtt.	3	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Nagercoil, Tamil Nadu, India.
		1	Andaman and Nicobar Islands, India.
2.	*M. malabarica* Lam.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Community Agro Biodiversity Center, Wayanad, Kerala, India.
		1	Nagercoil, Tamil Nadu, India.
		1	Ernakulam, Kerala, India.
		1	Kerala Forest Research Institute, Thrissur, Kerala.

(a)

(b)

(c)

(d)

Figure 6. Representative pictures of *Myristica* reference species. (a) branch of *Myristica fragrans,* (b) mace of *M. fragrans*, (c) branch of *Myristica malabarica,* (d) mace of *M. malabarica*

3.1.2.2.2. Market samples of nutmeg mace

Five traded nutmeg mace samples were collected from different shops in Kozhikode, Kerala, India (Figure 7).

Figure 7. Representative pictures of traded nutmeg mace samples. MS1 - MS5 - traded nutmeg mace samples

3.1.2.2. Phylogenetic studies

Leaves were collected from 18 accessions representing 6 species of genus *Myristica,* 1 species each of the genus *Knema* and genus *Gymnocranthera* from different locations in India (Table 6) (Figure 8).

Table 6. Details of *Myristica, Knema* and *Gymnocranthera* spp. used in phylogenetic studies

Sl. No	Species	No. of Accession	Place of collection
1.	*M. fragrans* Houtt.	3	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Nagercoil, Tamil Nadu, India.
		1	Andaman and Nicobar Islands, India.
2.	*M. malabarica* Lam.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Community Agro Biodiversity Center, Wayanad, Kerala, India.
		1	Nagercoil, Tamil Nadu, India.
		1	Ernakulam, Kerala, India.
		1	Kerala Forest Research Institute, Thrissur, Kerala.
3.	*M. andamanica* Hook.f.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Andaman and Nicobar Islands, India.
4.	*M. fatua* Houtt. var. *magnifica* (Bedd.) Sinclair	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Kerala Forest Research Institute, Thrissur, Kerala, India.
5.	*M. amygdalina* (Wall.) Warb.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
6.	*M. beddomei* King	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
		1	Ernakulam, Kerala, India.

		1	Kerala Forest Research Institute, Thrissur, Kerala, India.
7.	*Knema attenuata* (Hook. f. & Th.) Warb.	1	Kerala Forest Research Institute, Thrissur, Kerala, India.
8.	*Gymnocranthera farquhariana* (Hook. f. & Th.) Warb.	1	Kerala Forest Research Institute, Thrissur, Kerala, India.

(a)

(b)

(c)

(a)

(b)

(c)

Figure 8A. *Myristica fragrans.* (a) habitat (b) close up of tree (c) branch

Figure 8B. *Myristica malabarica.* (a) habitat (b) close up of tree (c) branch

(a)

(b)

(c)

(a)

(b)

(c)

Figure 8C. *Myristica fatua*. (a) habitat (b) close up of tree (c) branch

Figure 8D. *Myristica andamanica*. (a) habitat (b) close up of tree (c) branch

(a)

(b)

(c)

Figure 8E. *Myristica beddomei*. (a) habitat (b) close up of tree (c) branch

(a)

(b)

(c)

(a)

(b)

(c)

Figure 8F. *Knema attenuata*. (a) habitat (b) close up of tree (c) branch
Source : India Biodiversity Portal

Figure 8G. *Gymnocranthera farquhariana*. (a) habitat (b) close up of tree (c) branch.
Source : India Biodiversity Portal

3.2. Methods

3.2.1. Group I –Cinnamon

3.2.1.1. Commodity Authentication

3.2.1.1.1. Genomic DNA Isolation

3.2.1.1.1.1. Isolation of DNA from leaves of *Cinnamomum* spp.

The collected leaves were cleaned by washing under running tap water followed by washes with distilled water. They were then wiped with ethanol and used for isolation.

High quality genomic DNA was isolated from the leaves of the reference *Cinnamomum* spp. viz., *C. verum, C. aromaticum* and *C. malabatrum* using Qiagen DNeasy kit (Qiagen, Germany) as per the manufacturer's protocol as given below:

i. 100 mg of the leaf tissue was ground using liquid nitrogen in a mortar and pestle.

ii. 400 µl of buffer AP1 and 4 µl RNase A were added, vortexed and incubated for 10 minutes at 65ºC. The tubes were inverted 2-3 times during incubation.

iii. 130 µl buffer P3 was added, mixed and incubated on ice for 5 minutes.

iv. The lysate was centrifuged for 5 minutes at 20,000 g.

v. The lysate was then pipetted into a QIA shredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at 20,000 g.

vi. The flow through was transferred into a new tube without disturbing the pellet, added 1.5 volumes of buffer AW1, and mixed by pipetting.

vii. 650 µl of the mixture was transferred into a DNeasy mini spin column placed in a 2 ml collection tube and centrifuged for 1 minute at > 6000 g.

viii. The flow through was discarded and the above step was repeated.

ix. The column was placed in a new 2 ml collection tube, added 500 µl buffer AW2 and centrifuged for 1 minute at > 6000 g.

x. 500 µl buffer AW2 was added again and centrifuged for 2 minutes at 20,000 g.

xi. The spin column was removed from the collection tube carefully to avoid its contact with the flow through and transferred to a new 2 ml microcentrifuge tube.

xii. 100 µl of buffer AE was added to elute the DNA. It was incubated for 5 minutes at room temperature (15-25°C) and centrifuged for 1 minute at >6000 g.

xiii. The above step was repeated.

3.2.1.1.1.2. Isolation of DNA from traded cinnamon barks

The list of reagents used for DNA isolation and their preparation are given in Annexure 1.

Genomic DNA was isolated from traded cinnamon bark samples using a modification of Asif and Cannon (2005) protocol.

i. The traded cinnamon barks procured were made into fine powder using Cyclotech 1083 sample mill.

ii. 1 gm of the powdered sample was homogenized in 10 ml of preheated extraction buffer (100 mM Tris base, 20 mM Ethylenediaminetetracetic acid (EDTA), 3 M sodium chloride (NaCl), 5% cetyl trimethyl ammonium bromide (CTAB), 0.3 % β-mercaptoethanol and 1% Polyvinyl pyrrolidone) and transferred to tubes.

iii. The tubes were incubated at 65°C in a water bath for 2 hours with intermittent shaking.

iv. The tubes were cooled to room temperature and an equal volume of chloroform: isoamylalcohol (24:1) was added.

v. The tubes were centrifuged at 1118 g for 15 minutes at 4°C and the aqueous phase obtained was transferred to another tube.

vi. One-third volume of 3 M sodium acetate (pH-5.2) and two third volume of chloroform : isoamylalcohol (24:1) were added to the tubes and centrifuged at 1118 g for 15 minutes at 4°C.

vii. The aqueous phase was taken in to a fresh tube and an equal volume of ice cold isopropanol was added.

viii. The tubes were kept for incubation at -40°C overnight and centrifuged at 4472 g for 20 minutes at 4°C.

ix. The pellet was washed with 70% ethanol, dried, dissolved in sterile nuclease free water and stored at -20°C.

3.2.1.1.2. Quantification and Quality Analysis of DNA.

3.2.1.1.2.1. Spectrometric Analysis

The concentration of the isolated DNA was assessed by measuring the absorbance of DNA at 260 nm and its quality was determined by the ratio of absorbances at 260 nm and 280 nm (A_{260}/A_{280})

in a Biophotometer (Eppendorf, Germany) by the following steps:

i. The lid with the optical path length of 0.2 mm was selected.

ii. The zero value was set by using the liquid used to dissolve the DNA as blank.

iii. 1 µl of the sample was added on to the inner side of the mirror of the 0.2 mm lid and the lid was placed on the Helma tray cell.

iv. The concentration and purity ratio was recorded.

v. The inner side of the lid and the Helma tray cell was cleaned with lint free litmus paper between and after measurements.

vi. The Helma tray cell was cleaned using distilled water before and after use.

3.2.1.1.2.2. Agarose gel electrophoresis

The quality and quantity of genomic DNA was also assessed by 0.8% agarose gel electrophoresis by comparing band intensity of the samples with that of a standard Human Genomic DNA (blood cells) (Genei, Bangalore). The reagents used in gel preparation are detailed in Annexure 1. Electrophoresis was done in a Maxi plus submarine unit SUB25 (Hoefer, USA) with a programmable Hoefer power supply PS300-B using the following protocol:

i. The gel casting trays and combs were wiped using 70% ethanol and they were placed in the casting unit.

ii. 0.8% agarose solution was prepared in 1X Tris Borate EDTA buffer (TBE) by boiling in a microwave oven until it dissolved completely.

iii. The gel solution was cooled to room temperature and ethidium bromide was added to it at a final concentration of 0.5 µg ml^{-1} and mixed.

iv. The gel solution was poured in to the gel casting tray and allowed to set.

v. The combs were removed and the gel trays were placed in the electrophoresis tank containing 1X TBE buffer.

vi. 5 µl of DNA was mixed with 2 µl of 1X gel loading dye and loaded in to wells along with Human genomic DNA.

vii. The samples were run at 100 V for 1 hour.

viii. The bands were visualized under ultra violet light (UV) and gels were photographed using Syngene gel documentation system.

3.2.1.1.3. PCR amplification of barcoding loci

Amplification of genomic DNA was done using the universal primers of barcoding loci viz. *rbcL, matK, psbA-trnH* and ITS synthesized by Integrated DNA Technologies (IDT) (USA) (Table 7). Two sets of primers were tested for *matK* and ITS loci.

Table 7. Primers used for PCR amplification

Primer	Sequence (5′- 3′)	Reference
rbcL af *rbcL* ar	5′ ATG TCA CCA CAA ACA GAG ACT AAA GC 3′ 5′ GTA AAA TCA AGT CCA CCG CG 3′	Kress & Erickson, 2007
matK360F *matK 1326R*	5′ CGA TCT ATT CAT TCA ATA TTC 3′ 5′ TCT AGC ACA CGA AAG TCG AAGT 3′	Cuénoud et al., 2002
matK 3F *matK* 1R	5′ CGT ACA GTA CTT TTG TGT TTA CGA G 3′ 5′ ACC CAG TCC ATC TGG AAA TCT TGG TTC 3′	Vijayan & Tsou, 2010
psbA f *trnH* R	5′ GTT ATG CAT GAA CGT AAT GCTC 3′ 5′ CGT AAC AAG GTT TCC GTA GGT GAA 3′	Yang et al., 2011
ITS F ITS R	5′ GCT GCG TTC TTC ATC GAT GC 3′ 5′ GCA TCG ATG AAG AAC GCA GC 3′	Vijayan & Tsou, 2010
ITS 4 ITS 5A	5′ TCC TCC GCT TAT TGA TAT GC 3′ 5′ CCT TAT CAT TTA GAG GAA GGA G 3′	Abeysinghe et al., 2009.

The lyophilised primer stocks were diluted 100 times to make the final concentration to 1 µM. Primers were further diluted 10 times to get 10 µM or 10 pmol μl^{-1} working concentration.

The optimum concentration of reaction mixture components like *Taq* buffer, dNTP, primers, *Taq* DNA polymerase and template DNA was standardized by keeping one component constant and varying the other components (Dhanya, 2009). The concentration range that was tried for the different PCR components are shown below:

a) dNTPs (mM) – 0.5 to 2

b) *Taq* polymerase (U) – 0.5 to 2

c) Primer (pmol $\mu l\text{-}^{1}$) – 5 to 20

d) Template (ng) – 10-50

The reaction mixture that produced amplicons devoid of any non specific products was selected for each primer pair. Optimisation of the temperature profile was carried out by setting a gradient PCR using the temperature range of 50°C - 60°C in the Agilent Sure cycler 8800 thermocycler for all loci. The amplicons were resolved in 1% agarose gel using the electrophoretic procedure described in section 3.2.1.1.2.2. Amplification success was evaluated by calculating the percentage of successfully amplified products.

3.2.1.1.4. PCR product purification

The amplified products were purified using QIA quick PCR Purification kit (Qiagen, Germany) following the manufacturer's protocol as described below:

i. 5 volumes of buffer PB was added to 1 volume of the PCR product and mixed. If the mixture turned orange or violet, 10 µl of 3 M sodium acetate (pH-5) was added and mixed till its colour became yellow.

ii. A QIA quick column was placed in a 2 ml collection tube provided.

iii. The sample was applied to the column to bind DNA and centrifuged at 17,900 g for 60 seconds. The flow through was discarded and the column was placed in to the same tube.

iv. 750 µl buffer PE was added to the QIA quick column to wash DNA. The flow through was discarded and the column was placed in to the same tube.

v. The QIA quick column was centrifuged once more at 17,900 g to remove the residual wash buffer and was placed in a clean 1.5 ml microcentrifuge tube.

vi. 50 µl buffer EB (10 mM Tris-Cl, pH-8.5) was added to the centre of the QIA quick membrane, the column kept for 1 minute at room temperature and then centrifuged at 17,900 g for 1 minute.

vii. The purified DNA was stored at -20°C.

3.2.1.1.5. PCR product sequencing

The purified products were sequenced at the DNA sequencing unit of SciGenome Labs Pvt Ltd (Cochin) Kerala by Sanger method. Amplicons were sequenced bidirectionally in ABI 3730XL DNA Analyzer (Applied Biosystems, USA) using the Bigdye terminator V.3 cycle sequencing kit with the same primers used for the loci amplification.

3.2.1.1.6. Data Analysis

The forward and reverse sequence reads were assembled using the Cap 3 software (Huang and Madan, 1999) to generate their consensus sequences and were queried against the GenBank nucleotide database of National Centre for Biotechnology Information (NCBI) to confirm the species identity using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). Sequencing success was determined by calculating the percentage of successfully assembled contigs (Olivar et al., 2014). Sequences obtained for the coding loci *rbcL* and *matK* were translated using the translate tool of ExPASY, a Swiss Institute of Bioinformatics (SIB) Resource Portal (Gasteiger et al., 2003) and their identity was checked by Protein BLAST tool. The nucleotide sequences generated were then deposited in the nucleotide database GenBank.

3.2.1.1.6.1. GenBank submission

Submission of sequences was done as described below:

Nucleotide sequences were submitted in GenBank through Bankit, a web based sequence submission tool (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank). Bankit could be accessed by registering and creating a login id and password by submitting the contact information of the submitter. A Bankit submission was completed after giving the following details:

- Names of the person submitting the sequence.

- Reference information – Title of the paper in which the sequences appear and the publishing status of the paper.
- Sequence information – Raw sequences/trimmed sequences, sequenced directly by submitter/ created by submitter, nucleotide sequence and its general information.
- Source information – Information regarding the sample from which the sequence was generated.
- Information regarding PCR primers.
- Feature information – Information regarding the part of the sequence that has a specific function like exon, intron, and non-coding region.

After submission of the above details a flat file displaying the information provided will be generated which will mark the end of the submission. A submission number was assigned initially and the GenBank accession number was provided after the GenBank team checked, clarified and confirmed the data submitted.

3.2.1.1.6.2. Ideal barcode identification

The potential of an ideal barcode was assessed by calculating the interspecific and intraspecific distances. These distances were computed using Kimura 2 parameter model (K2P) of MEGA 7 software (Kumar et al., 2016). The positions containing gaps and missing data were excluded from the analysis using pairwise deletion option as described by Wong et al., (2013).The intraspecific distances were assessed by two parameters:

- All intraspecific distance - mean of all intraspecific K2P distances between all samples collected within each species with more than one individual.
- Maximum intraspecific distance or coalescent depth - maximum intraspecific distance within each species with at least two individuals.

Similarly the interspecific distance was calculated using two parameters:

- All interspecific distance - mean of all interspecific K2P distances between all species in the genus with at least two species
- Minimum interspecific distance- minimum interspecific distance within each genus with at least two species.

3.2.1.1.6.3. Detection of polymorphic sites and NJ tree analysis

The sequences obtained for the individual loci were aligned using CLUSTAL W (Thompson et al., 1994) and trimmed using Bioedit (Hall, 1999). The trimmed sequences were analyzed for the detection of polymorphic sites using Mega 7 (Kumar et al., 2016). A neighbor joining tree was constructed using the Kimura 2 parameter (K2P) model of Mega 7 software with a bootstrap support of 1000 replicates to assess the confidence estimate for the topology of the tree. Positions containing gaps and missing data were removed from analysis by complete deletion option (Nithaniyal et al., 2014).

3.2.1.1.6.4. Validation of SNPs by cloning

The polymorphic sites found between the plant species were validated by cloning in a pTZ57R/T vector (Thermoscientific, USA).

The amplicons obtained were purified by gel extraction, ligated to vector and cloned as detailed below:

3.2.1.1.6.4.1. Gel extraction

The gel elution of the DNA fragments was done using QIA quick gel extraction kit following the manufacturer's protocol as detailed below:

i. The desired DNA fragment was excised from the agarose gel using a clean scalpel.

ii. The gel slice was weighed in a colourless tube and 3 volumes of buffer QG was added to 1 volume gel (100 µl buffer added to approximately 100 mg gel).

iii. The tubes were incubated at 50°C for 10-15 minutes with intermittent vortexing until the gel slice got completely dissolved.

iv. One volume of isopropanol was added to the samples and mixed.

v. A QIA quick column was placed in a 2 ml collection tube and the sample was applied to the column to bind DNA and centrifuged for 1 minute at 17,000 g.

vi. The flow through was discarded and the column was placed back in the same tube.

vii. 500 µl of buffer QG was added to the column and centrifuged at 17,000 g for 1 minute. Flow through was discarded and column placed back in the same tube.

viii. 750 µl buffer PE was added to wash the column and the column was allowed to stand for 2-3 minutes. It was then centrifuged for 1 minute at 17,000 g.

ix. The flow through was discarded and the column was placed in a clean 1.5 ml microcentrifuge tube.

x. 50 µl of EB was added to the centre of the QIA quick membrane to elute DNA. Column was allowed to stand for 1 minute and then centrifuged at 17,000 g for 1 minute.

3.2.1.1.6.4.2. Ligation of DNA to cloning vector

The eluted bands were ligated into cloning vector pTZ57R/T vector (Figure 9). The optimal insert to vector ratio was 3: 1.

Figure 9. pTZ57R/T vector system

The amount of PCR product required for efficient ligation with the vector was calculated (Table 8).

Table 8. Recommended amount of PCR product for ligation

Length of PCR product (bp)	Optimal PCR product quantity for ligation reaction (ng)
100 bp	17 ng
300 bp	51 ng
500 bp	86 ng
1000 bp	172 ng
2000 bp	343 ng
3000 bp	515 ng
4000 bp	686 ng
5000 bp	858 ng

The ligation reaction was set up in 0.2 ml tubes (Table 9).

Table 9. Ligation mixture composition

Sl.No.	Component	Volume
1	5 X ligation buffer	3 µl
2	PCR Product (0.52 pmol ends)	1 µl*
3	pTZ57R/T (0.17 pmol ends)	1.5 µl
4	T4 DNA ligase	0.5 µl
5	Nuclease free water	10 µl*
	Total volume	15 µl

* variable

The ligation mixture was vortexed briefly and incubated at room temperature (22°C) for one hour and kept for overnight incubation at 4°C. It was stored at -20°C until transformation.

3.2.1.1.6.4.3. Transformation

Bacterial transformation was done using calcium chloride transformation method as described below

i. 60 ml of LB broth was inoculated with 6 colonies of *E. coli* JM109 a day before transformation and was incubated at 37°C in a shaker for 4 to 6 hours.
ii. The bacterial cells were pelleted by centrifugation at 3593 g for 3 minutes.
iii. The cells were resuspended in 15 ml cold 0.1 M calcium chloride (anhydrous) and the tubes were incubated on ice for 45 minutes.
iv. The tubes were centrifuged at 3593 g for 3 minutes and cells pelleted again.
v. The cells were resuspended in 1.5 ml cold 0.1 M calcium chloride and 100 µl of the cell suspension was aliquotted into 0.5 ml microcentrifuge tubes.
vi. The ligated product and controls were added to the cell suspension and incubated on ice for 30 minutes.
vii. The mixture was given heat shock for 90 seconds at 42°C and the tubes were incubated on ice for 10 minutes.
viii. 350 µl LB broth was added to the tubes and incubated at 37°C for 40 minutes.
ix. The tubes were centrifuged at 6781 g for 1 minute and 900 µl of the supernatant was discarded.
x. The cells were resuspended in the remaining supernatant and it was then plated on pre-warmed LB-ampicillin-X-gal/IPTG agar plates and incubated overnight at 37°C.

3.2.1.1.6.4.4. Colony screening and selection

The bacterial colonies were selected by blue-white screening method. Grids were drawn and numbered on the bottom side of LB-amp-X-gal-IPTG plates. The light blue colored and white colonies were streaked in these grided regions using a sterile tooth pick. The plates were incubated overnight at 37°C.

3.2.1.1.6.4.5. Identification of positive clones by colony PCR.

Positive clones were identified by colony PCR (Gussow and Clackson, 1989) using M13 forward and reverse primers. A single colony was dissolved in 50 µl of sterile nuclease free water and mixed by spinning. 1 µl of this bacterial suspension was used as a template in colony PCR. The reaction mixture composition and temperature profile for colony PCR are given in Tables 10 and 11, respectively

Table 10. Reaction mixture composition for colony PCR

Component	Volume
10 X *Taq* buffer	1.25 µl
25 mM $MgCl_2$	0.75 µl
10 mM dNTP mix	0.75 µl
10 pmol $µl^{-1}$ M13 forward primer	0.5 µl
10 pmol $µl^{-1}$ M13 reverse primer	0.5 µl
Taq DNA polymerase (5U)	0.15 µl
Template DNA	1 µl
Nuclease free water	7.6 µl
Total volume	12.5 µl

Table 11. Temperature profile for colony PCR

Reaction condition	Temperature profile
Initial denaturation	95°C – 3 minutes
Denaturation	95°C – 30 seconds
Annealing	50°C – 1 minute
Extension	72°C – 1 minute
Final extension	72°C – 5 minutes
Number of cycles	30

The amplicons were electrophoresed on 1.5% agarose gels and documented using Syngene gel documentation system. The positive clones were identified based on their amplicon size and further characterized.

3.2.1.1.6.4.6. Isolation of plasmid DNA from the positive clones

A single bacterial colony from the selected positive clones was inoculated in 5 ml of LB broth supplemented with ampicillin (50 $µgml^{-1}$) and incubated overnight at 37°C in a shaker. Plasmids were isolated from the inoculated cultures using QIA prep spin mini prep kit (Qiagen, Germany) following the instructions given below:

i. 5 ml of overnight bacterial culture was pelleted by centrifugation at 6800 g for 3 minutes at room temperature.

ii. The pelleted cells were resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube.

iii. 250 µl buffer P2 was added and mixed thoroughly by inverting the tubes for 4-6 times until the solution became clear.

iv. 350 µl buffer N3 was then added and mixed thoroughly by inverting for 4-6 times and centrifuged for 10 minutes at 17,900 g.

v. The supernatant obtained from the above step was applied to the QIA prep spin column and centrifuged at 17,900 g for 1 minute and the flow-through was discarded.

vi. The column was washed with 500 µl buffer PB, centrifuged for 1 minute and the flow-through was discarded.

vii. The QIA prep spin columns were then washed by adding 750 µl buffer PE, centrifuged at 17,900 g for 1 minute and the flow-through was discarded.

viii. The column was transferred to a collection tube and centrifuged at 17,900 g for 1 minute to remove the residual wash buffer.

ix. A clean 1.5 ml microcentrifuge tube was taken and the column was placed in it. 50 µl of buffer EB was added to the center of the QIA prep spin column, kept for 1 minute and centrifuged at 17,900 g for 1 minute.

x. The eluted plasmid DNA was stored at -20°C.

3.2.1.1.6.4.7. Sequencing and analysis of plasmid DNA

The isolated plasmids were bidirectionally sequenced at SciGenome Pvt Labs, (Cochin) Kerala, using M13 forward and reverse primers. Forward and reverse reads were assembled in Cap 3 software (Huang and Madan, 1999). The vector sequences were identified and trimmed using VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) and Bioedit respectively. Sequence homology of the trimmed sequences was checked using nucleotide BLAST and SNP analysis was done using Mega 7.

3.2.1.2. Phylogenetic study

3.2.1.2.1. Isolation of DNA

Genomic DNA was isolated from all the *Cinnamomum* species given in Table 3 using Qiagen DNeasy kit (Qiagen, Germany) as detailed in section 3.2.1.1.1.1. The qualitative and quantitative estimation of the isolated DNA was done as in section 3.2.1.1.2

3.2.1.2.2. PCR amplification

Amplification of genomic DNA was carried out using the universal primers of barcoding loci viz. *rbcL, matK, psbA-trnH* and ITS synthesized by Integrated DNA Technologies (IDT) (USA) (Table 5). The reaction mixture composition of all loci was optimized as described in section 3.2.1.1.3.

3.2.1.2.3. PCR product purification and sequencing

The amplified products were purified using QIA quick PCR Purification kit (Qiagen, Germany) following the manufacturer's protocol as described in section 3.2.1.1.4. The purified products were sequenced at the DNA sequencing unit of SciGenome Labs Pvt Ltd (Cochin) Kerala by Sanger method. Amplicons were sequenced bidirectionally in ABI 3730XL DNA Analyzer (Applied Biosystems, USA) using the Bigdye terminator V.3 cycle sequencing kit with the same primers used for the loci amplification.

3.2.1.2.4. Data Analysis and submission of sequences in GenBank

The forward and reverse sequence reads were assembled using the Cap 3 software (Huang and Madan, 1999) to generate their consensus sequences and were queried against the GenBank nucleotide database of National Centre for Biotechnology Information (NCBI) to confirm the species identity using the Basic Local Algorithm Search Tool (BLAST) (Altschul et al., 1997). Sequences obtained for the coding loci *rbcL* and *matK* were translated using the translate tool of ExPASY, a Swiss Institute of Bioinformatics (SIB) Resource Portal (Gasteiger et al., 2003) and their identity was checked by Protein BLAST tool. The nucleotide sequences generated were then deposited in the nucleotide database GenBank as detailed in section 3.2.1.1.6.1.

3.2.1.2.5. Phylogenetic analysis

In addition to the amplified *Cinnamomum* sequences, sequences that gave an e-value of zero on BLAST analysis for each locus were also included in the sequence file for phylogenetic analysis. *Phoebe zhennan,* a member of the *Persea* group was selected as the outgroup for the phylogenetic analysis based on the previous study in *Cinnamomum* species (Huang et al., 2016). Single locus (*rbcL, matK* and *psbA-trnH)* and multi locus barcode (*rbcL* + *matK*, *rbcL* + *trnH-psbA*, *matK* + *trnH-psbA* and *rbcL* + *matK* + *trnH-psbA*) approaches were tried for phylogenetic studies. The sequences were aligned using MUSCLE algorithm (Edger, 2004) and manually trimmed using BioEdit software (Hall, 1999). Multilocus barcodes were created for those loci that generated sequences for all loci by concatenating the sequence alignment files into one with FASconCATv1.0 tool (Kück and Longo, 2014*).*

The evolutionary history was inferred using the Bayesian analysis. Bayesian analysis was performed in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) with two searches run simultaneously for at least two million generations. Flat Dirichlet priors were used for the gamma shape parameter and the proportion of invariable sites. Three heated chains (temperature 0.2) and one cold chain were used in each search. The parameter was then fixed for a bootstrap analysis with 10,000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated.

3.2.2. Group II- Nutmeg

3.2.2.1. Commodity Authentication

3.2.2.1.1. Genomic DNA Isolation

3.2.2.1.1.1. Isolation of genomic DNA from reference samples of *Myristica* spp.

Fresh leaves of the reference *Myristica* spp. viz. *M. fragrans* and *M. malabarica* were cleaned as mentioned in section 3.2.1.1.1.1. and genomic DNA was isolated using the protocol of Sheeja et al., (2008) as detailed below:-

i. The extraction buffer (100 mM Tris, 20 mM EDTA, 1.5 M NaCl and 3% CTAB) was preheated at 65°C in a waterbath and 0.3% β-mercaptoethanol was added to it.
ii. Two grams of the leaf sample was ground using 15 ml of the extraction buffer and transferred to a tube containing 1.5% PVP.
iii. The tubes were kept for incubation at 65°C for 1.5 hours.

iv. After the tubes were brought to room temperature, equal volume of chloroform: isoamyl alcohol (24:1) was added to it, mixed by inversion and kept at room temperature for 10 minutes.

v. The tubes were centrifuged at 12,000 g for 10 minutes at 4°C.

vi. The aqueous phase was transferred to another tube, added 2/3rd volume of ice cold isopropanol, mixed by inversion and kept for overnight incubation at -20°C.

vii. The tubes were centrifuged at 12,000 g for 15 minutes at 4°C.

viii. The supernatant was discarded and the pellet obtained was washed using 70% ethanol, dried and dissolved in sterile distilled water.

ix. Ten μgml^{-1} RNase was added to the tubes and kept for incubation at 37°C for one hour.

x. Equal volume of Tris saturated phenol:chloroform:isoamylalcohol (25:24:1) was added to the above tubes, mixed by inversion and incubated at room temperature for 10 minutes.

xi. The tubes were centrifuged at 12,000g for 15 minutes at 4°C and the supernatant was transferred to a fresh tube, and equal volume of chloroform: isoamylalcohol (24:1) was added.

xii. The tubes were then centrifuged at 12,000g for 15 minutes at 4°C.

xiii. The aqueous phase obtained on chloroform:isoamylalcohol (24:1) extraction was taken in fresh tubes.

xiv. One-tenth volume of sodium acetate (3 M, pH-5.2) and ice cold isopropanol was added to the aqueous phase and the tubes were kept for incubation at -20°C for 1 hour.

xv. The tubes were centrifuged at 12,000 g for 15 minutes at 4°C.

xvi. The pelleted DNA was washed using 70% ethanol, air dried and dissolved in 100 µl of sterile distilled water and reprecipitated using 100% ethanol.

xvii. The tubes were centrifuged at 12,000 g for 15 minutes at 4°C and the pellet obtained was washed using 70% ethanol.

xviii. The pellet was air dried and dissolved in sterile nuclease free water. It was stored at -20°C until use.

3.2.2.1.1.2. Isolation of DNA from traded nutmeg mace.

Genomic DNA was isolated from traded nutmeg mace samples using the modified protocol of Dhanya et al., (2007) as described below:

i. The nutmeg mace was powdered using Cyclotech 1083 sample mill and soaked in distilled water overnight.The water was poured off and the mace samples were sundried.

ii. Two grams of the sample was homogenised using 15 ml of preheated extraction buffer (100 mM Tris, 20 mM EDTA, 2 M NaCl, 2% SDS, 2% PVP and 1% β-mercaptoethanol) in a prechilled mortar and pestle and transferred to oakridge tubes.

iii. The tubes were incubated at 65°C in a water bath with intermittent shaking for 1.5 hours.

iv. The tubes were plunged in ice and brought to room temperature. One-third volume of 6 M potassium acetate solution was added and the tubes were incubated in ice for 1 hour.

v. An equal volume of chloroform:isoamylalcohol (24:1) was added and the tubes were centrifuged at 10,000 g for 15 minutes at 4°C.

vi. The aqueous phase was taken in new tubes and chloroform:isoamylalcohol extraction was repeated.The aqueous phase was then transferred to fresh tubes, an equal volume of 30% PEG 8000 was added and incubated in ice for 1 hour.

vii. The tubes were centrifuged at 12,000 g for 20 minutes at 4ºC.

viii. The pellet obtained was washed with 70% ethanol, air dried, dissolved in sterile nuclease free water and stored at -20°C until use.

3.2.2.1.2. Quantification and quality analysis of DNA.

Qualitative and quantitative estimation of isolated DNA was done as mentioned in section 3.2.1.1.2.

3.2.2.1.3. PCR amplification of barcoding loci

Amplification of genomic DNA was carried out using the universal primers of barcoding loci viz. *rbcL, matK, psbA-trnH* and ITS synthesized by Integrated DNA Technologies (IDT) (USA) (Table 3). The reaction mixture composition of all loci was optimized as described in section 3.2.1.1.3. The temperature profile was optimized for all loci except *matK* by putting a gradient PCR as detailed in 3.2.1.1.3. *matK* was amplified using the following condition : 95°C for 2.5 minutes; 95°C for 30 seconds; 56°C for 1 minute; 72°C for 1 minute; 88°C for 30 seconds; 56°C for 1 minute; 72°C for 1 minute and 72°C for 10 minutes (Stoeckle et al., 2011).

3.2.2.1.4. PCR product purification

The amplified products were purified using QIA quick PCR Purification kit (Qiagen, Germany) following the manufacturer's protocol as described in section 3.2.1.1.4.

3.2.2.1.5. PCR Product sequencing

The purified products were sequenced at the DNA sequencing unit of SciGenome Labs Pvt. Ltd (Cochin), Kerala by Sanger method. Amplicons were sequenced bidirectionally in ABI 3730XL DNA Analyzer (Applied Biosystems, USA) using the Bigdye terminator V.3 cycle sequencing kit with the same primers used for the loci amplification.

3.2.2.1.6. Data Analysis

Data analysis was carried out as described in section 3.2.1.1.6.

3.2.2.2. Phylogenetic study

3.2.2.2.1. Isolation of DNA

Genomic DNA was isolated from all the *Myristica* species given in Table 6 using the protocol of Sheeja et al., (2008) as detailed in section 3.2.2.1.1.1. The qualitative and quantitative estimation of the isolated DNA was done as in section 3.2.1.1.2.

3.2.2.2.2. PCR amplification

Amplification of genomic DNA was carried out using the universal primers of barcoding loci viz. *rbcL, matK, psbA-trnH* and ITS synthesized by Integrated DNA Technologies (IDT) (USA) (Table 3). The reaction mixture composition of all loci was optimized as described in section 3.2.1.1.3.

3.2.2.2.3. PCR product purification and sequencing

The amplified products were purified using QIA quick PCR Purification kit (Qiagen, Germany) following the manufacturer's protocol as described in section 3.2.1.1.4. The purified products were sequenced at the DNA sequencing unit of SciGenome Labs Pvt Ltd (Cochin) Kerala by Sanger method. Amplicons were sequenced bidirectionally in ABI 3730XL DNA Analyzer (Applied Biosystems, USA) using the Bigdye terminator V.3 cycle sequencing kit with the same primers used for the loci amplification.

3.2.2.2.4. Data Analysis and submission of sequences in GenBank

The forward and reverse sequence reads were assembled using the Cap 3 software (Huang and Madan, 1999) to generate their consensus sequences and were queried against the GenBank nucleotide database of National Centre for Biotechnology Information (NCBI) to confirm the species identity using the Basic Local Algorithm Search Tool (BLAST) (Altschul et al., 1997). Sequences obtained for the coding loci *rbcL* and *matK* were translated using the translate tool of ExPASY, a Swiss Institute of Bioinformatics (SIB) Resource Portal (Gasteiger et al., 2003) and their identity was checked by Protein BLAST tool. The nucleotide sequences generated were then deposited in the nucleotide database GenBank as detailed in section 3.2.1.1.6.1.

3.2.2.2.5. Phylogenetic analysis

In addition to the amplified *Myristica* sequences, sequences that gave an e-value of zero on BLAST analysis for each locus were also included in the sequence file for phylogenetic analysis. *Magnolia acuminata* was selected as the outgroup for the phylogenetic analysis based on a previous study in *Myristica* species (Sheeja et al., 2014). Single locus (*rbcL, matK* and *psbA-trnH)* and multi locus barcode (*rbcL* + *matK*, *rbcL* + *trnH-psbA*, *matK* + *trnH-psbA* and *rbcL* + *matK* + *trnH-psbA*) approaches were tried for phylogenetic studies. The sequences were aligned using MUSCLE algorithm (Edger, 2004) and manually trimmed using BioEdit software (Hall, 1999). Multilocus barcodes were created for those loci that generated sequences for all loci by concatenating the sequence alignment files into one with FASconCATv1.0 tool (Kück and Longo, 2014*).*

The evolutionary history was inferred using the Bayesian analysis, maximum likelihood and maximum parsimony methods. Bayesian analysis was performed in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) with two searches run simultaneously for at least two

million generations. Flat Dirichlet priors were used for the gamma shape parameter and the proportion of invariable sites. Three heated chains (temperature 0.2) and one cold chain were used in each search. The parameter was then fixed for a bootstrap analysis with 10,000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated.

Chapter 4

Results

4.1. Commodity Authentication

4.1.1. Group I - Cinnamon

4.1.1.1. Isolation of genomic DNA from leaves of *Cinnamomum* species

Genomic DNA was isolated from the leaves of the different accessions of the 3 reference *Cinnamomum* species viz. *C. verum, C. aromaticum* and *C. malabatrum* using Qiagen DNeasy kit. The yield of DNA isolated from the different *Cinnamomum* species was in the range of 132-145 $\mu g g^{-1}$. An absorbance ratio (A260/A280) in the range of 1.8-1.9 and conspicuous bands on 0.8% agarose gel were obtained indicating the high quality of genomic DNA free from protein, polysaccharide and RNA contamination (Figures 10a-c).

4.1.1.2. Isolation of genomic DNA from traded cinnamon barks

Isolation of genomic DNA from barks of cinnamon was attempted using Qiagen DNeasy kit, Doyle and Doyle method (1990), protocols of DNA isolation from other recalcitrant tissues like rhizomes of ginger and turmeric (Syamkumar et al., 2003), dried capsules of cardamom (Syamkumar et al., 2005), processed wood of *Gonystylus bacanus* (Asif and Cannon, 2005), dried berries of black pepper (Dhanya et al., 2007), and stem bark of leguminosae trees (Novaes et al., 2009). None of these protocols could yield DNA from cinnamon barks. Modifications of all these protocols were tried out by changing the buffer composition. But among these even then only the modifications introduced in the Asif and Canon (2005) method could yield high quality genomic DNA.

Asif and Cannon protocol was modified by introducing the following changes: The solid to liquid ratio taken for DNA isolation was 1: 10 (10 ml extraction buffer for 1g powdered cinnamon bark). CTAB concentration in the DNA extraction buffer was increased from 3% to 5% and sodium chloride concentration was changed from 1.4 M to 3 M. Sodium acetate (3 M, pH-5.2) was added during chloroform – isoamyl alcohol extraction for the efficient removal of polysaccharides and proteins. Isopropanol was used for precipitating DNA and overnight incubation was done at -40°C in order to increase the yield of genomic DNA.

The yield of DNA obtained was in the range of 150-350 $\mu g g^{-1}$. An absorbance ratio (A260/A280) of 1.8-1.9 was obtained for the DNA isolated from all the traded samples indicating its purity. Conspicuous bands were also obtained for the traded bark samples on 0.8% agarose gel that further attested the quality of the genomic DNA (Figure 11).

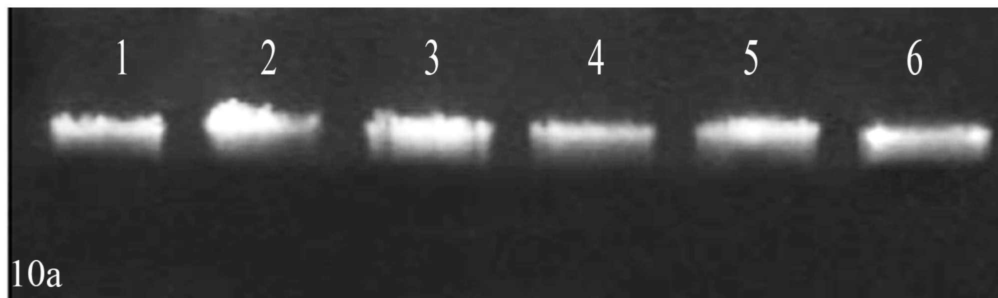
4.1.1.3. PCR amplification of barcoding loci

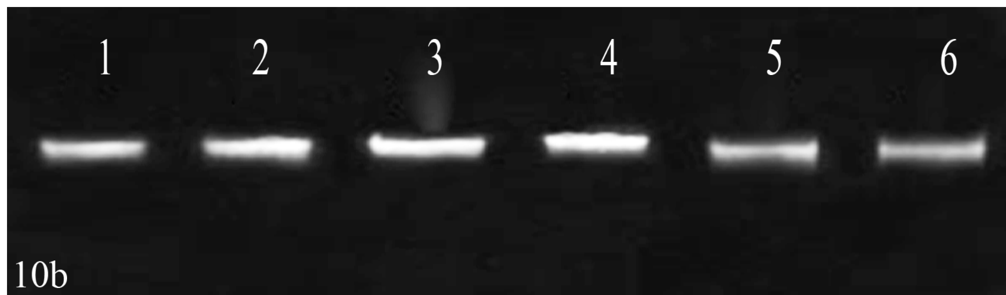
The composition of the PCR reaction mixture was optimised as 1 mM *Taq* assay buffer with 1.5 mM $MgCl_2$, 1 pmole μl^{-1} of forward and reverse primers, 1 U *Taq* DNA polymerase and 50 ng of genomic DNA in a reaction volume of 50 µl to yield specific PCR products. The temperature profile for the barcoding loci was optimised by putting gradient PCR between 50°C and 60°C. Two sets of primers were employed for *matK* and ITS locus out of which only *matK* 3F, 1R and ITS 4, 5A generated amplicons. The optimised reaction condition for each locus is given in Table 12.

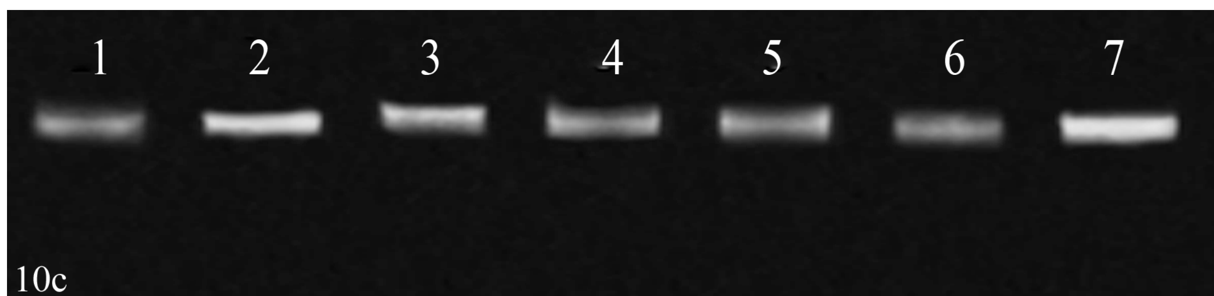
Table 12. Temperature profile for amplification of barcoding loci in *Cinnamomum* spp.

Reaction condition	Locus			
	rbcL	*psbA-trnH*	*matK*	ITS
Initial denaturation	95°C - 4 min	92°C - 1 min	94°C - 1 min	95°C - 4 min
Denaturation	94°C - 30 sec	94°C - 1 min	94°C - 30 sec	95°C - 1 min
Annealing	55°C - 1 min	52°C - 1 min	52°C - 20 sec	52.9°C - 1 min
Extension	72°C - 1 min	64°C - 1 min	72°C - 50 sec	68°C - 2 min
Final extension	72°C - 10 min	64°C - 8 min	72°C - 5 min	68°C - 15 min
Total cycles	35	35	35	35

All the barcoding loci exhibited 100% amplification for the *Cinnamomum* species. But only *rbcL* and *psbA-trnH* genes could be amplified from the traded bark samples. *rbcL, matK, psbA-trnH* and ITS generated amplicons of size 600bp (Figures 12a-b and 13), 900bp (Figures 14a-b), 550bp (Figures 15a-b and 16) and 750bp (Figures 17a-b), respectively in the samples. The inability of *matK* and ITS to amplify the traded samples resulted in their exclusion from further analysis.







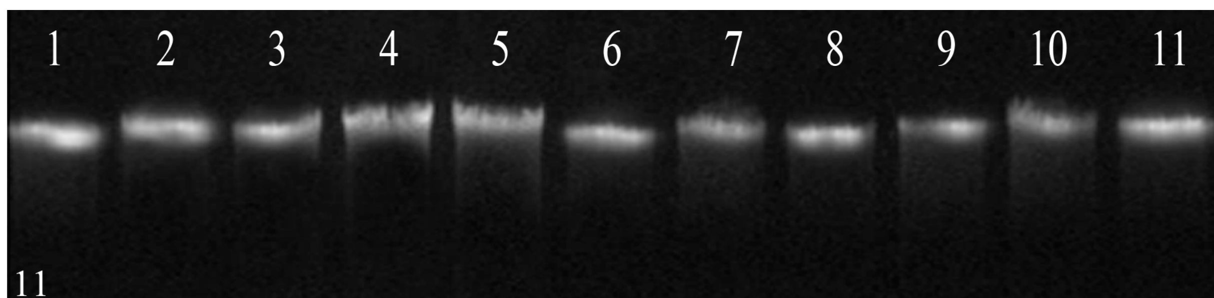
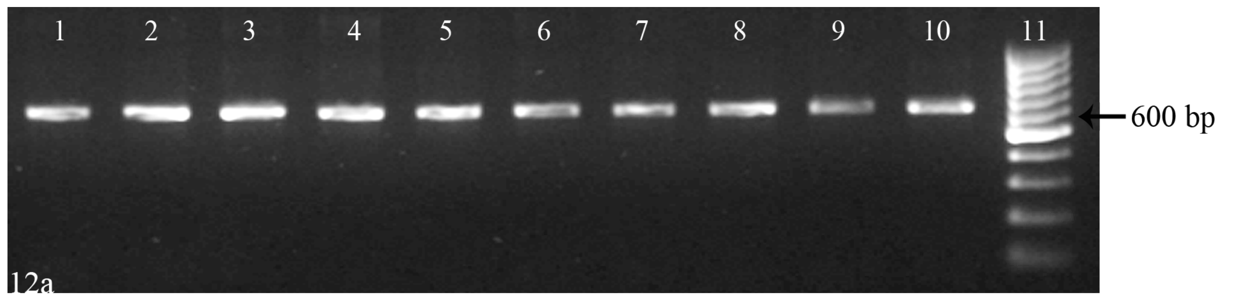
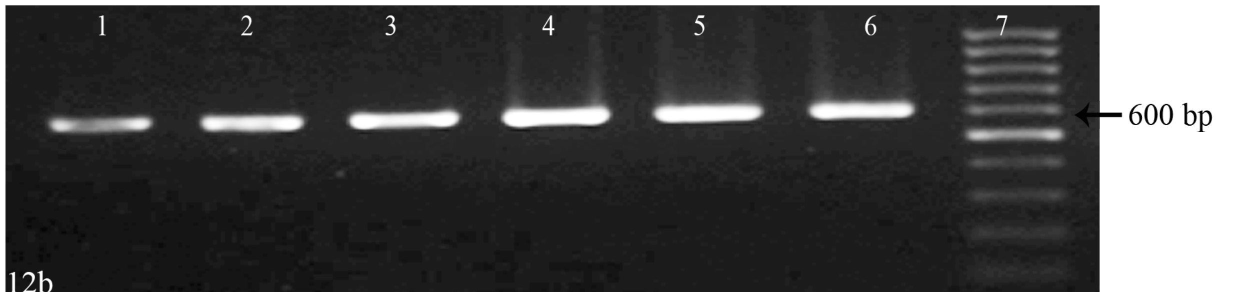
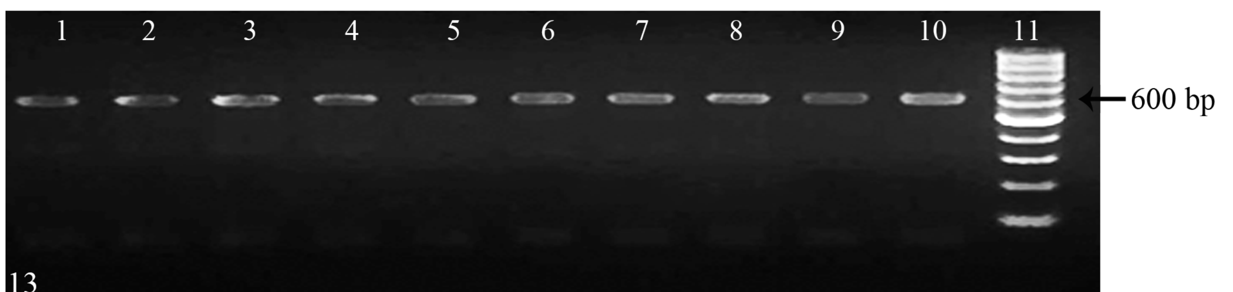
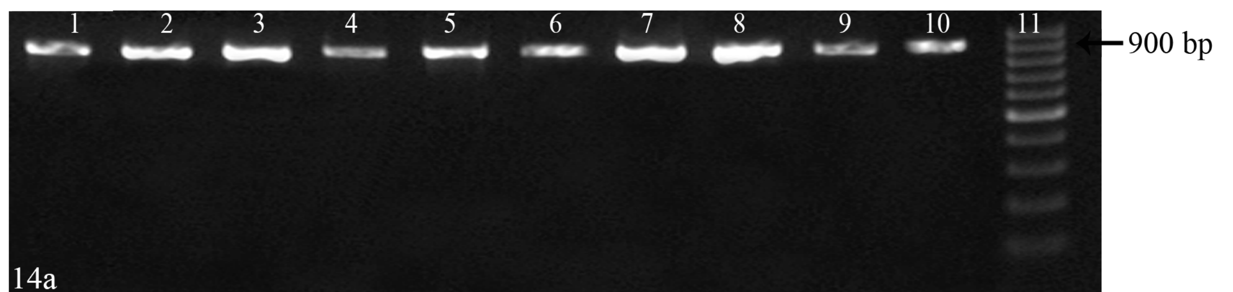


Figure 10a. DNA isolated from *C. verum* accessions (reference species). Lanes 1-5- *C. verum* accessions, lane 6- Human Genomic DNA (blood cells) (Genei).
Figure 10b. DNA isolated from *C. aromaticum* accessions (reference species).. Lanes 1-5 – *C. aromaticum* accessions, lane 6- Human Genomic DNA (blood cells) (Genei).
Figure 10c. DNA isolated from *C. malabatrum* accessions (reference species).. Lanes 1-6 – *C. malabatrum*, lane 7 – Human genomic DNA (blood cells) (Genei).
Figure 11. DNA isolated from traded cinnamon bark samples. Lanes 1-10 – traded cinnamon bark samples, lane 11- Human Genomic DNA (blood cells) (Genei).









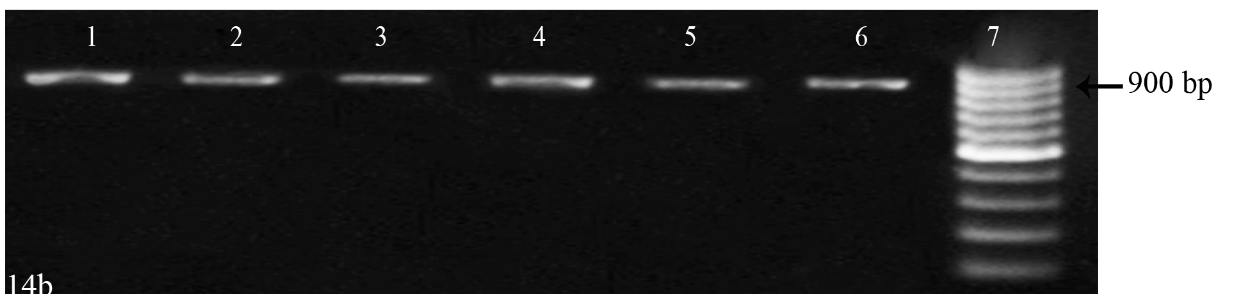


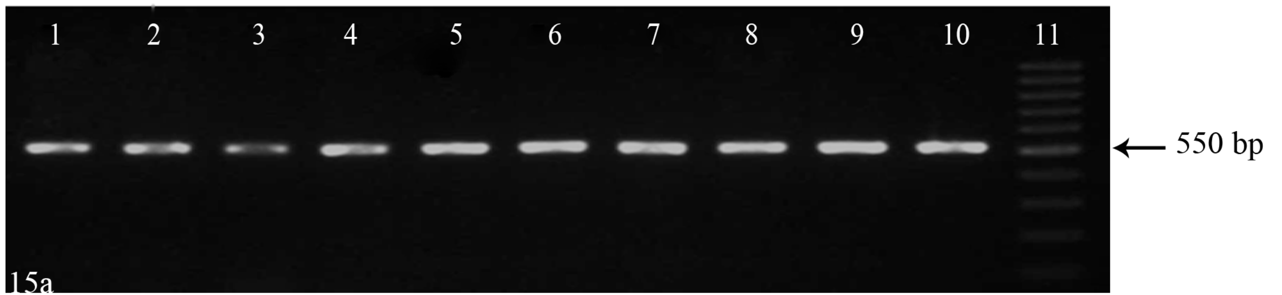
Figure 12a. Amplification of *rbcL* locus in *Cinnamomum* reference species. Lanes 1-5-*C. verum* accessions, lanes 6-10- *C. aromaticum* accessions, lane 11- 100bp ladder (Fermentas).

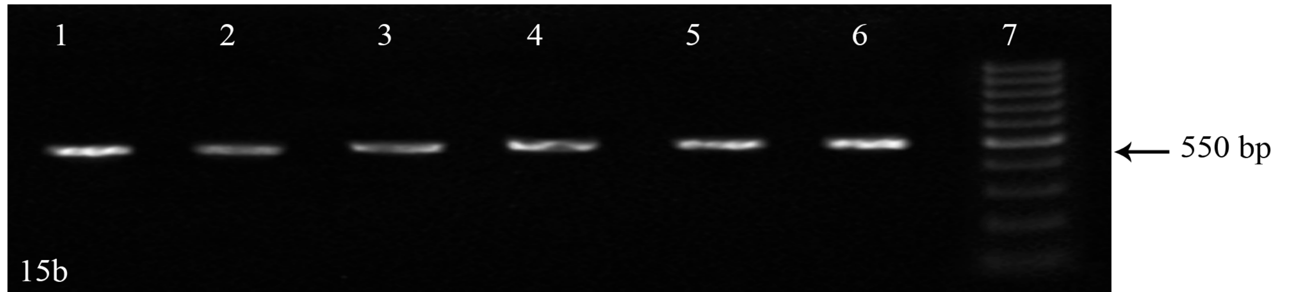
Figure 12b. Amplification of *rbcL* locus in *Cinnamomum* reference species. Lanes 1-6- *C. malabatrum* accessions, lane 7 – 100 bp ladder (Fermentas).

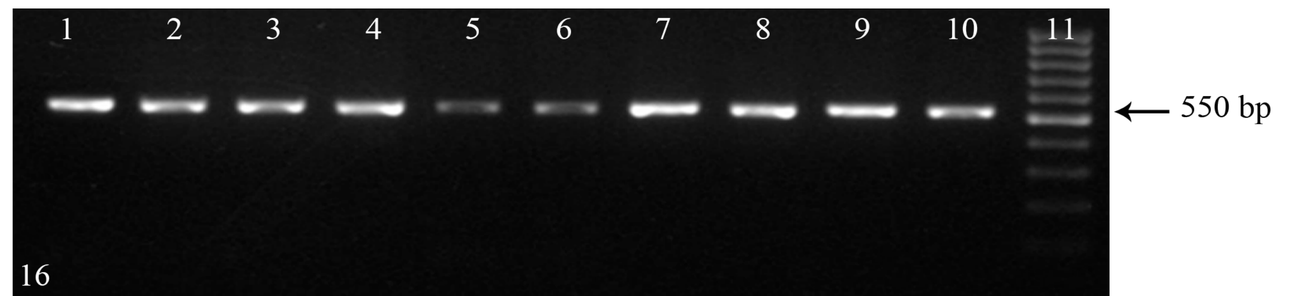
Figure 13. Amplification of *rbcL* locus in traded cinnamon bark samples. Lanes 1-10 – Traded cinnamon bark samples, lane 11- 100 bp ladder (Fermentas).

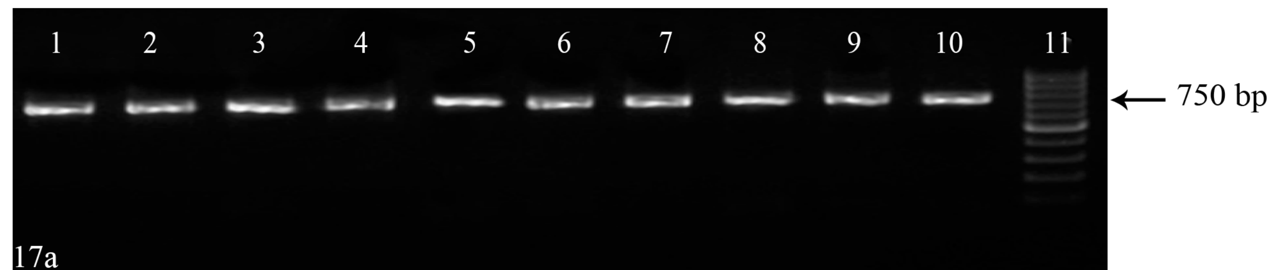
Figure 14a. Amplification of *matK* locus in *Cinnamomum* reference species. Lanes 1-5- *C. verum* accessions, lanes 6-10- *C. aromaticum* accessions, lane 11- 100 bp ladder (Fermentas).

Figure 14b. Amplification of *matK* locus in *Cinnamomum* reference species. Lanes 1-6 - *C. malabatrum* accessions, lane 7 – 100 bp ladder (Fermentas).









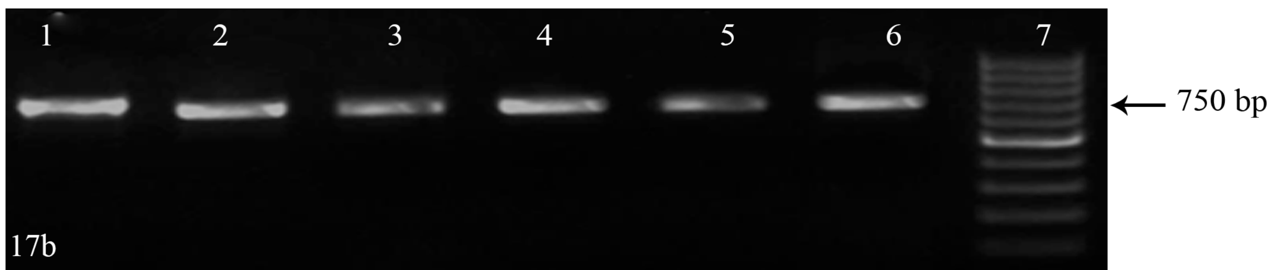


Figure 15a. Amplification of *psbA-trnH* locus in *Cinnamomum* reference species. Lanes 1-5- *C. verum* accessions, lanes 6-10- C. aromaticum accessions, lane 11- 100 bp ladder (Fermentas).

Figure 15b. Amplification of *psbA-trnH* locus in *Cinnamomum* reference species. Lanes 1-6 - *C. malabatrum* accessions, lane 7 – 100 bp ladder (Fermentas).

Figure 16. Amplification of *psbA-trnH* locus in traded cinnamon bark samples. Lanes 1-10 – Traded cinnamon bark samples, lane 11- 100 bp ladder (Fermentas).

Figure 17a. Amplification of ITS locus in *Cinnamomum* reference species. Lanes 1-5- *C. verum* accessions, lanes 6-10- C. aromaticum accessions, lane 11- 100 bp ladder (Fermentas).

Figure 17b. Amplification of ITS locus in *Cinnamomum* reference species. Lanes 1-6 - *C. malabatrum* accessions, lane 7 – 100 bp ladder (Fermentas).

4.1.1.4. Sequencing of PCR products and Data Analysis

Sequencing success of 100% was obtained for *rbcL* and *psbA-trnH* amplicons. The forward and reverse reads of sequences were assembled to generate contigs. The length of sequences was in the range of 510 bp to 579 bp and 402 bp to 501 bp for *rbcL* and *psbA-trnH*, respectively. BLAST analysis of the contig sequences obtained for *rbcL* and *psbA-trnH* exhibited maximum similarity to sequences of the respective locus of *Cinnamomum* species (Tables 13 and 14). *rbcL* sequences were translated to protein sequences and they showed 100% identity to ribulose bisphosphate sequences of the genus *Cinnamomum*.

Table 13. BLAST analysis of *rbcL* sequences of *Cinnamomum* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E-value
C. verum 1	*Cinnamomum* spp	AB936048	100	100	0
C. verum 2	*Cinnamomum* spp	AB936048	100	100	0
C. verum 3	*Cinnamomum* spp	AB936048	100	100	0
C. verum 4	*C. pauciflorum*	KP095065	100	100	0
C. verum 5	*Cinnamomum* spp	AB936048	100	100	0
C. aromaticum 1	*C. camphora*	GU135257	99	99	0
C. aromaticum 2	*C. aromaticum*	KP094936	99	100	0
C. aromaticum 3	*C. camphora*	GU135257	99	99	0
C. aromaticum 4	*C. camphora*	GU135257	99	99	0
C. aromaticum 5	*C. aromaticum*	KP094936	99	100	0
C. malabatrum 1	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 2	*C. baileyanum*	KU564775	100	100	0
C. malabatrum 3	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 4	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 5	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 6	*Cinnamomum* spp	AB936048	100	100	0

Table 14. BLAST analysis of *psbA-trnH* sequences of *Cinnamomum* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E-value
C. verum 1	*C. verum*	KX509887	100	99	0
C. verum 2	*C. insularimontanum*	AF268782	100	97	0
C. verum 3	*C. verum*	KX509887	100	99	0
C. verum 4	*C. bejolghota*	GQ248266	100	99	0
C. verum 5	*C. bejolghota*	KU160275	97	99	0
C. verum 6	*C. verum*	KU160291	100	99	1e-148
C. aromaticum 1	*C. aromaticum*	KU160278	97	100	0
C. aromaticum 2	*C. aromaticum*	KU160278	98	100	0
C. aromaticum 3	*C. aromaticum*	KU160278	97	100	0
C. aromaticum 4	*C. aromaticum*	KU160278	98	100	0
C. aromaticum 5	*C. bejolghota*	KU160275	99	98	0
C. malabatrum 1	*C. verum*	KX509887	100	99	0
C. malabatrum 2	*C. verum*	KX509887	100	99	0
C. malabatrum 3	*C. verum*	KX509887	100	99	0
C. malabatrum 4	*Cinnamomum* spp	JN988467	99	100	1e-153
C. malabatrum 5	*Cinnamomum* spp	JN988467	99	99	1e-152
C. malabatrum 6	*Cinnamomum* spp	JN988467	100	97	0

4.1.1.5. Construction of reference library

A reference library was constructed by depositing the *rbcL* and *psbA-trnH* sequences obtained for *Cinnamomum* species in the GenBank database of National Centre for Biotechnology Information (NCBI) (Table 15).

Table 15. GenBank Accession numbers for *Cinnamomum* reference species

Species	Accession Number	
	rbcL	*psbA-trnH*
C. verum 1	KF744226	KF978091
C. verum 2	KF744227	MG209137
C. verum 3	KF744230	KF978095
C. verum 4	KF744228	KF978093
C. verum 5	KF744229	KF978092
C. aromaticum 1	KF878109	KF978096
C. aromaticum 2	KF878110	KF978097
C. aromaticum 3	KF878111	KF978098
C. aromaticum 4	KF878112	KF978099
C. aromaticum 5	KF878113	MG209138
C. malabatrum 1	KY945242	KY966336
C. malabatrum 2	KY945243	KY966337
C. malabatrum 3	KY945244	KY966338
C. malabatrum 4	KY945245	KY966339
C. malabatrum 5	KY945246	KY966340
C. malabatrum 6	KY945247	KY966341

4.1.1.6. Identification of ideal barcode locus.

A barcode locus is said to be ideal if it is easy to amplify, sequence, have high interspecific divergence and possess sufficient variation to distinguish between closely related species.

4.1.1.6.1. Analysis of intraspecific and interspecific divergence

rbcL and *psbA-trnH* were assessed for their barcoding potential by calculating the interspecific divergence. A favourable barcode should possess higher interspecific distances than the intraspecific distances. The interspecific and intraspecific divergences were assessed by calculating two parameters each for them. The intraspecific divergence was assessed by two parameters- all intraspecific distance (mean of all intraspecific K2P

distances between all samples collected within each species with more than one representative) and maximum intraspecific distance or coalescent depth (maximum intraspecific distance within each species). Two parameters viz., all interspecific distance (mean of all K2P distances between all species in the genus with atleast two species) and minimum interspecific distance (minimum interspecific distance within each genus with at least two species) was calculated to determine the interspecific divergence. The distance values for *rbcL* and *psbA-trnH* loci are given in Table 16.

Table 16. Average intraspecific and interspecific distance of *rbcL* and *psbA-trnH*

Distance	*rbcL*	*psbA-trnH*
All intraspecific distance	0	0.013±0.005
Coalescent depth (Maximum intraspecific distance)	0	0.042±0.012
All interspecific distance	0.004±0.002	0.025±0.008
Minimum interspecific distance	0	0

rbcL locus exhibited higher interspecific divergence than its corresponding intraspecific divergence thereby fulfilling the criteria for an ideal barcode. All interspecific distance parameter of *psbA-trnH* was higher than its corresponding intraspecific distance, but the coalescent depth was higher than the minimum interspecific distance value. *psbA-trnH* failed as a barcode as the intraspecific distance was greater than interspecific distance, which is contradictory to the ideal barcode criteria.

Wilcoxon two sample tests also showed that the interspecific distances of *rbcL* locus was significantly greater than its intraspecific distances; the z-value obtained was 6.415 (p<0.0001).

4.1.1.6.2. Identification of polymorphic sites

rbcL and *psbA-trnH* sequences were aligned and trimmed to 508 bp and 307 bp, respectively. Sequence analysis of *rbcL* locus showed the presence of three single nucleotide polymorphic (SNP) sites specific to *C. aromaticum* at positions 54, 55 and 304 (Table 17). In seven out of the ten market samples studied, these specific SNPs were found. But we could not detect any sites to discriminate *C. verum* from *C. malabatrum.*

Table 17. Polymorphic sites in *rbcL* locus

Species	Position		
	54	55	304
C. verum	A	G	T
C. aromaticum	G	A	C
C. malabatrum	A	G	T

Though the sequence alignment of *psbA-trnH* showed polymorphic sites they were not informative enough to differentiate between the three species.

4.1.1.7. Validation of SNPs by cloning

rbcL amplicons of *C. verum, C. aromaticum* and the market samples were purified, ligated in to pTZ57R/T vector and cloned in to *E. coli* JM 109 cells that was made competent by calcium chloride method. Blue and white colonies were obtained on the plates indicating the cloning success (Figure 18). About 20 white and light blue colonies were subcultured and the positive recombinants were identified by colony PCR (Figure 19). Two clones were selected for each sample and the plasmids were isolated from these positive recombinants. The sequences retrieved from the plasmids isolated from cloned samples exhibited the same polymorphic sites initially obtained on direct sequencing of the samples. *C. aromaticum* specific SNPs were found in all the clones of the seven traded bark samples.

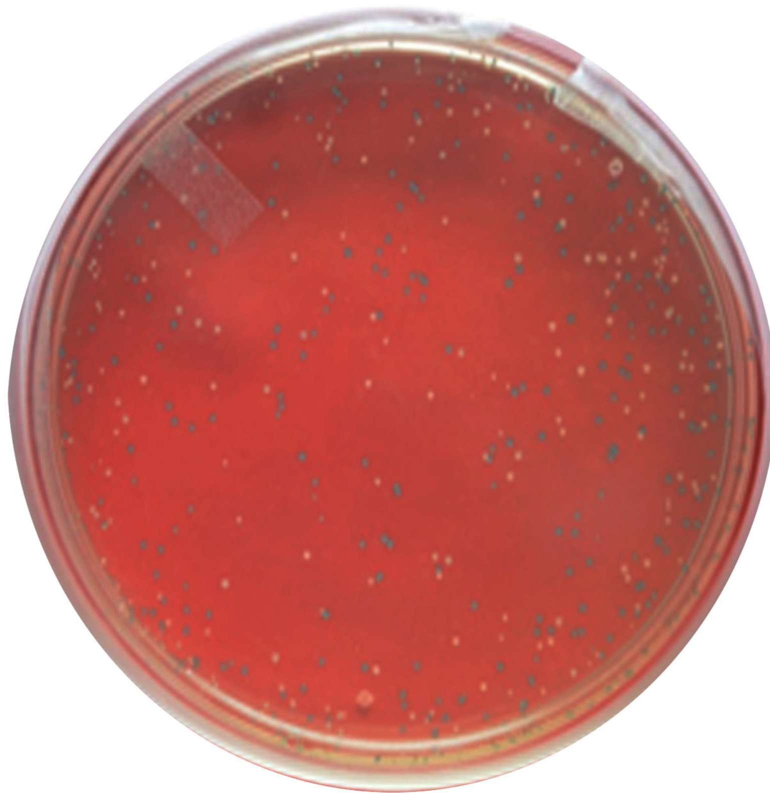


Figure 18. Blue white screening of *rbcL* clones

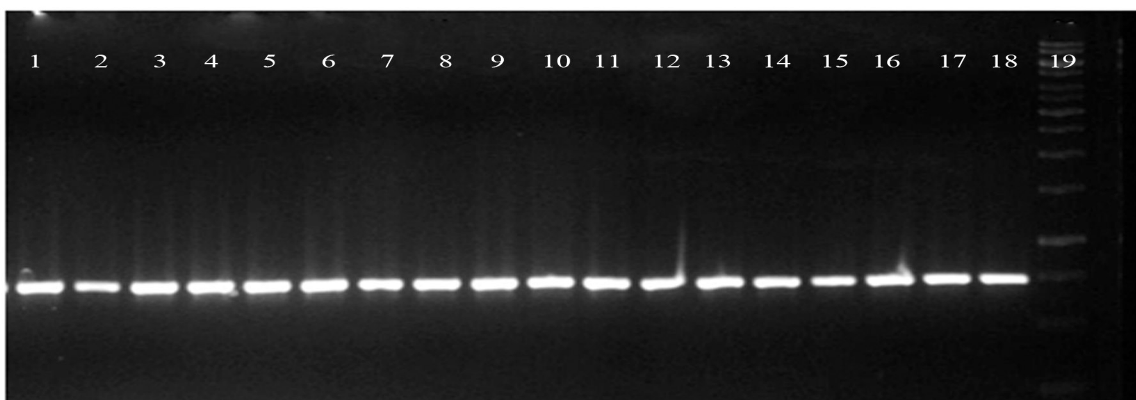


Figure 19. Colony PCR. Lanes 1-18 - *rbcL* clones, lane 19 - 1 kb ladder (Fermentas)

4.1.1.8. NJ tree based analysis

NJ tree was drawn using the sequences of *C. verum, C. aromaticum*, *C. malabatrum* and the traded samples of cinnamon bark (Figure 20). The tree clustered *C. verum* and *C. malabatrum* sequences in to one cluster and *C. aromaticum* sequences in another cluster. Seven of the market samples clustered along with *C. aromaticum* while the remaining three formed a part of the *C. verum* - *C .malabatrum* cluster.

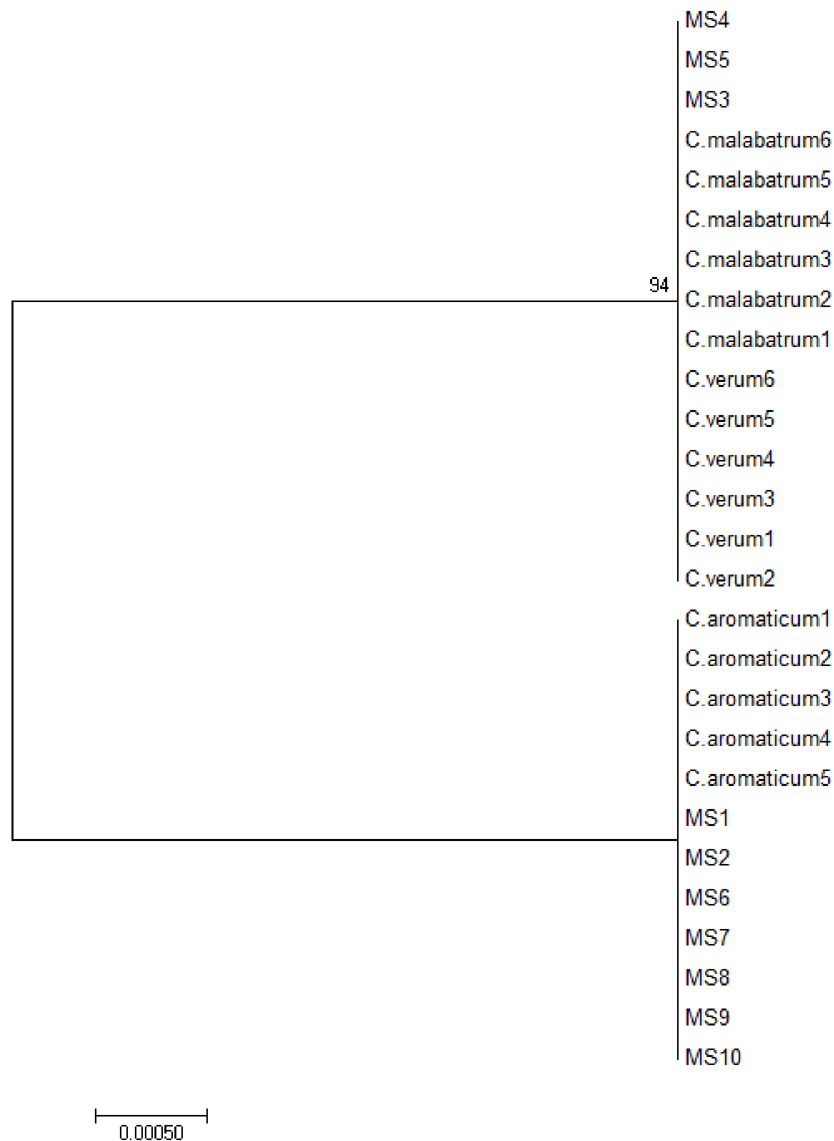


Figure 20. NJ tree constructed based on the *rbcL* sequences with a bootstrap of 1000 replicates

4.1.2. Group II - Nutmeg

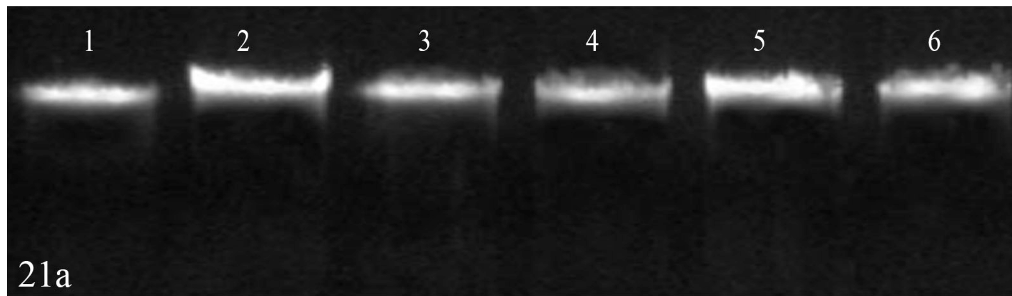
4.1.2.1. Isolation of genomic DNA from leaves of *Myristica* spp.

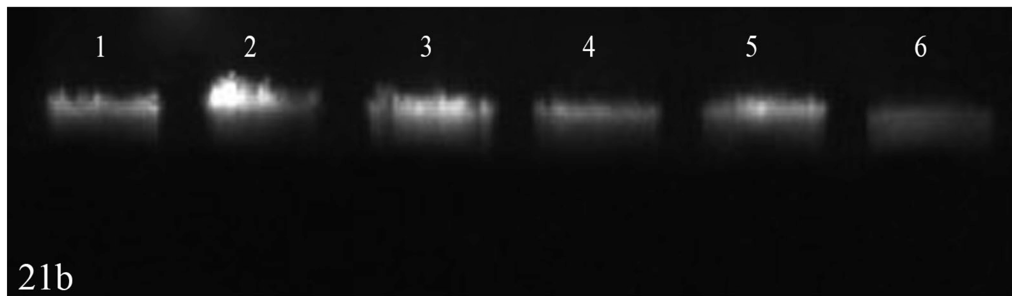
High quality genomic DNA was isolated from the leaves of reference *Myristica* species viz. *M. fragrans* and *M. malabarica* following the protocol of Sheeja et al., (2008). The yield of DNA obtained from the samples was in the range of 135-170 $\mu g g^{-1}$. Conspicuous bands were obtained on 0.8% agarose gel on electrophoresis (Figures 21a-b). Absorbance ratio (A260/A280) in the range of 1.8-1.9 indicated the high quality of DNA free from proteins, RNA and other contaminants.

4.1.2.2. Isolation of genomic DNA from nutmeg mace

DNA isolation from nutmeg mace was problematic due to the high amount of polysaccharides and polyphenols present in it. Though we attempted to isolate the mace DNA using protocols of nutmeg leaves (Sheeja et al., 2008), traded nutmeg seeds (Lele, 2011), okra (Singh and Kumar, 2012), hibiscus (Reddy, 2009), recalcitrant black pepper berries (Dhanya et al., 2007) they were not successful. So the protocol of Dhanya et al., (2007) was modified to isolate genomic DNA from nutmeg mace. Some modifications such as overnight soaking of the mace in water, replacing CTAB with 2% SDS, incorporating 2% PVP and 1% β-mercaptoethanol in extraction buffer and increasing the concentration of potassium acetate to 6 M, helped to yield good quality DNA.

The yield of DNA obtained from the traded samples was in the range of 231.4-306.8 $\mu g g^{-1}$. The absorbance ratio (A260/A280) in the range of 1.79-1.83 indicated the purity of the DNA samples. DNA was obtained as conspicuous bands on 0.8% agarose gel electrophoresis (Figure 22).





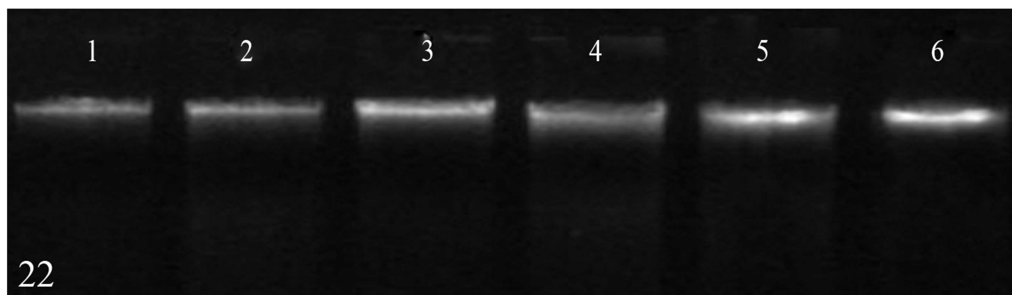


Figure 21a. DNA isolated from *M. fragrans* accessions (reference species). Lane 1 - Human Genomic DNA (blood cells) (Genei), lanes 2-6 - *M. fragrans* accessions

Figure 21b. DNA isolated from *M. malabarica* accessions (reference species). Lane 1 - Human Genomic DNA (blood cells) (Genei), lanes 2-6 - *M. malabarica* accessions

Figure 22. DNA isolated from traded nutmeg mace samples. Lane 1-5 - Traded nutmeg mace samples, lane 6 - Human Genomic DNA (blood cells) (Genei)

4.1.2.3. PCR amplification of barcoding loci

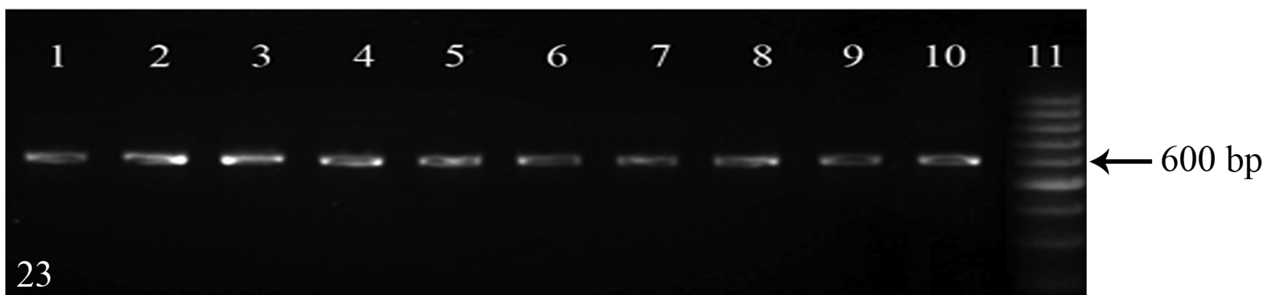
The reaction mixture components were standardized as 1 mM *Taq* assay buffer with 1.5 mM $MgCl_2$, 1 pmole μl^{-1} of forward and reverse primers, 1 U *Taq* DNA polymerase and 50 ng of genomic DNA in a reaction volume of 50 µl to yield specific PCR products.

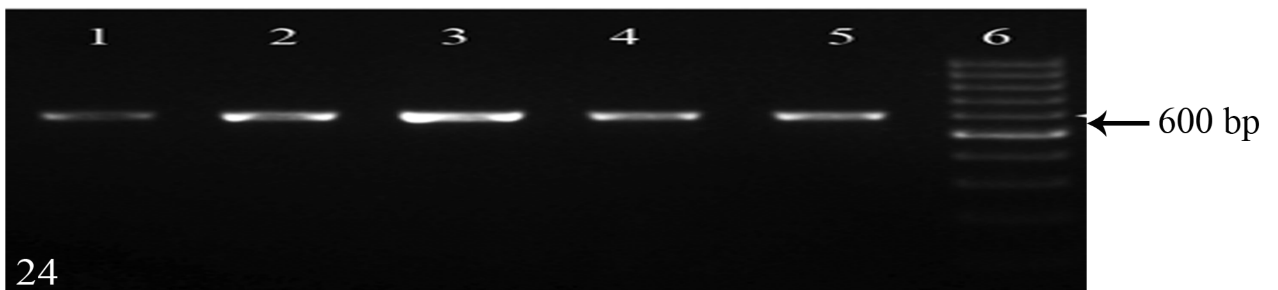
The reaction conditions were standardized by setting a gradient PCR in the temperature range of 50°C-60°C for *rbcL, psbA-trnH* and ITS. The optimized reaction condition for each locus is given in Table 18. *matK* was amplified following the protocol of Stoeckle et al., (2011).

Table 18. Temperature profile for amplification of barcoding loci in *Myristica* spp.

Reaction condition	Locus		
	rbcL	*psbA-trnH*	ITS
Initial denaturation	95°C – 4 min	94°C – 3 min	94°C – 5 min
Denaturation	94°C – 30 sec	94°C – 1 min	94°C – 1 min
Annealing	52.5°C – 1 min	54.5°C – 1 min	56°C – 1 min
Extension	72°C – 1 min	72°C – 1 min	72°C – 1 min
Final Extension	72°C – 10 min	72°C – 10 min	72°C – 10 min
Number of cycles	35	35	40

PCR amplification success was 100% for *rbcL, matK*, *psbA-trnH* and ITS in *Myristica* species. In case of traded mace samples all the loci except *matK* showed 100% amplification success. Amplicons of size 600 bp for *rbcL* (Figures 23-24), 900 bp for *matK* (Figure 25) 750 bp for ITS (Figures 26-27) and 450 bp for *psbA-trnH* (Figures 28-29) were obtained in the samples. *matK* produced amplicons of size 900 bp in *Myristica* species but failed to amplify traded samples. So it was excluded from further analysis.





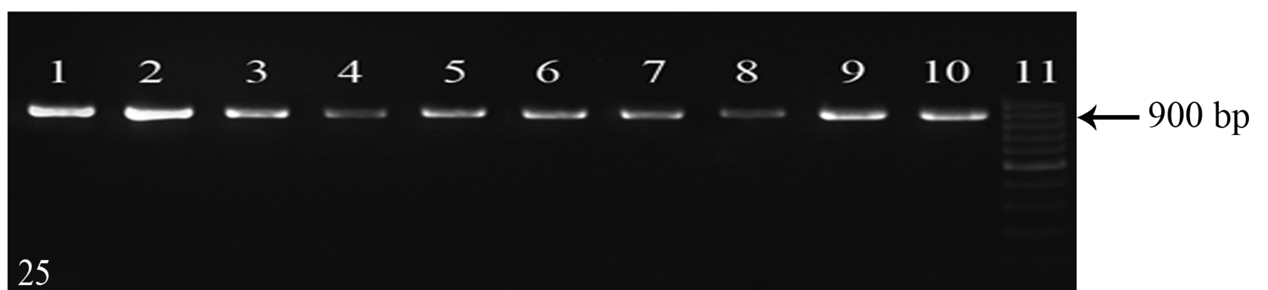
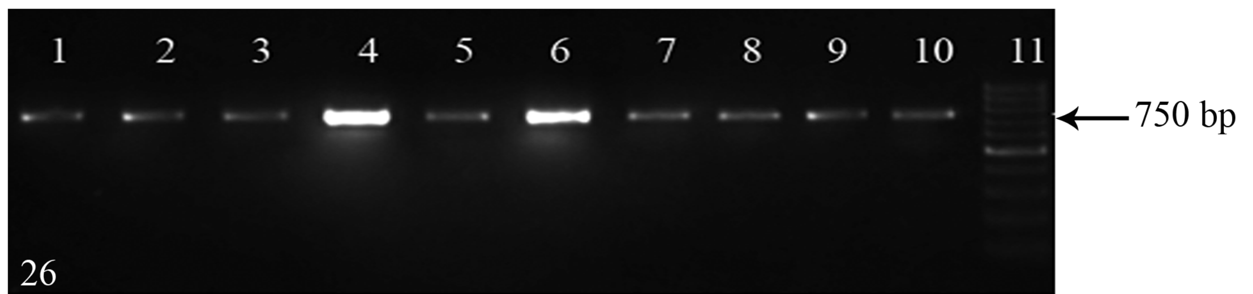
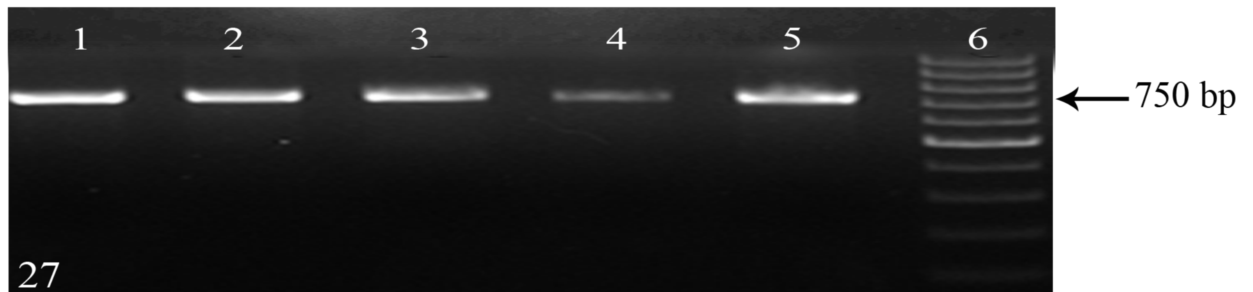
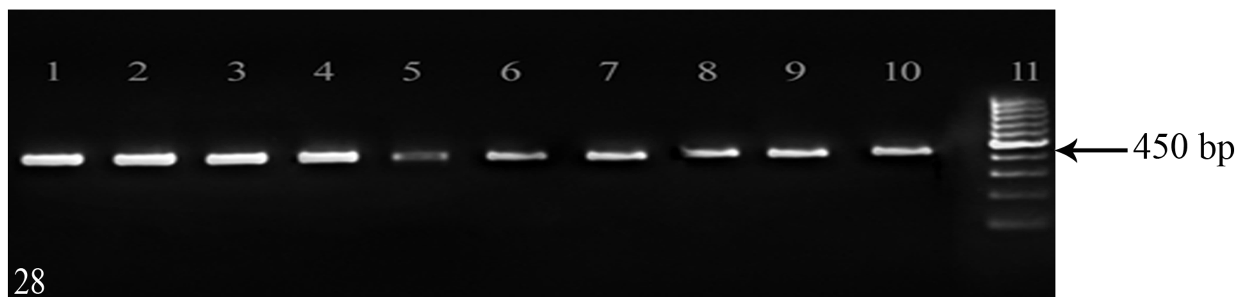


Figure 23. Amplification of *rbcL* locus in *Myristica* reference species. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100 bp ladder (Fermentas)
Figure 24. Amplification of *rbcL* locus in traded nutmeg mace samples. Lanes 1 – 5 - Market samples 1-5, lane 6- 100 bp ladder (Fermentas).
Figure 25. Amplification of *matK* locus in *Myristica* reference samples. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100 bp ladder (Fermentas).







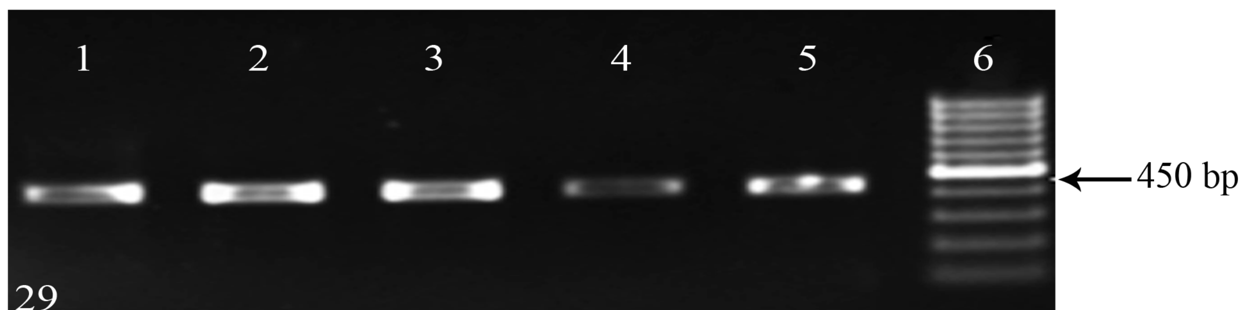


Figure 26. Amplification of ITS locus in *Myristica* reference species. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100 bp ladder (Fermentas)
Figure 27. Amplification of ITS locus in traded nutmeg mace samples. Lanes 1-5 - Market samples 1-5, lane 6- 100 bp ladder (Fermentas).
Figure 28. Amplification of *psbA-trnH* locus in *Myristica* reference species. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100 bp ladder (Fermentas)
Figure 29. Amplification of *psbA-trnH* locus in traded nutmeg mace samples. Lanes 1-5 – Market samples 1-5, lane 6- 100 bp ladder (Fermentas).

4.1.2.4. Sequencing of PCR products and Data Analysis

Sequencing was 100% successful for *rbcL* and *psbA-trnH* but ITS locus generated mixed sequence data in most of the samples thus restricting its further use. The length of sequences obtained was in the range of 426-589 bp, and 204-412 bp for *rbcL* and *psbA-trnH*, respectively. *rbcL* and *psbA-trnH* sequences queried against the nucleotide database using BLAST showed maximum identity to *rbcL,* and *psbA-trnH* sequences of genus *Myristica* and other genus of family Myristicaceae (Tables 19-20). *rbcL* sequences were translated to protein sequences and they showed 100% identity to ribulose bisphosphate sequences of the genus *Myristica* and other genus of family Myristicaceae.

Table 19. BLAST analysis of *rbcL* sequences of *Myristica* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E - value
M. fragrans 1	*M. fragrans*	KT758178	99	99	3e-154
M. fragrans 2	*M. fragrans*	KT758178	86	98	7e-161
M. fragrans 3	*M. fragrans*	KT758178	99	99	1e-162
M. fragrans 4	*M. fragrans*	KT758178	100	99	1e-168
M. fragrans 5	*M. fragrans*	KT758178	99	99	4e-158
M. malabarica 1	*Coelocaryon preussi*	KC688811	100	94	7e-151
M. malabarica 2	*M. fragrans*	KT758178	82	86	6e-92
M. malabarica 3	*M. fragrans*	KT758178	98	95	1e-143
M. malabarica 4	*C. preussi*	KC688811	98	94	1e-153
M. malabarica 5	*C. preussi*	KC688811	98	94	2e-156

Table 20. BLAST analysis of *psbA-trnH* sequences of *Myristica* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query Coverage	% Identity	E -value
M. fragrans 1	*Mauloutchia chapelieri*	AF197594	99	99	0
M. fragrans 2	*M. chapelieri*	AF197594	100	99	0
M. fragrans 3	*M. chapelieri*	AF197594	100	99	0
M. fragrans 4	*M. chapelieri*	AF197594	98	99	0
M. fragrans 5	*M. chapelieri*	AF197594	98	99	0
M. malabarica 1	*Horsefieldia amygdalina*	KR529437	96	99	0
M. malabarica 2	*M. chapelieri*	AF197594	98	99	0
M. malabarica 3	*M. chapelieri*	AF197594	97	98	0
M. malabarica 4	*M. chapelieri*	AF197594	99	100	0
M. malabarica 5	*M. chapelieri*	AF197594	100	99	0

4.1.2.5. Construction of reference libray

A reference library was constructed by depositing the *rbcL* and *psbA-trnH* sequences obtained for *Myristica* species in the GenBank database of National Centre for Biotechnology Information (NCBI). (Table 21).

Table 21. GenBank Accession numbers of *Myristica* reference species

Species	Accession Number	
	rbcL	*psbA-trnH*
M. fragrans 1	KT367808	KY966347
M. fragrans 2	KT380141	KY966348
M. fragrans 3	KT445277	KY966349
M. fragrans 4	KY945257	KY966350
M. fragrans 5	KY945258	KY966351
M. malabarica 1	KY945259	KY966342
M. malabarica 2	KY945260	KY966343
M. malabarica 3	KY945261	KY966344
M. malabarica 4	KY945262	KY966345
M. malabarica 5	KY945263	KY966346

4.1.2.6 Identification of ideal barcode locus.

An ideal DNA barcode should have higher interspecific divergence than intraspecific divergence, and sufficient nucleotide variation to distinguish between species.

4.1.2.6.1. Analysis of interspecific and intraspecific divergence.

The intraspecific divergence was assessed by two parameters- all intraspecific distance (mean of all intraspecific K2P distances between all samples collected within each species with more than one representative) and maximum intraspecific distance or coalescent depth (maximum intraspecific distance within each species). Two parameters viz., all interspecific distance (mean of all K2P distances between all species in the genus with at least two species) and minimum interspecific distance (minimum interspecific distance within each genus with at least two species) were calculated to determine the interspecific divergence (Table 22).

Table 22. Average intraspecific and interspecific distance of *rbcL* and *psbA-trnH*

Distance	*rbcL*	*psbA-trnH*
All intraspecific distance	0.003±0.002	0.057±0.011
Coalescent depth	0.011±0.004	0.166±0.024
All interspecifc distance	0.004±0.002	0.771±0.098
Minimum interspecific distance	0	0.748±0.092

From Table 22 it is evident that the coalescent depth parameters of *rbcL* locus was greater than the minimum interspecific distances. *rbcL* failed as a barcode as the intraspecific distance was greater than interspecific distance, which is contradictory to the ideal barcode criteria.

The intraspecific variation of *psbA-trnH* sequences of *M. fragrans* and *M. malabarica* was less than the interspecific distance parameters (Table 20). Thus this locus met the requirement of an ideal barcode.

Wilcoxon two sample tests also showed that the interspecific distances of *psbA-trnH* locus was significantly greater than its intraspecific distances; the z value obtained was 5.710. ($p<0.0001$).

4.1.2.6.2. Identification of polymorphic sites

rbcL and *psbA-trnH* sequences of the reference and market samples were aligned and trimmed to a final length of 425 bp and 217 bp, respectively. *rbcL* locus did not exhibit much sequence variation between *M. fragrans* and *M. malabarica*. But it showed intraspecific variations in two samples of *M. malabarica* at two positions and in one sample of *M. fragrans* at a single site. However, these polymorphisms were not informative in the context of this study and could not be used for analyzing the market samples.

psbA-trnH exhibited high sequence variation between *M. fragrans* and *M. malabarica* species. Sixty polymorphic sites and 9 indels (positions 25-37, 44-47, 59-61, 101-103, 115-118, 171-174, 186-188, 192 and 210) were identified in the alignment between these two species (Tables 27-29). Out of the five market samples analyzed, three showed SNPs and indels similar to *M. malabarica* thus pointing to a possible substitution of *M. fragrans* with *M. malabarica* samples.

Table 23. Polymorphic sites in *psbA-trnH* locus

Species	Position																			
	2	3	4	6	7	9	11	12	17	19	20	38	41	49	54	56	57	75	77	80
M.fragrans	T	A	C	C	T	T	T	A	T	A	C	T	A	A	C	T	A	T	A	A
M.malabarica	G	C	T	T	A	C	A	C	G	C	T	C	C	G	T	C	G	A	C	T

Table 24. Polymorphic sites in *psbA-trnH* locus

Species	Position																
	81	82	83	84	86	89	90	91	108	109	111	112	120	121	124	134	139
M.fragrans	T	T	T	A	A	A	A	A	G	A	C	A	A	C	A	A	C
M.malabarica	A	A	A	C	G	G	T	G	A	C	A	G	C	T	C	T	T

Table 25. Polymorphic sites in *psbA-trnH* locus

Species	Position														
	142	147	150	155	157	158	159	160	165	168	169	177	178	180	181
M. fragrans	A	G	A	A	A	A	T	C	G	A	G	C	T	G	T
M.malabarica	C	T	G	T	T	T	A	A	T	G	T	T	G	T	C

Table 26. Polymorphic sites in *psbA-trnH* locus

Species	Position							
	196	200	203	205	206	207	213	215
M. fragrans	C	C	C	G	G	G	G	T
M.malabarica	T	T	T	T	T	T	T	A

Table 27. Indels in *psbA-trnH* locus

Species	Position																
	25	26	27	28	29	30	31	32	33	34	35	36	37	44	45	46	47
M.fragrans	G	G	A	A	A	A	A	A	T	G	C	A	T	-	-	-	-
M.malabarica	-	-	-	-	-	-	-	-	-	-	-	-	-	T	A	T	T

Table 28. Indels in *psbA-trnH* locus

Species	Position													
	59	60	61	101	102	103	115	116	117	118	171	172	173	174
M.fragrans	-	-	-	-	-	-	-	-	-	-	A	A	A	C
M.malabarica	G	T	C	C	T	G	G	T	T	T	-	-	-	-

Table 29. Indels in *psbA-trnH* locus

Species	Position				
	186	187	188	192	210
M.fragrans	C	A	G	A	-
M.malabarica	-	-	-	-	T

4.1.2.7. Validation of SNPs by cloning

psbA-trnH amplicons of *M. fragrans, M. malabarica* and the market samples were purified, ligated in to pTZ57R/T vector and cloned in to *E. coli* JM 109 cells that was made competent by calcium chloride method. Blue and white colonies were obtained on the plates indicating the cloning success (Figure 30). White and light blue colonies were subcultured and the positive recombinants gave amplicons of size 650 bp on colony PCR (Figure 31). Plasmids isolated from these positive recombinants were sequenced. The sequences obtained exhibited the same polymorphic sites initially obtained on direct sequencing of the samples. *M. malabarica* specific SNPs were found in all clones of the three traded mace samples.

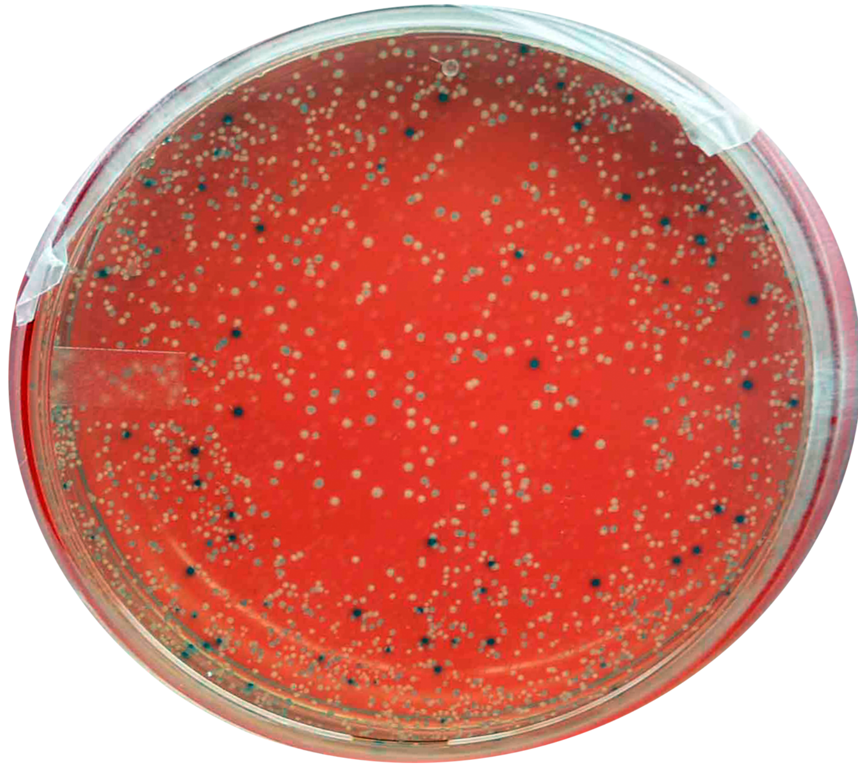


Figure 30. Blue white screening of *psbA-trnH* clones.

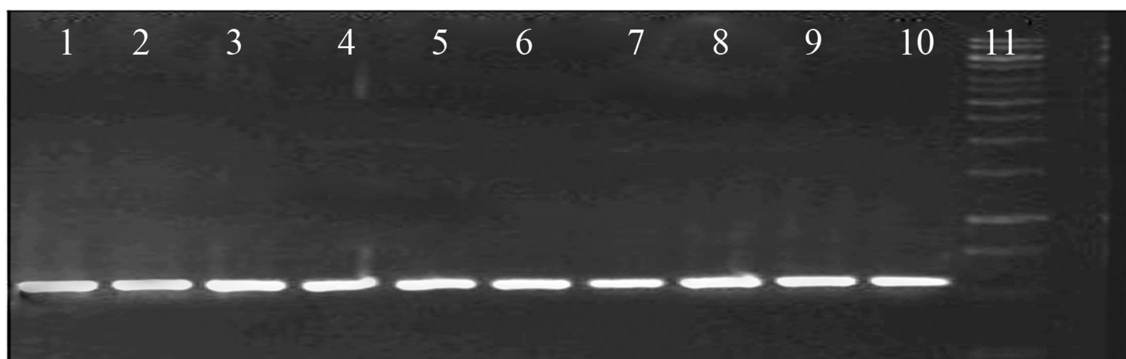


Figure 31. Colony PCR. Lanes 1-10 - *psbA-trnH* clones, lane 11- 1 kb ladder.

4.1.2.8. NJ tree based analysis

A neighbour joining tree constructed based on bootstrap support of 1000 replicates clustered *M. fragrans* and *M. malabarica* into two well separated clades (Figure 32). Two market samples clustered along with *M. fragrans* proving its authenticity while three of them clustered with *M. malabarica* further confirming the substitution of *M. fragrans* mace with inferior quality *M. malabarica* mace.

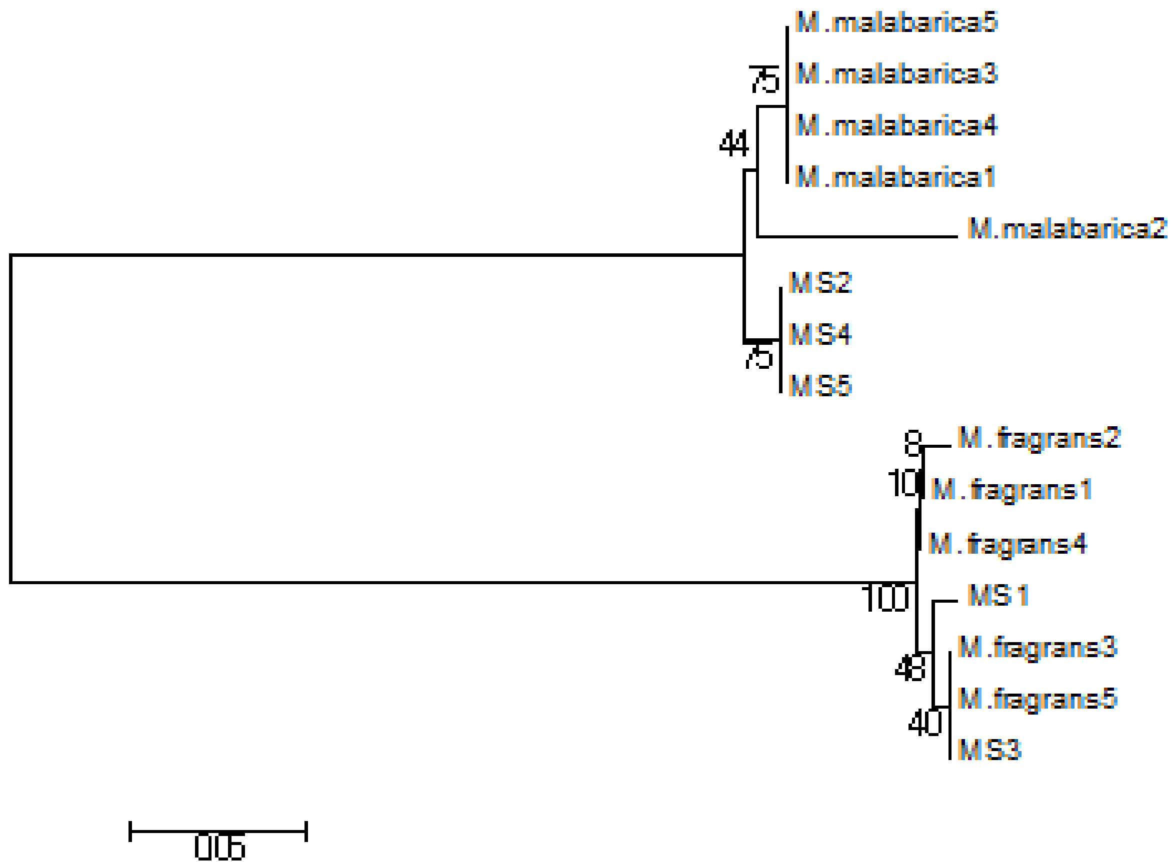


Figure 32. NJ tree cons tructed based on *psbA-trnH* sequences with a bootstrap support of 1000 replicates.

4.2 Phylogenetic Analysis

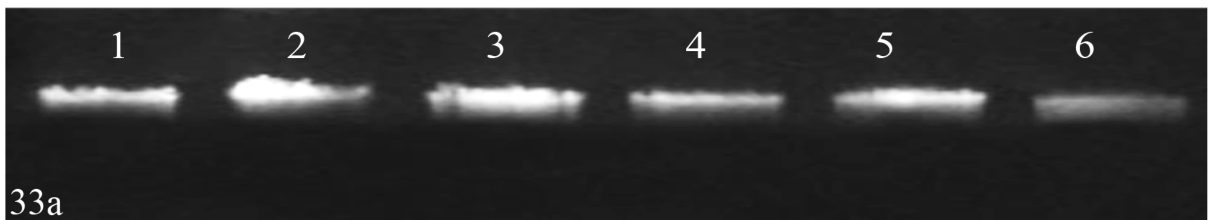
4.2.1. Group I – Cinnamon

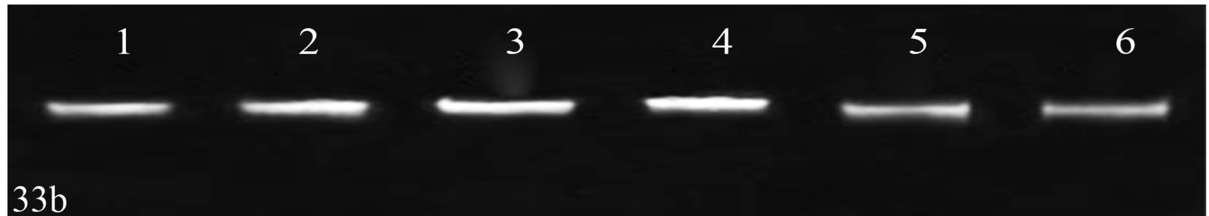
4.2.1.1. Isolation of DNA

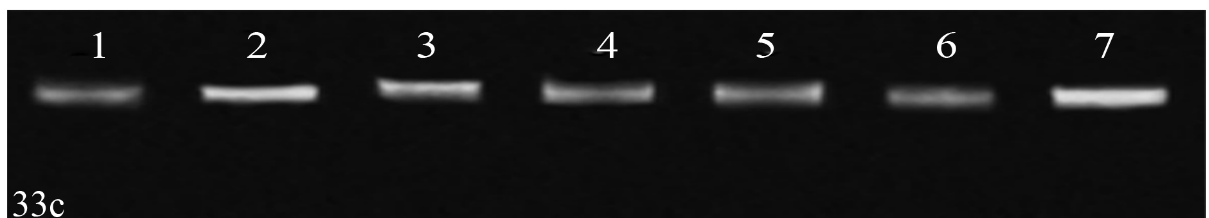
High quality genomic DNA was obtained from the leaves of the different accessions of *Cinnamomum* species using Qiagen DNeasy kit. The yield of DNA was in the range of 132-185 $\mu g g^{-1}$ with A260/A280 ratio in the range 1.8-1.9 indicating its purity (Figures 33a-e).

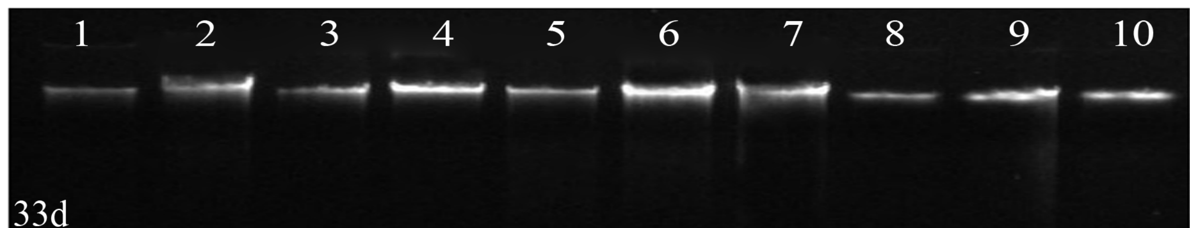
4.2.1.2. PCR amplification and sequencing of barcoding loci

The four loci *rbcL, matK, psbA-trnH* and ITS were amplified using a 50 µl reaction mixture containing 1 mM *Taq* assay buffer with 1.5 mM $MgCl_2$, 1 pmole μl^{-1} of forward and reverse primers, 1 U *Taq* DNA polymerase and 50 ng of genomic DNA. They were amplified using the reaction conditions mentioned in Table 12 of section 4.1.1.3. All the four loci gave 100% amplification success with amplicons of size 600bp (Figures 34a-d), 900bp (Figures 35a-d), 550 bp (Figures 36a-d) and 750bp (Figures 37a-d), for *rbcL, matK, psbA-trnH* and ITS loci, respectively.









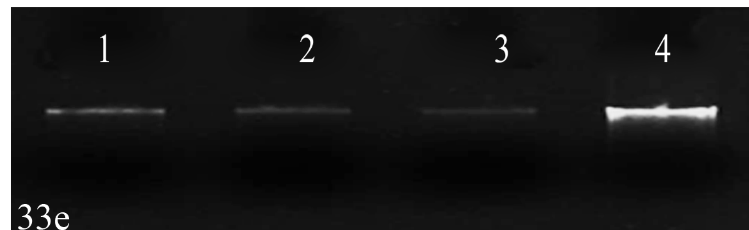
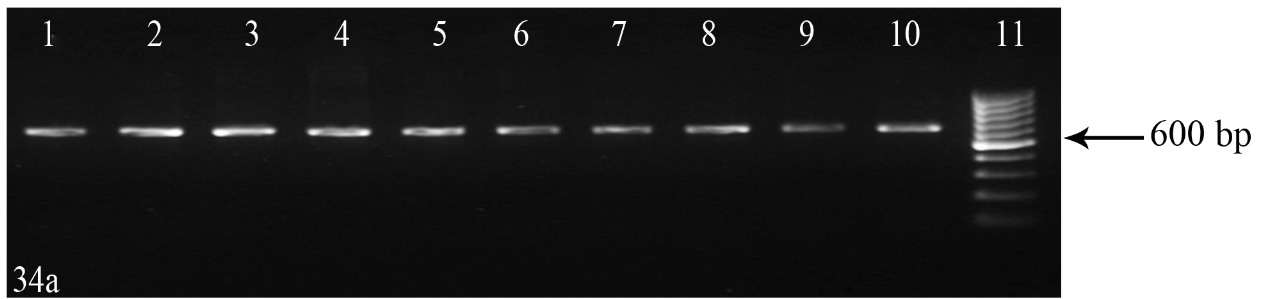
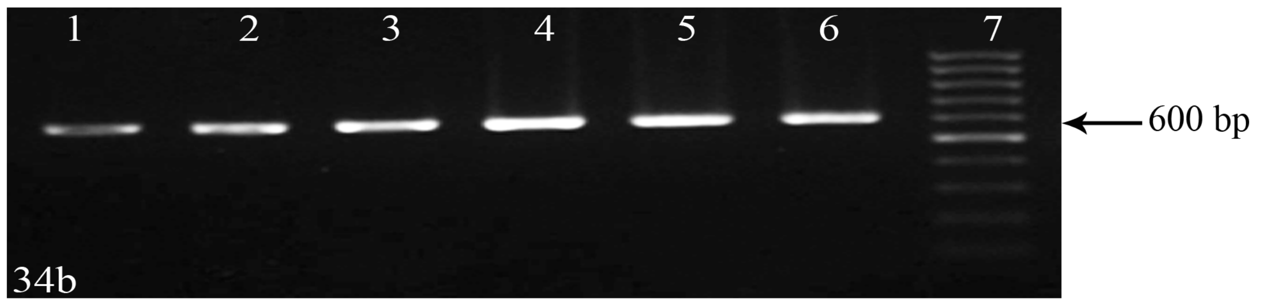
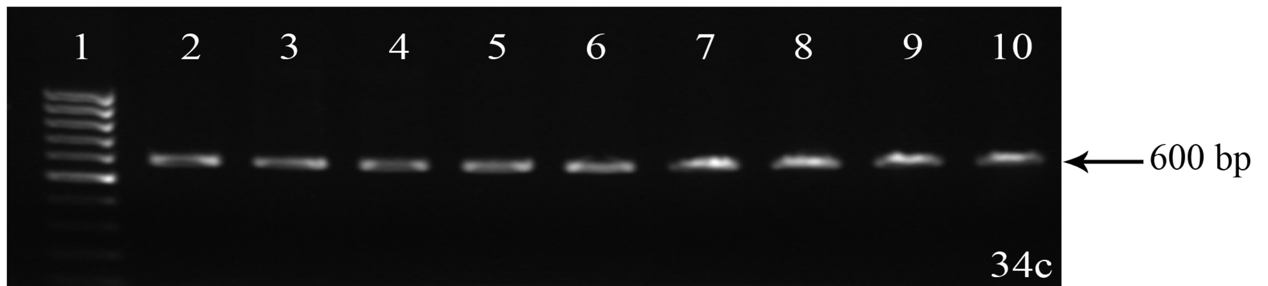


Figure 33a. DNA isolated from *Cinnamomum* species. Lanes 1-5- *C. verum* accessions, lane 6- Human Genomic DNA (blood cells) (Genei).
Figure 33b. DNA isolated from *Cinnamomum* species. Lanes 1-5 – *C. aromaticum* accessions, lane 6- Human Genomic DNA (blood cells) (Genei).
Figure 33c. DNA isolated from *Cinnamomum* species. Lanes 1-6 – *C. malabatrum*, lane 7 – Human genomic DNA (blood cells) (Genei).
Figure 33d. DNA isolated from *Cinnamomum* species. Lane 1- *C. citriodorum*, lane 2-4 - *C. tamala*, lane 5 – *C. glaucescens*, lane 6 – *C. sulphuratum*, lane 7-8 – *C. camphora*, lane 9 – *C. alexei*, lane 10 – Human genomic DNA (blood cells) (Genei).
Figure 33e. DNA isolated from *Cinnamomum* species. Lane 1- *C. heyneanum*, lane 2 – *C. riparium*, lane 3 - *C. travancoricum*, lane 4 – Human genomic DNA (blood cells) (Genei).







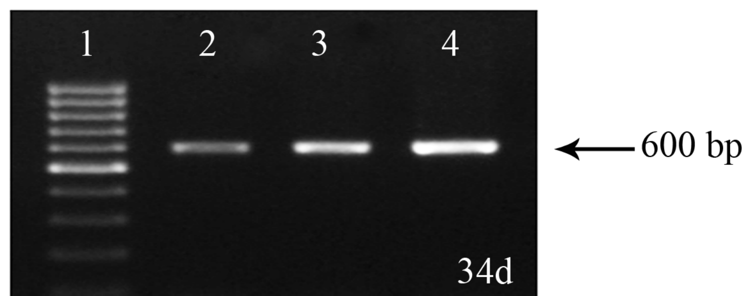
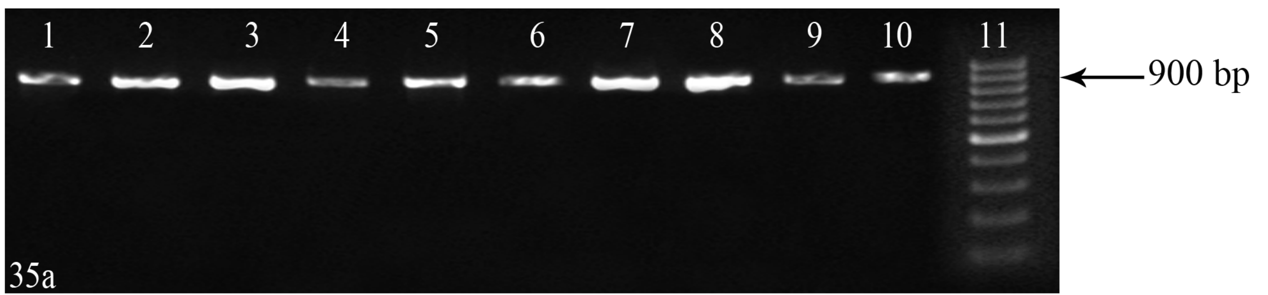
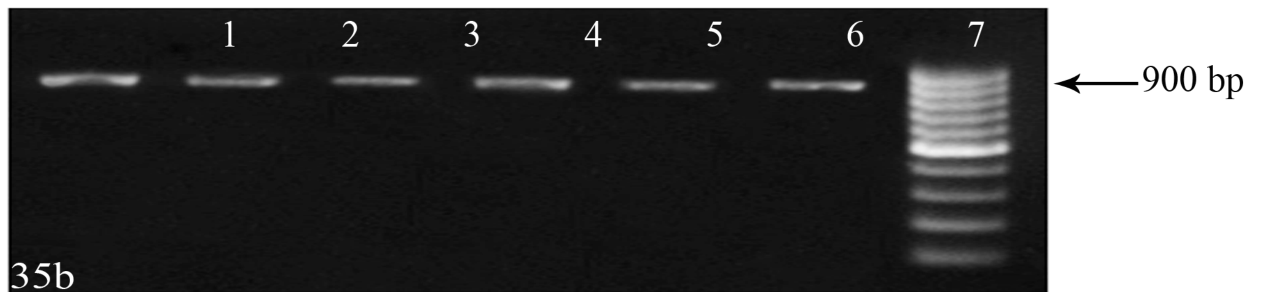
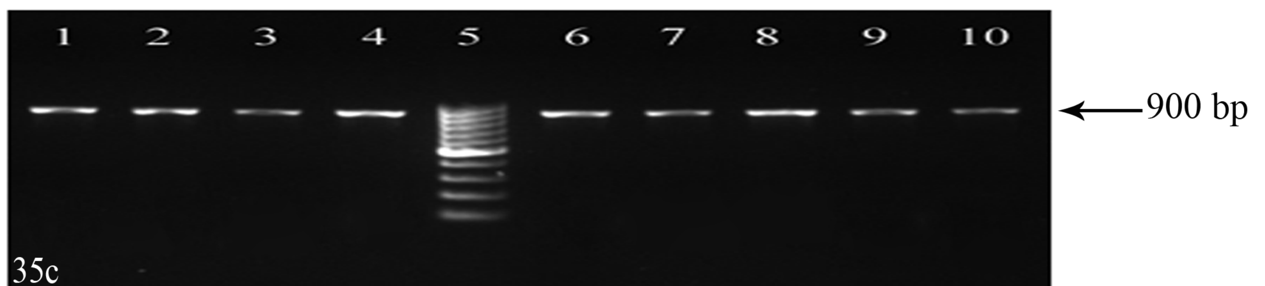


Figure 34a. Amplification of *rbcL* locus in *Cinnamomum* species. Lanes 1-5- *C. verum* accessions, lanes 6-10- *C. aromaticum* accessions, lane 11- 100bp ladder (Fermentas).
Figure 34b. Amplification of *rbcL* locus in *C. malabatrum* accessions. Lanes 1-6 - *C. malabatrum* accessions, lane 7 – 100 bp ladder (Fermentas).
Figure 34c. Amplification of *rbcL* locus in *Cinnamomum* species. Lane 1- 100 bp ladder (Fermentas), lane 2 – *C. citriodorum*, lanes 3-5 – *C. tamala*, lanes 6 - *C. sulphuratum*, lane 7- *C. glaucescens*, lanes 8 – 9 – *C. camphora*, lane 10 - *C. alexei*
Figure 34d. Amplification of *rbcL* locus in *Cinnamomum* species. Lane 1 – 100 bp ladder (Fermentas). lane 2 – *C. heyneanum*, lane 3 – *C. riparium*, lane 4 - *C. travancoricum*.







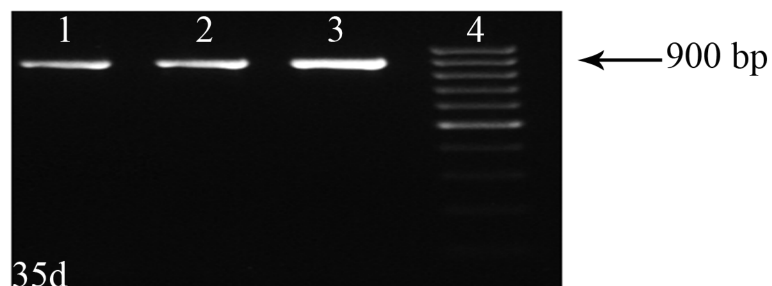
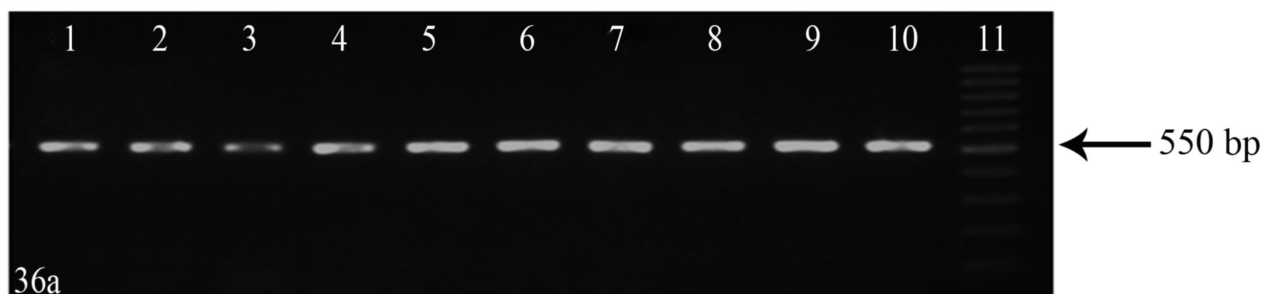


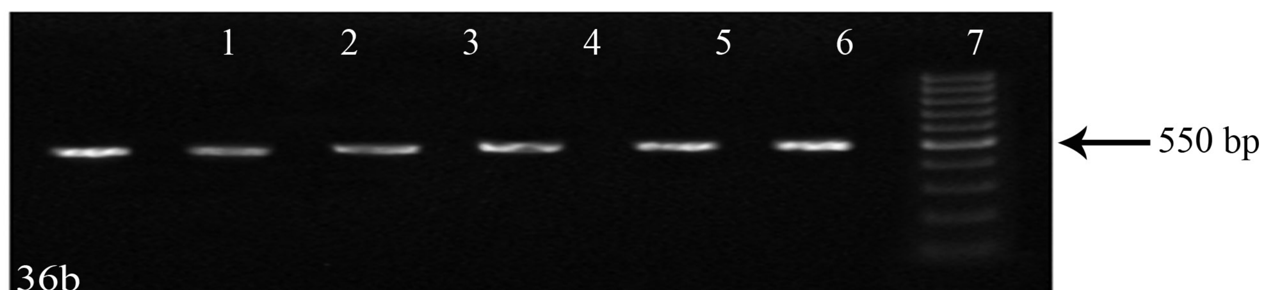
Figure 35a. Amplification of *matK* locus in *Cinnamomum* species. Lanes 1-5- *C. verum* accessions, lanes 6-10- *C. aromaticum* accessions, lane 11- 100 bp ladder (Fermentas).

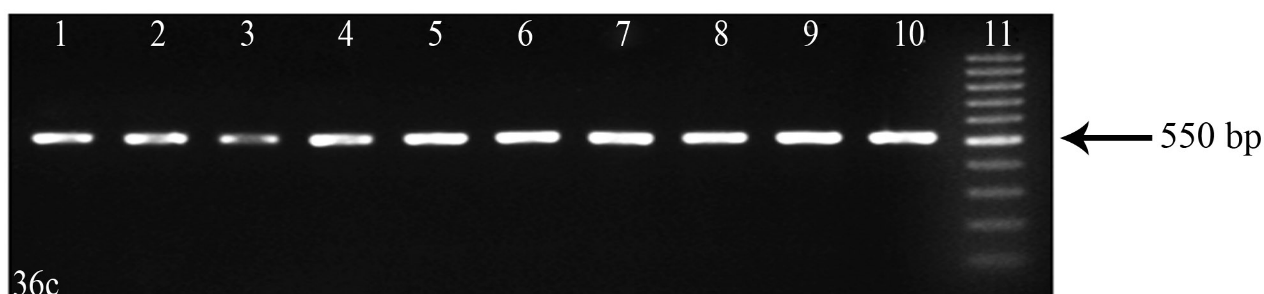
Figure 35b. Amplification of *matK* locus in *Cinnamomum* species. Lanes 1-6 - *C. malabatrum* accessions, lane 7 - 100 bp ladder (Fermentas).

Figure 35c. Amplification of *matK* locus in *Cinnamomum* species. Lane 1- *C. citriodorum*, lanes 2-4 – *C. tamala*, lane 5- 100bp ladder (Fermentas), lanes 6 - *C. sulphuratum*, lane 7 - *C. glaucescens*, lanes 8-9 – *C. camphora*, lane 10 - *C. alexei*.

Figure 35d. Amplification of *matK* locus in *Cinnamomum* species. Lane 1- *C. heyneanum*, lane 2 - *C. riparium*, lane 3 - *C. travancoricum*, lane 4 – 100 bp ladder (Fermentas).







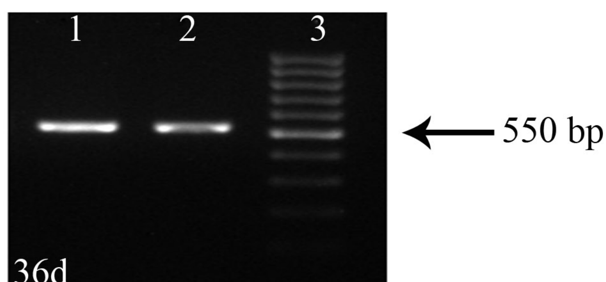
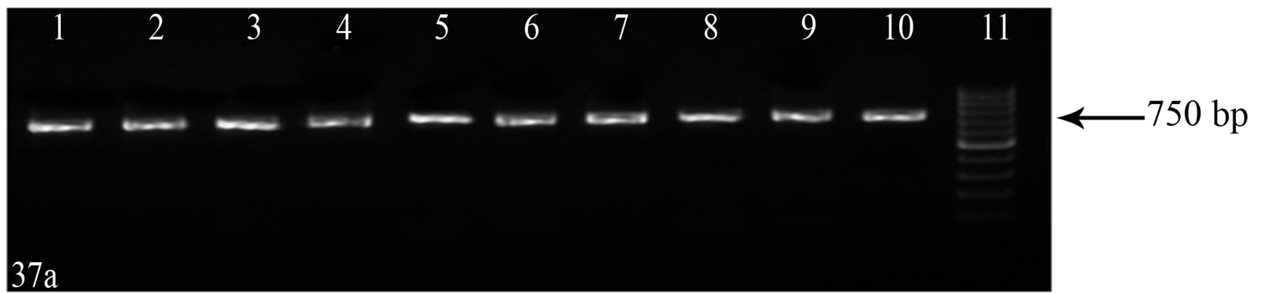


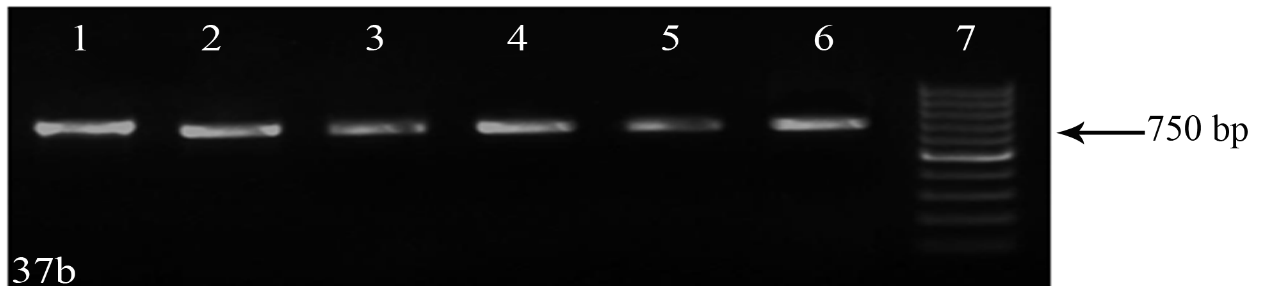
Figure 36a. Amplification of *psbA-trnH* locus in *Cinnamomum* species. Lanes 1-5- *C. verum* accessions, lanes 6-10- *C. aromaticum* accessions, lane 11- 100 bp ladder (Fermentas).

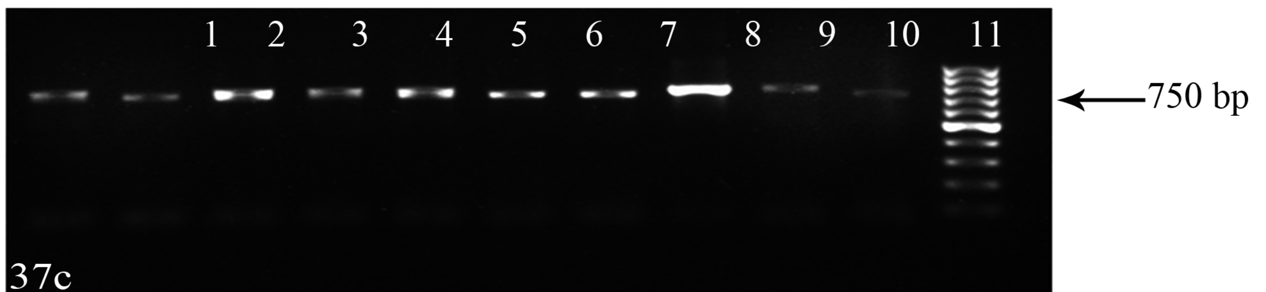
Figure 36b. Amplification of *psbA-trnH* locus in *Cinnamomum* species. Lanes 1-6 - *C. malabatrum* accessions, lane 7- 100 bp ladder (Fermentas).

Figure 36c. Amplification of *psbA-trnH* locus in *Cinnamomum* species. Lane 1- *C. citriodorum*, lanes 2-4 - *C. tamala*, lane 5 – *C. sulphuratum*, lane 6 – *C. glaucescens*, lanes 7-8- *C. camphora*, lane 9 – *C. alexei,* lane 10 – *C. heyneanum*, lane 11 - 100 bp ladder (Fermentas).

Figure 36d. Amplification of *psbA-trnH* locus in *Cinnamomum* species. Lane 1 – *C. riparium*, lane 2 – *C. travancoricum*, lane 3 - 100 bp ladder (Fermentas).







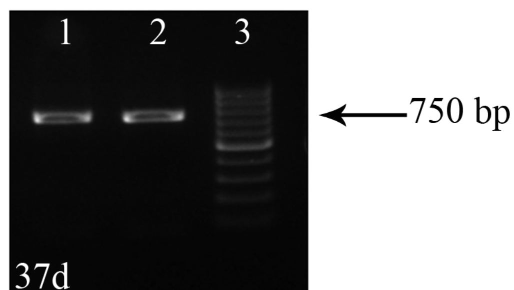


Figure 37a. Amplification of ITS locus in *Cinnamomum* species. Lanes 1-5- *C. verum* accessions, lanes 6-10- *C. aromaticum* accessions, lane 11- 100 bp ladder (Fermentas).
Figure 37b. Amplification of ITS locus in *Cinnamomum* species. Lanes 1-6 - *C. malabatrum* accessions, lane 7 – 100 bp ladder (Fermentas).
Figure 37c. Amplification of ITS locus in *Cinnamomum* species. Lane 1 – *C. citriodorum*, lanes 2-4 – *C. tamala*, lanes 5 – *C. sulphuratum*, lane 6 – *C. glaucescens*, lanes 7 - 8- *C. camphora*, lane 9 – *C. alexei*, lane 10 – *C. heyneanum*, lane 11 - 100 bp ladder (Fermentas).
Figure 37d. Amplification of ITS locus in *Cinnamomum* species. Lane 1 – *C. riparium*, lane 2 - *C. travancoricum*, lane 3 – 100 bp ladder (Fermentas).

Sequencing success was 100% for *rbcL*, *matK* and *psbA-trnH* while in case of ITS the forward and reverse reads could not be assembled to contigs due to poor sequence quality. The sequence length obtained for *rbcL* was 510 bp to 579 bp. *matK* sequences obtained were in the range of 695 bp to 782 bp and *psbA-trnH* sequences obtained were in the range of 299bp to 413bp. BLAST analysis of the contig sequences obtained for *rbcL, matK* and *psbA-trnH* exhibited maximum similarity to sequences of the respective locus of *Cinnamomum* species (Tables 30-32). The generated sequences were deposited in the GenBank database of National Centre for Biotechnology Information (NCBI) (Table 33).

Table 30. BLAST analysis of *rbcL* sequences of *Cinnamomum* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E-value
C. verum 1	*Cinnamomum* sp	AB936048	100	100	0
C. verum 2	*Cinnamomum* sp	AB936048	100	100	0
C. verum 3	*Cinnamomum* sp	AB936048	100	100	0
C. verum 4	*C. pauciflorum*	KP095065	100	100	0
C. verum 5	*Cinnamomum* sp	AB936048	100	100	0
C. aromaticum 1	*C. camphora*	GU135257	99	99	0
C. aromaticum 2	*C. aromaticum*	KP094936	99	100	0
C. aromaticum 3	*C. camphora*	GU135257	99	99	0
C. aromaticum 4	*C. camphora*	GU135257	99	99	0
C. aromaticum 5	*C. aromaticum*	KP094936	99	100	0
C. malabatrum 1	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 2	*C. baileyanum*	KU564775	100	100	0
C. malabatrum 3	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 4	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 5	*C. pauciflorum*	KP095065	100	100	0
C. malabatrum 6	*Cinnamomum* sp	AB936048	100	100	0
C. citriodorum	*Cinnamomum* sp	AB936048	100	100	0
C. tamala 1	*Cinnamomum* sp	AB936048	99	100	0
C. tamala 2	*Cinnamomum* sp	AB936048	100	100	0
C. tamala 3	*Cinnamomum* sp	AB936048	100	100	0
C. glaucescens	*C. burmanni*	KP094209	100	100	0
C. sulphuratum 1	*Cinnamomum* sp	AB936048	100	100	0
C. camphora 1	*C. camphora*	GU135257	98	100	0
C. camphora 2	*Cinnamomum* sp	AB936048	100	99	0
C. alexei	*Cinnamomum* sp	AB936048	100	100	0
C. heyneanum	*Cinnamomum* sp	AB936048	100	100	0
C. riparium	*Cinnamomum* sp	AB936048	100	100	0
C. travancoricum	*Cinnamomum* sp	AB936048	100	100	0

Table 31. BLAST analysis of *matK* sequences of *Cinnamomum* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E-value
C. verum 1	*Cinnamomum* sp	AB936045	100	100	0
C. verum 2	*C. bejolghota*	AB925175	99	99	0
C. verum 3	*Cinnamomum* sp	AB936045	100	99	0
C. verum 4	*C. bejolghota*	AB925175	99	100	0
C. verum 5	*Cinnamomum* sp	AB936045	100	99	0
C. aromaticum 1	*C. polyadelphum*	AB924731	100	100	0
C. aromaticum 2	*C. polyadelphum*	AB924731	100	99	0
C. aromaticum 3	*C. triplinerve*	EU153832	100	99	0
C. aromaticum 4	*C. polyadelphum*	AB924731	100	100	0
C. aromaticum 5	*C. polyadelphum*	AB924731	100	100	0
C. malabatrum 1	*C. citriodorum*	JQ843687	100	99	0
C. malabatrum 2	*C. bejolghota*	AB925175	99	99	0
C. malabatrum 3	*Cinnamomum* sp	AB936045	100	99	0
C. malabatrum 4	*Cinnamomum* sp	AB936045	100	99	0
C. malabatrum 5	*C. citriodorum*	JQ843687	100	99	0
C. malabatrum 6	*C. bejolghota*	AB925175	99	99	0
C. citriodorum	*Cinnamomum* sp	AB936045	100	99	0
C. tamala 1	*Cinnamomum* sp	AB936045	100	99	0
C. tamala 2	*Cinnamomum* sp	AB936045	100	100	0
C. tamala 3	*C. citriodorum*	JQ843687	99	99	0
C. glaucescens	*C. camphora*	AJ247154	100	100	0
C. sulphuratum 1	*C. bejolghota*	AB925175	99	99	0
C. sulphuratum 2	*C. bejolghota*	AB925175	99	99	0
C. camphora 1	*C. camphora*	AJ247154	100	100	0
C. camphora 2	*C. camphora*	AJ247154	100	100	0
C. alexei	*C. bejolghota*	AB925175	99	99	0
C. heyneanum	*C. bejolghota*	AB925175	99	100	0
C. riparium	*C. citriodorum*	JQ843687	100	99	0
C. travancoricum	*C. bejolghota*	AB925175	100	100	0

Table 32. BLAST analysis of *psbA-trnH* sequences of *Cinnamomum* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E-value
C. verum 1	*C. verum*	KX509887	100	99	0
C. verum 2	*C. insularimontanum*	AF268782	100	97	0
C. verum 3	*C. verum*	KX509887	100	99	0
C. verum 4	*C. bejolghota*	GQ248266	100	99	0
C. verum 5	*C. bejolghota*	KU160275	97	99	0
C. aromaticum 1	*C. aromaticum*	KU160278	97	100	0
C. aromaticum 2	*C. aromaticum*	KU160278	98	100	0
C. aromaticum 3	*C. aromaticum*	KU160278	97	100	0
C. aromaticum 4	*C. aromaticum*	KU160278	98	100	0
C. aromaticum 5	*C. bejolghota*	KU160275	99	98	0
C. malabatrum 1	*C. verum*	KX509887	100	99	0
C. malabatrum 2	*C. verum*	KX509887	100	99	0
C. malabatrum 3	*C. verum*	KX509887	100	99	0
C. malabatrum 4	*Cinnamomum* spp	JN988467	99	100	1e-153
C. malabatrum 5	*Cinnamomum* spp	JN988467	99	99	1e-152
C. malabatrum 6	*C. bejolghota*	KU160275	95	99	2e-151
C. citriodorum	*C. verum*	KF978093	98	100	3e-151
C. tamala 1	*C. verum*	KF978093	99	99	2e-156
C. tamala 2	*C. iners*	KU160282	100	100	0
C. tamala 3	*C. verum*	KF978093	99	99	3e-154
C. glaucescens	*C. verum*	KF978093	100	100	8e-155
C. sulphuratum 1	*C. camphora*	KT634240	100	97	0
C. camphora 1	*C. camphora*	KT634240	100	99	2e-160
C. camphora 2	*C. camphora*	KT634240	100	99	5e-162
C. alexei	*C. verum*	KF978095	100	99	5e-157
C. heyneanum	*C. verum*	KU160291	99	100	4e-153
C. riparium	*C. verum*	KF978095	100	96	0
C. travancoricum	*C. verum*	KX509887	100	97	0

Table 33. GenBank Accession numbers for *Cinnamomum* spp.

Species	Accession Number		
	rbcL	*psbA-trnH*	*matK*
C. verum 1	KF744226	KF978091	KP318139
C. verum 2	KF744227	MG209137	KP318142
C. verum 3	KF744230	KF978095	MF962747
C. verum 4	KF744228	KF978093	MF991903
C. verum 5	KF744229	KF978092	MF962748
C. aromaticum 1	KF878109	KF978096	MF627715
C. aromaticum 2	KF878110	KF978097	MF627716
C. aromaticum 3	KF878111	KF978098	MF627717
C. aromaticum 4	KF878112	KF978099	MF627718
C. aromaticum 5	KF878113	MG209138	MF627719
C. malabatrum 1	KY945242	KY966336	MF662762
C. malabatrum 2	KY945243	KY966337	MF662763
C. malabatrum 3	KY945244	KY966338	MF662764
C. malabatrum 4	KY945245	KY966339	MF662765
C. malabatrum 5	KY945246	KY966340	MF662766
C. malabatrum 6	KY945247	KY966341	MF662767
C. citriodorum	KY945248	MF072386	MF589647
C. tamala 1	KY945249	MF072383	MF685880
C. tamala 2	KY945250	MF072384	MF685881
C. tamala 3	KY945251	MF072385	MF685882
C. glaucescens	MF186598	MF072393	KP318145
C. sulphuratum 1	MF158637	MF072387	MF589650
C. camphora 1	KY945252	MF072391	MF589651
C. camphora 2	KY945253	MF072392	MF589649
C. alexei	KY945254	MF072388	MF589646
C. heyneanum	KY945255	MG209136	MF627720
C. riparium	KY945256	MF072390	MF589648
C. travancoricum	MF547521	MF547522	MF547522

4.2.1.3. Sequence characteristics

Sequences of individual loci viz. *rbcL, matK, psbA-trnH* and concatenated sequences of multilocus combinations viz. *rbcL+matK, rbcL+psbA-trnH*, *matK+psbA-trnH* and *rbcL+matK+psbA-trnH* were aligned and trimmed to a uniform size. Sequences downloaded from GenBank database of NCBI were included in the phylogenetic analysis of individual loci. *rbcL* sequences taken from GenBank are *C. aromaticum* (KP094936), *C. citriodorum* (JQ843681), *C. camphora* (GQ436752), *C. camphora* (GU135257), *C. tamala* (KT354297) *C. burmannii* (KP094209) and *C. loureiroi* (JX843241). *matK* sequences taken from GenBank are *C. verum* (JX495693), *C. aromaticum* (KJ510893), *Cinnamomum polyadelphum* (AB924685), *C. polyadelphum* (AB924731), *C. citriodorum* (JQ843687), *C. citriodorum* (JQ843686), *Cinnamomum brenesii* (JQ589829), *C. brenesii* (JQ589828), *Cinnamomum bejolghota* (GQ248098), *C. bejolghota* (EU153831), *C. camphora* (KJ510888), *C. camphora* (HQ427401), *C. loureiroi* (JQ435500), *C. burmannii* (KF740402), *C. burmannii* (HM019315). *psbA-trnH* sequences taken from GenBank are *C. verum* (KX509887), *C. aromaticum* (KU160278), *C. burmannii* (KU160276), *C. burmannii* (KP095534), *Cinnamomum contractum* (KR533096), *C. contractum* (KR533097), *Cinnamomum insularimontanum* (KX509885), *C. insularimontanum* (AF268782), *Cinnamomum glanduliferum* (KU160280), *C. camphora* (KJ5686728), *C. camphora* (HQ415574), *C. bejolghota* (KU160275), *C. bejolghota* (KR533133) and *Cinnamomum parthenoxylon* (KP095538). Multilocus combinations were done using those species that generated sequences for all the loci. The characteristics of the aligned sequences that are used in phylogenetic analysis are given in Table 34.

Table 34. Sequence characteristics of the loci for *Cinnamomum* species

Sequence Characteristics	*rbcL*	*matK*	*psbA-trnH*	*rbcL+ matK*	*rbcL+ psbA-trnH*	*matK+ psbA-trnH*	*rbcL+matK+ psbA-trnH*
Number of sequences	36	44	43	20	29	29	29
Aligned Length (bp)	462	697	296	1413	731	998	1428
Number of variable sites	22	26	152	21	31	40	44
Number of Parsimony Informative sites	4	4	20	8	17	18	21
Number of indels	0	0	35	0	1	14	5

4.2.1.4. Phylogenetic analysis

Phylograms were constructed using single locus and multilocus combinations. The phylogenetic relationship between *Cinnamomum* species was studied using the individual (*rbcL, matK* and *psbA-trnH*) and concatenated (*rbcL+matK*, *rbcL+psbA-trnH*, *matK+psbA-trnH*, *rbcL+matK+psbA-trnH*) sequences with an outgroup using Bayesian inference (BI) methods. The phylogeny inferred from the BI tree for each individual locus and their combination with bootstrap (BS) support is given below. Bootstrap values shown on the trees indicate the number of times the node of the tree is repeated in the replicates.

4.2.1.4.1. Phylogenetic analysis based on *rbcL* gene analysis.

Phylogenetic analysis was conducted using the *rbcL* sequences of *Cinnamomum* species along with sequences taken from GenBank viz. *C. aromaticum* (KP094936), *C. citriodorum* (JQ843681), *C. camphora* (GQ436752), *C. camphora* (GU135257), *C. tamala* (KT354297) *C. burmannii* (KP094209), *C. loureiroi* (JX843241) and *P. zhennan* (HM019515) as the outgroup (Figure 38).

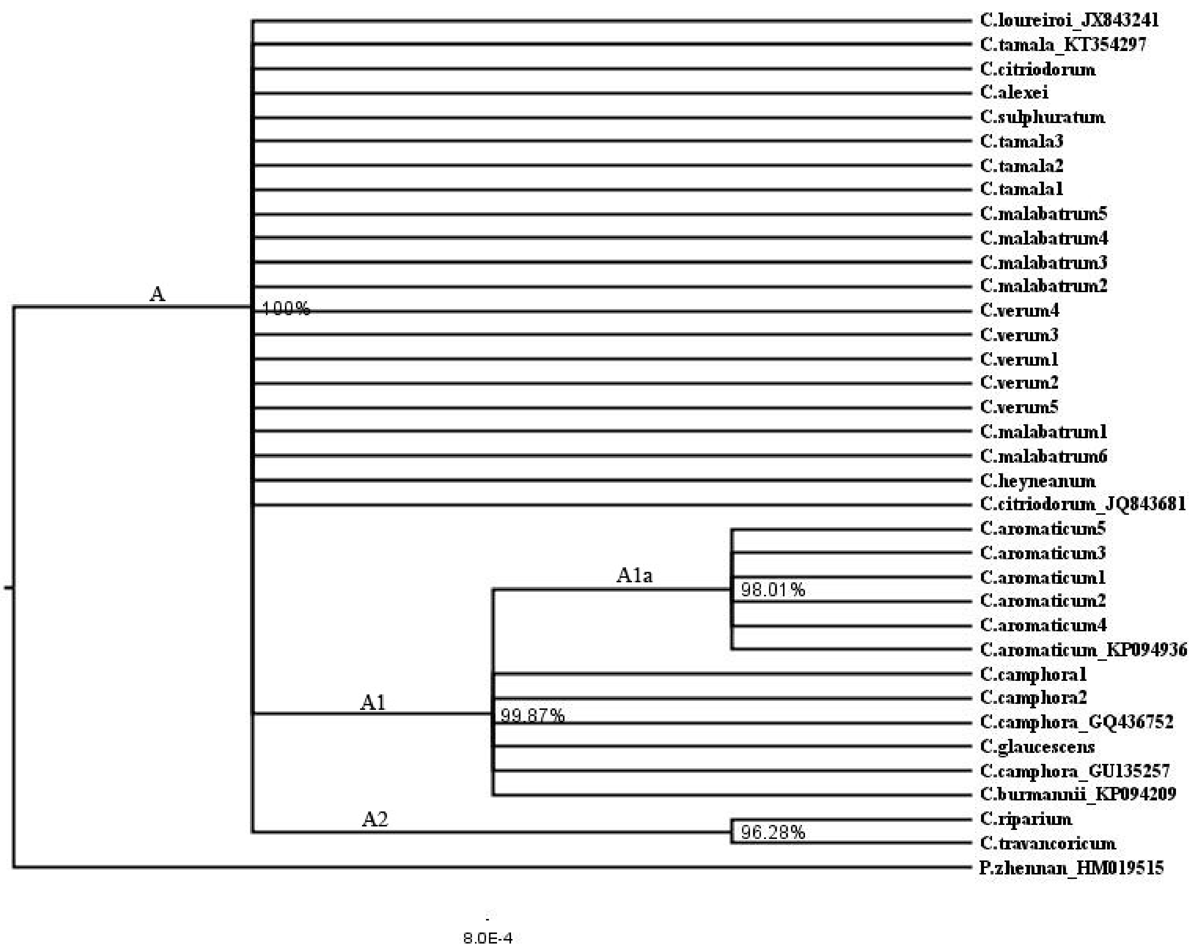


Figure 38. Bayesian phylogram for *rbcL* sequences in *Cinnamomum* spp.

The tree was rooted with *P. zhennan* that branched before the main ingroup comprising the *Cinnamomum* species. The clades formed had a high bootstrap support. But the species resolution in the phylogram was low. Clade A was formed with a bootstrap support of 100%. Sequences of *C. verum, C. malabatrum*, *C. tamala*, *C. sulphuratum*, *C. citriodorum*, *C. heyneanum* and *C. loureiroi* were unresolved within clade A. Two subclades A1 and A2 were formed in clade A. *C. camphora, C. glaucescens, C. burmannii* and *C. aromaticum* formed a strongly supported subclade A1. Within this subclade, *C. aromaticum* accessions formed a monophyletic subclade A1a with high bootstrap value (98.01%). In clade A2, *C. riparium* and *C. travancoricum* formed a paraphyletic group with bootstrap support of 96.28%.

4.2.1.4.2. Phylogenetic analysis based on *matK* gene analysis.

Phylogenetic analysis was conducted using the *matK* sequences of *Cinnamomum* species along with sequences taken from GenBank viz. *C. verum* (JX495693), *C. aromaticum* (KJ510893), *C. polyadelphum* (AB924685), *C. polyadelphum* (AB924731), *C.citriodorum* (JQ843687), *C.citriodorum* (JQ843686), *Cinnamomum brenesii* (JQ589829), *C. brenesii* (JQ589828), *Cinnamomum bejolghota* (GQ248098), *C. bejolghota* (EU153831), *C. camphora* (KJ510888), *C. camphora* (HQ427401), *C. loureiroi* (JQ435500), *C. burmannii* (KF740402), *C. burmannii* (HM019315) and *P. zhennan* (KF740391) as the outgroup (Figure 39).

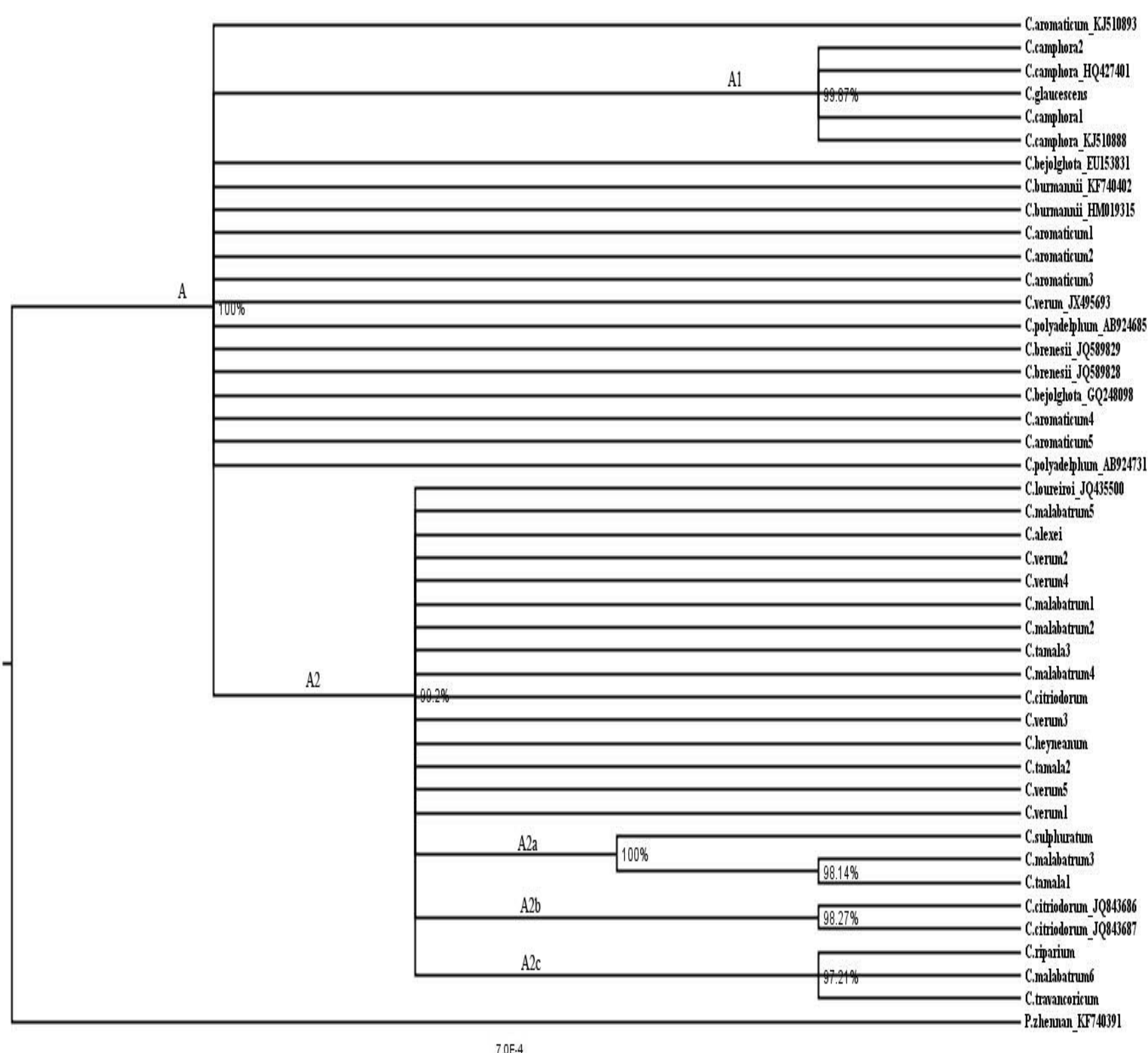


Figure 39. Bayesian phylogram for *matK* sequences in *Cinnamomum* spp.

A final alignment of 697 bp was used to generate the phylogram with overlaid bootstrap support. The *matK* phylogram was rooted with *P. zhennan* branched before the ingroup containing *Cinnamomum* sequences. Two clades A1 and A2 were formed with a high

bootstrap support. Clade A formed with a bootstrap support of 100% was comprised of *C. aromaticum, C. brensii, C. polyadelphum, C. bejolghota* and *C. burmannii.* Species resolution in clade1 was low. Within clade A, *C. camphora* and *C. glaucescens* formed a subclade A1 with 99.87% bootstrap support. Subclade A2 had 10 species viz. *C. verum, C. malabatrum, C. alexei, C. heyneanum, C. riparium, C. travancoricum, C. sulphuratum, C. tamala, C. citriodorum* and *C. loureiroi* of which *C. malabatrum* and *C. tamala* formed a paraphyletic subclade A2a and *C. sulphuratum* was positioned as a sister group to it. *C. citriodorum* sequences downloaded from GenBank formed monophyletic subclade A2b and *C. riparium, C. travancoricum* and *C. malabatrum* formed a paraphyletic subclade A2c. All the three subclades had bootstrap support greater than 97%.

4.2.1.3.3. Phylogenetic analysis based on *psbA-trnH* gene analysis

Phylogram was constructed using the *psbA-trnH* sequences of *Cinnamomum* species along with sequences taken from GenBank viz. *C. verum* (KX509887), *C. aromaticum* (KU160278), *C. burmannii* (KU160276), *C. burmannii* (KP095534), *Cinnamomum contractum* (KR533096), *C. contractum* (KR533097), *Cinnamomum insularimontanum* (KX509885), *C. insularimontanum* (AF268782), *Cinnamomum glanduliferum* (KU160280), *C. camphora* (KJ5686728), *C. camphora* (HQ415574), *C. bejolghota* (KU160275), *C. bejolghota* (KR533133), *Cinnamomum parthenoxylon* (KP095538) and *P. zhennan* (KU160270) as the outgroup (Figure 40).

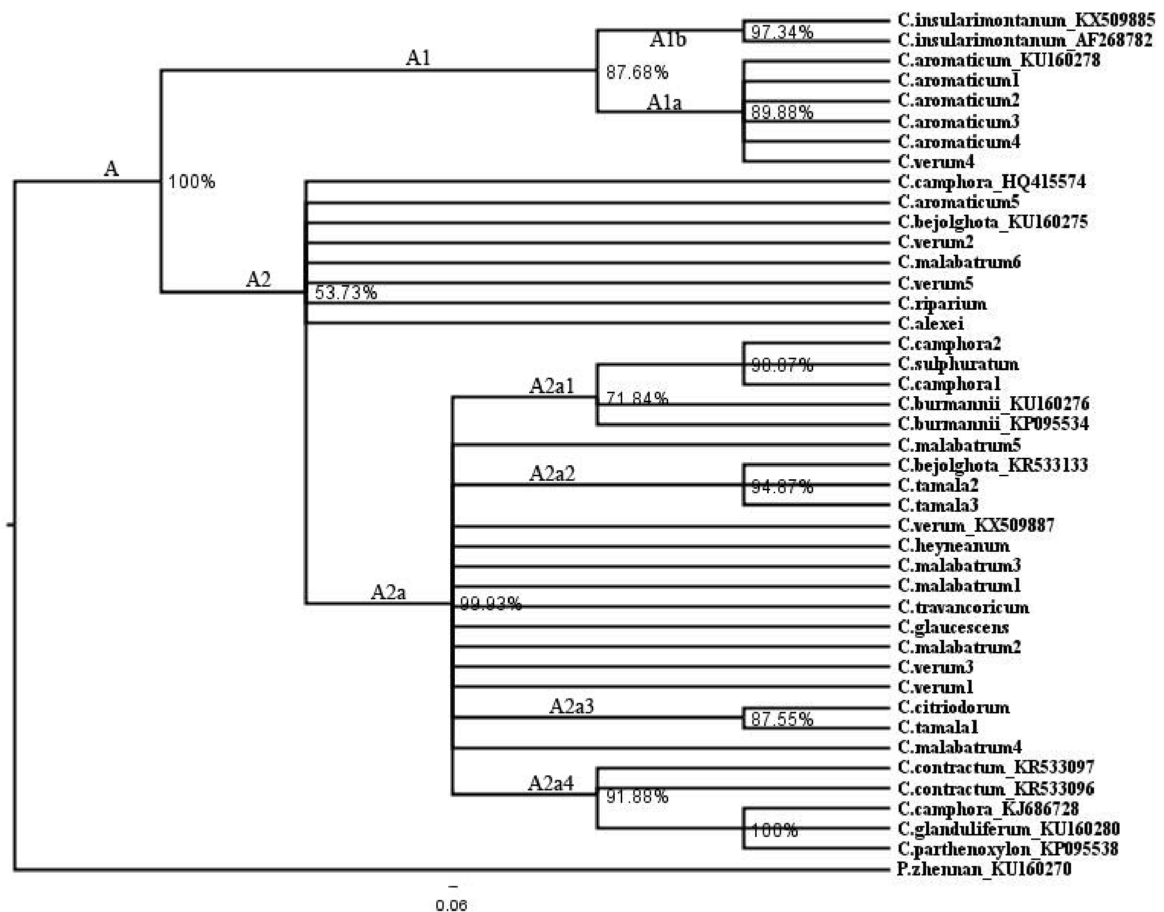


Figure 40. Bayesian phylogram for *psbA-trnH* sequences in *Cinnamomum* spp.

The tree was rooted with *P. zhennan*, which branched before the main ingroup comprising the *Cinnamomum* species. Different accessions of same species clustered with other species in this phylogram. *psbA-trnH* phylogram formed a clade A which divided *Cinnamomum* species into two major subclades A1 and A2. Subclade A1 with a bootstrap support of 87.68% was divided into two subclades A1a and A1b. A1a contains *C. aromaticum* sequences and a *C. verum* sequence (89.88% BS) while A1b (97.34% BS) is a monophyletic subclade of *C. insularimontanum* sequences downloaded from GenBank that is positioned sister to subclade A1a.

Subclade A2 was weakly supported with 53.73% bootstrap support and was comprised of sequences of different species like *C. verum, C. aromaticum, C. bejolghota, C. riparium, C.burmannii*, *C. alexei, C. camphora, C. sulphuratum, C. tamala, C. bejolghota, C. heyneanum, C. glaucescens, C. travancoricum, C. citriodorum*, *C. parthenoxylon*, *C. contractum* and *C. glanduliferum* which were divided into subclades with moderate to high bootstrap support. *C. camphora, C. sulphuratum, C. burmannii, C. tamala, C. bejolghota, C. heyneanum, C. glaucescens, C. travancoricum, C. citriodorum*, *C. parthenoxylon*, *C.*

contractum and *C. glanduliferum* formed a paraphyletic group A2a within clade A2 with a high bootstrap support of 99.93%. Within A2a group, species formed four other paraphyletic subclades. In subclade A2a1, *C. camphora* and *C. sulphuratum* clustered together (BS 90.07%) with *C. burmannii* positioned sister to it. Subclade A2a2 with a bootstrap support of 94.87% had *C. bejolghota* and two *C. tamala* sequences clustered together. *C. tamala* and *C. citriodorum* sequences formed a part of group A2a3 (87.55% BS) while *C. contractum* (91.88% BS), *C. camphora, C. glanduliferum,* and *C. parthenoxylon* sequences downloaded from GenBank formed group A2a4 (100% BS). Within A2a4, *C. camphora, C. glanduliferum,* and *C. parthenoxylon* clustered together with 100% BS.

4.2.1.3.4. Phylogenetic analysis based on multilocus approach of *rbcL+matK*

Phylogenetic trees were constructed using the *rbcL* and *matK* sequences of the *Cinnamomum* spp. with overlaid bootstrap support (Figure 41).

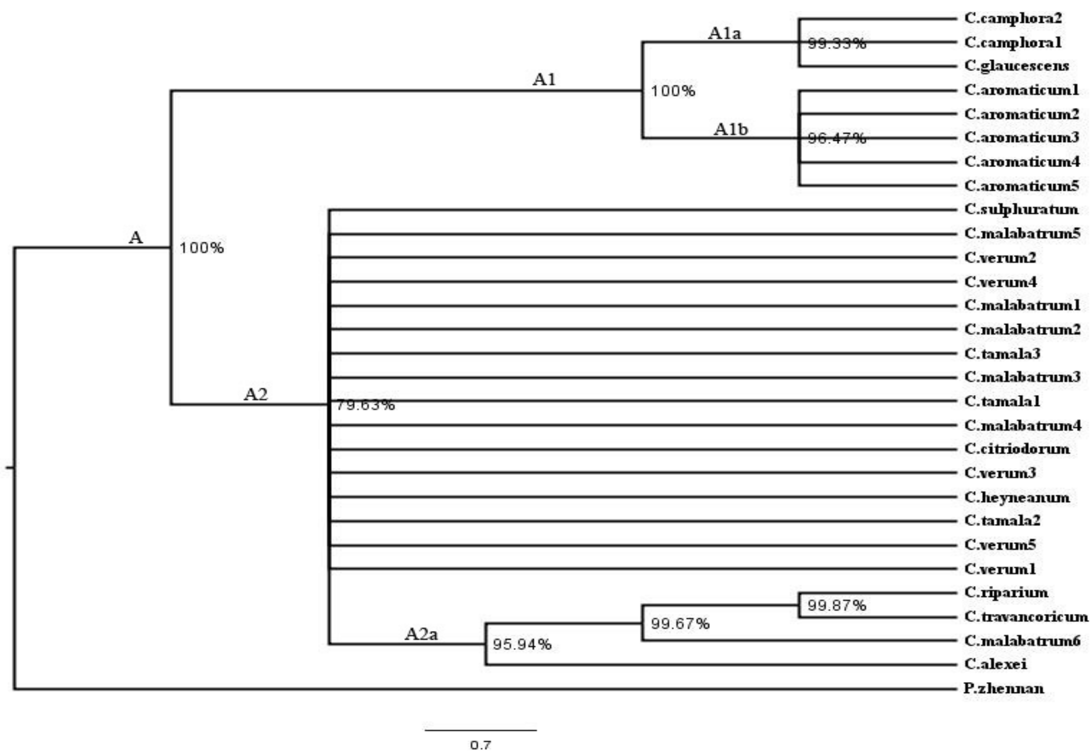


Figure 41. Bayesian phylogram for the combined *rbcL+matK* sequences in *Cinnamomum* spp.

Bayesian inference tree was drawn using concatenated sequences of *rbcL* and *matK* with overlaid bootstrap support. A main clade A was formed which was divided into two subclades A1 and A2 with 100% bootstrap support with *P. zhennan* branched separately from the inner *Cinnamomum* species. Subclade A1 was further divided in to A1a and A1b

with 100% bootstrap support. A1a had *C. camphora* and *C. glaucescens* sequences grouped together with a bootstrap support of 99.99% and A1b had the *C. aromaticum* sequences grouped together with a bootstrap support of 99.47%. Sequences of *C. verum, C. malabatrum, C. tamala, C. sulphuratum* and *C. heyneanum* arose from the node of clade A2 with a bootstrap support of 79.53%. Species resolution was low within this clade. Within subclade A2, *C. alexei, C. riparium, C. malabatrum* and *C. travancoricum* formed subclade A2a with very high bootstrap values (95.94%). Within A2a, *C. riparium* and *C. travancoricum* clustered together with high bootstrap support (99.87%) with *C. malabatrum* positioned sister to it.

4.2.1.3.5. Phylogenetic analysis based on multilocus approach of *rbcL+psbA-trnH*

Phylogenetic trees were constructed using *rbcL* and *psbA-trnH* sequences of the *Cinnamomum* spp. with overlaid bootstrap support (Figure 42).

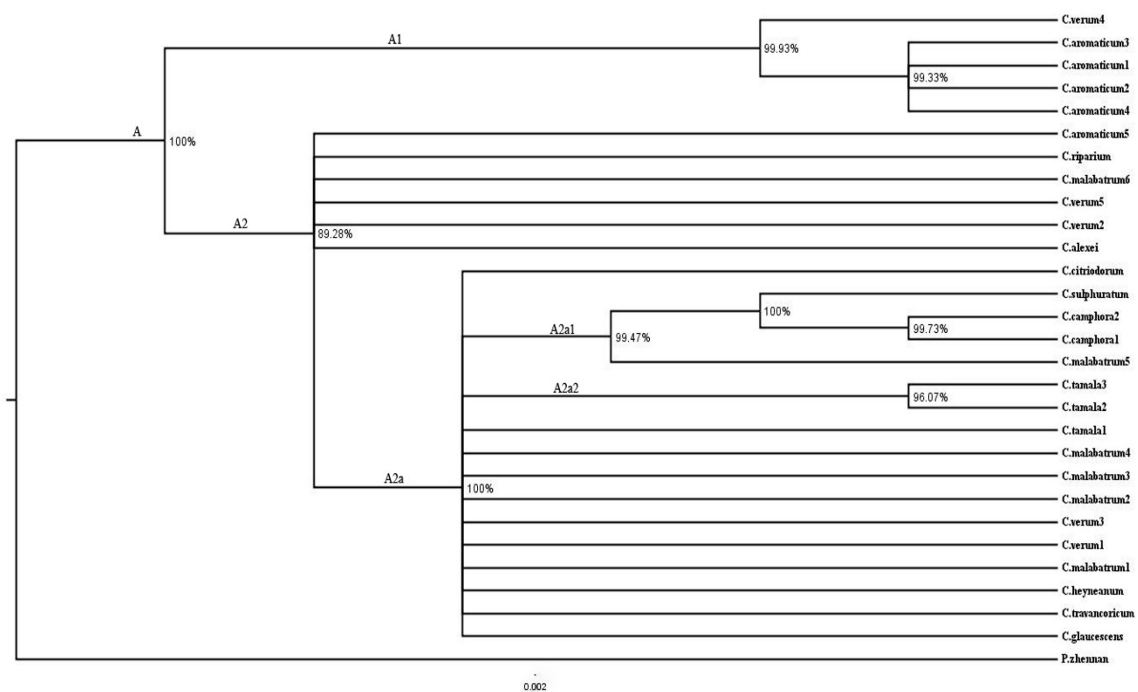


Figure 42. Bayesian phylogram for the combined *rbcL+psbA-trnH* sequences in *Cinnamomum* spp.

The tree drawn using the concatenated sequences of *rbcL* and *psbA-trnH* were divided into two clades with *P. zhennan* branched separately from the inner *Cinnamomum* species. Different accessions of the same species were seen clustered in different clades. Clade A was divided into two major subclades A1 and A2.

Subclade A1 formed with a bootstrap support of 100% had *C. verum* positioned sister to a monophyletic *C. aromaticum* group with 99% bootstrap support. Species resolution was low within subclade A2. Clade A2 (89.28% BS) had *C. aromaticum, C. verum, C. riparium* and *C. malabatrum* occupying an unresolved status. A subclade A2a was obtained within clade A2 that consisted of *C. citriodorum, C. verum, C. malabatrum, C. heyneanum, C. travancoricum, C.camphora, C. glaucescens* and *C. tamala.* Two subclades A2a1 and A2a2 with strong bootstrap were seen in subclade A2a. A2a1 (99.47% BS), consisted of a monophyletic *C. camphora* group to which *C. sulphuratum* was positioned as a sister group. *C. malabatrum* was also positioned in subclade A2a1. *C. tamala* formed the other subclade A2a2 (96.07% BS).

4.2.1.3.6. Phylogenetic analysis based on multilocus approach of *matK+psbA-trnH*

The phylogram was drawn using concatenated *matK* and *psbA-trnH* sequences of the *Cinnamomum* species with overlaid bootstrap support (Figure 43).

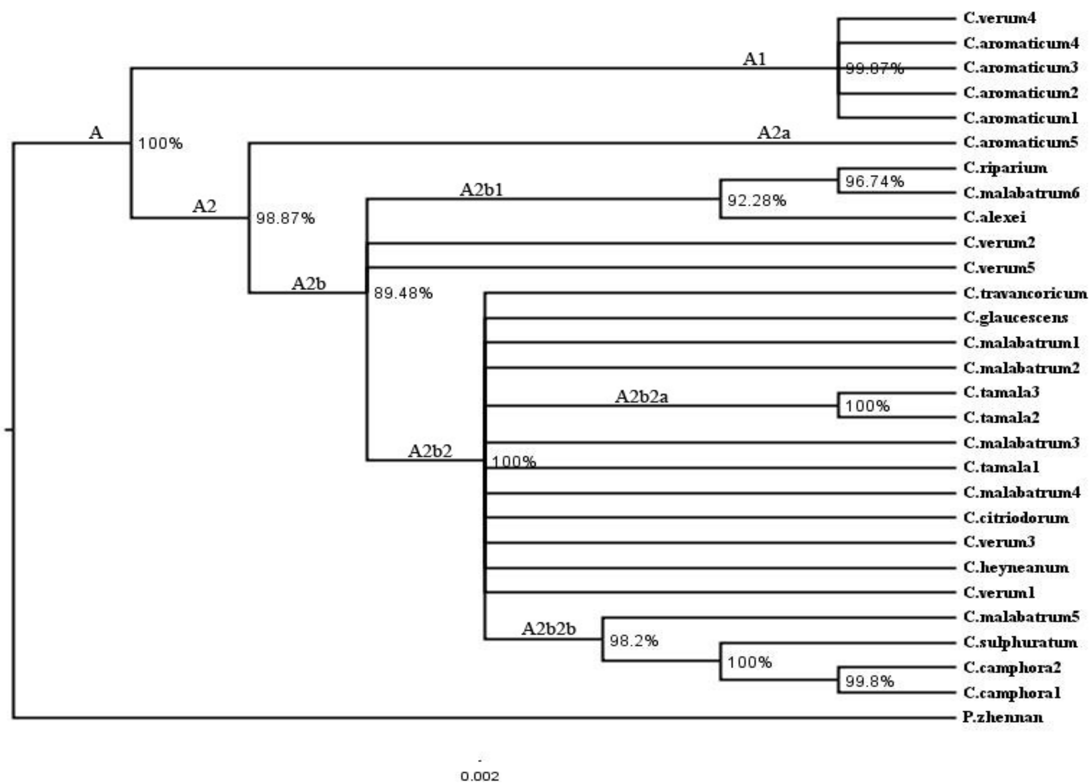


Figure 43. Bayesian phylogram for the combined *matK+psbA-trnH* sequences

The tree was drawn using concatenated *matK* and *psbA-trnH* sequences with overlaid bootstrap support. In this phylogram, different accessions of the same species clustered in different clades. Clade A formed in this tree was divided in to two main subclades A1 and A2. A1 had *C. verum*4 and *C. aromaticum* sequences grouped together with a bootstrap

support of 99.07%. Sublade A2 was divided in to two subclades. A2a had a *C. aromaticum* sample while A2b was further subdivided to 2 subclades A2b1 and A2b2. A2b1 with a bootstrap support of 96.78% had *C. riparium* and *C. malabatrum* clustered together with *C. alexei* as its sister group. Two *C. verum* sequences were also seen unresolved in A2b. A2b2 formed with a bootstrap value of 100% had *C. travancoricum, C. heyneanum, C. verum, C. malabatrum, C. citriodorum, C. camphora* and *C. sulphuratum.* Within subclade A2b2, *C. tamala* sequences formed a monophyletic cluster A2b2a with 100% bootstrap support, and another cluster A2b2b had *C. sulphuratum, C. camphora* and *C. malabatrum* grouped together with high bootstrap values.

4.2.1.3.7. Phylogenetic analysis based on multilocus approach of *rbcL+matK+psbA-trnH*

The phylogram was drawn using the concatenated *rbcL*, *matK* and *psbA-trnH* sequences of the *Cinnamomum* species with overlaid bootstrap support (Figure 44).

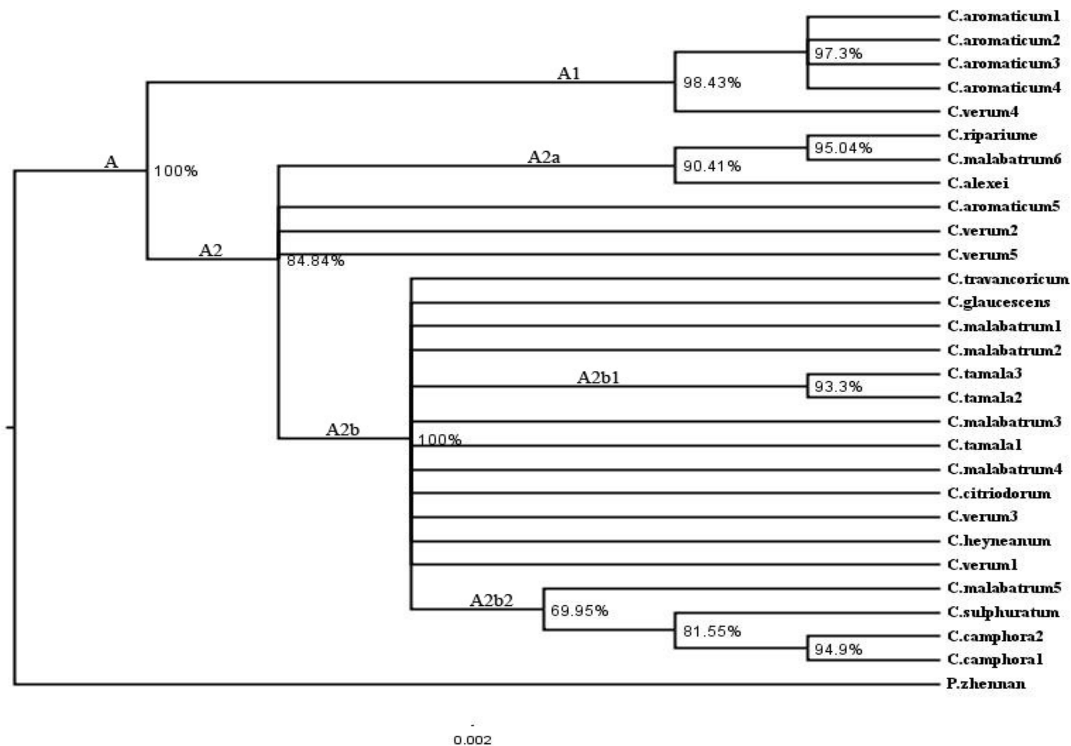


Figure 44. Bayesian phylogram for the combined *rbcL+matK+psbA-trnH* sequences

The outgroup *P. zhennan* was branched separately from the inner *Cinnamomum* group. Different accessions of the same species were positioned in different clades and groups. Clade A was divided into two major clades A1 and A2 with 100% BS. Subclade A1 consisted of *C. verum*4 positioned sister to *C. aromaticum* sequences (97.3% BS). In A2, *C. verum* and *C. aromaticum* was found to be unresolved and it also formed a subclade A2a that

contained *C. alexei* positioned sister to a cluster containing *C. riparium* and *C. malabatrum.* A2b contained subclades A2b1 and A2b2 in addition to *C. travancoricum, C. heyneanum, C. citriodorum, C. glaucescens, C. malabatrum* and *C. verum. C. tamala* sequences formed subclade A2b1 (93.3% BS). A2b2 consisted of a monophyletic group of *C. camphora* with *C. sulphuratum* and *C. malabatrum* positioned sister to it.

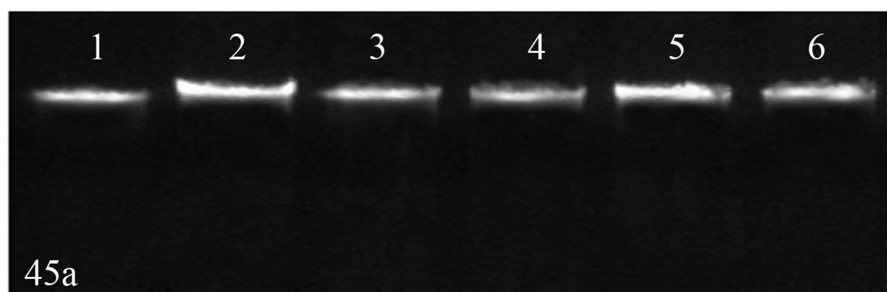
4.2.2. Group I – Nutmeg

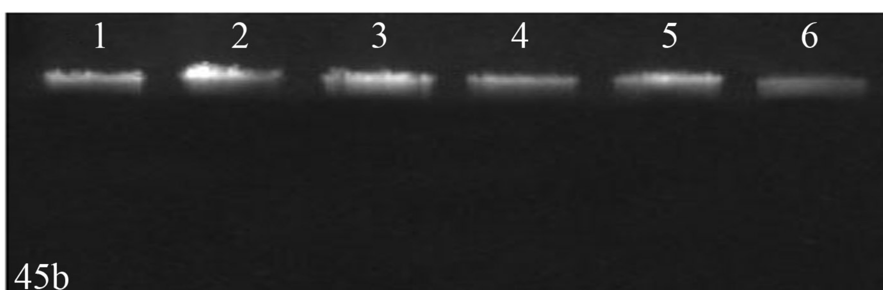
4.2.2.1. Isolation of DNA

Genomic DNA was isolated from the leaves of *Myristica* species using the protocol of Sheeja et al., (2008) with a yield of 135-271 $\mu g g^{-1}$ and purity ratio (A260/A280) in the range 1.8-1.9 (Figures 45a-c).

4.2.2.2. PCR amplification and sequencing of barcoding loci.

The four loci *rbcL, matK, psbA-trnH* and ITS were amplified using a 50 µl reaction mixture containing 1 mM *Taq* assay buffer with 1.5 mM $MgCl_2$, 1 pmole μl^{-1} of forward and reverse primers, 1 U *Taq* DNA polymerase and 50 ng of genomic DNA. They were amplified using the reaction conditions mentioned in Table 18 of section 4.1.2.3. *rbcL, matK* and ITS loci gave 100% amplification success with amplicons of size 600 bp (Figures 46a-b), 900 bp (Figures 47a-b) and 750 bp (Figures 48a-b) respectively. *psbA-trnH* exhibited 95% amplification success with an amplicon size of 450 bp (Figures 49a-b) .





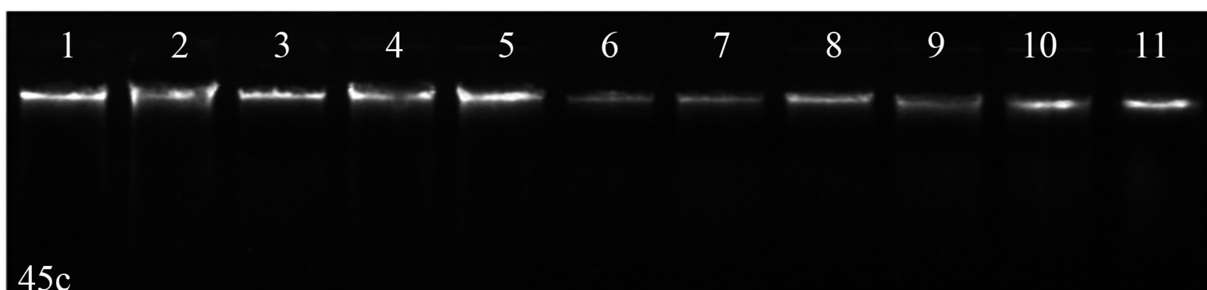
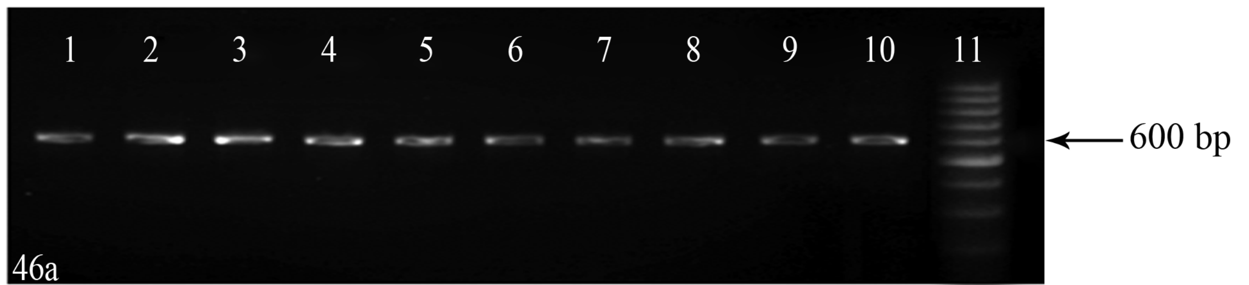
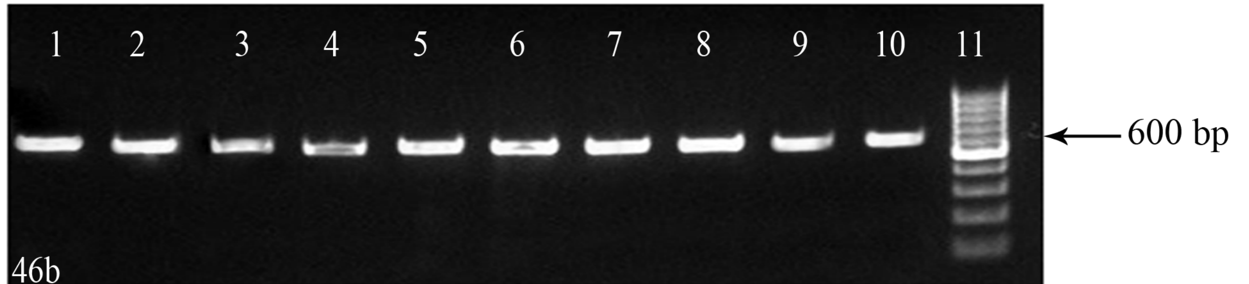
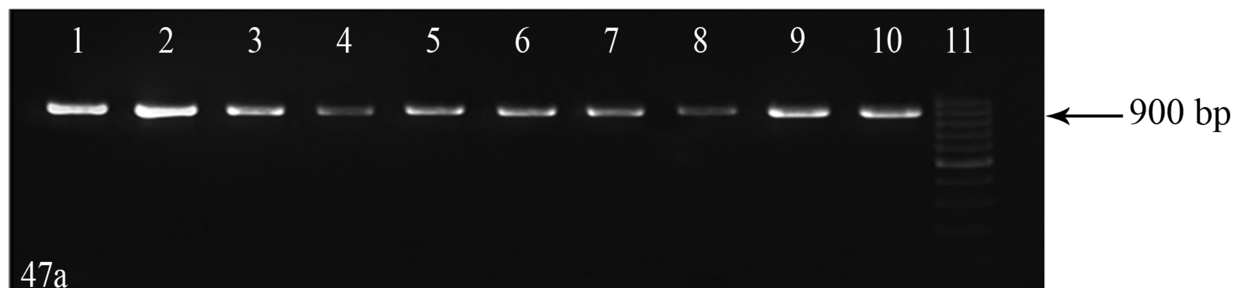


Figure 45a. DNA isolated from *Myristica* species. Lane 1 – Human Genomic DNA (blood cells) (Genei), lanes 2-6– *M. fragrans* accessions.
Figure 45b. DNA isolated from *Myristica* species. Lane 1 – Human Genomic DNA (blood cells) (Genei), lanes 2-6– *M. malabarica* accessions.
Figure 45c. DNA isolated from *Myristica*, *Knema* and *Gymnocranthera* species. Lanes 1-2 – *M. andamanica*, lanes 3-4 – *M. fatua*, lane 5 – *M. amygdalina*, lanes 6-8 – *M. beddomei*, lane 9 – *K. attenuata*, lane 10 - *G. farquhariana*, lane 11 – Human genomic DNA (Blood cells) (Genei).







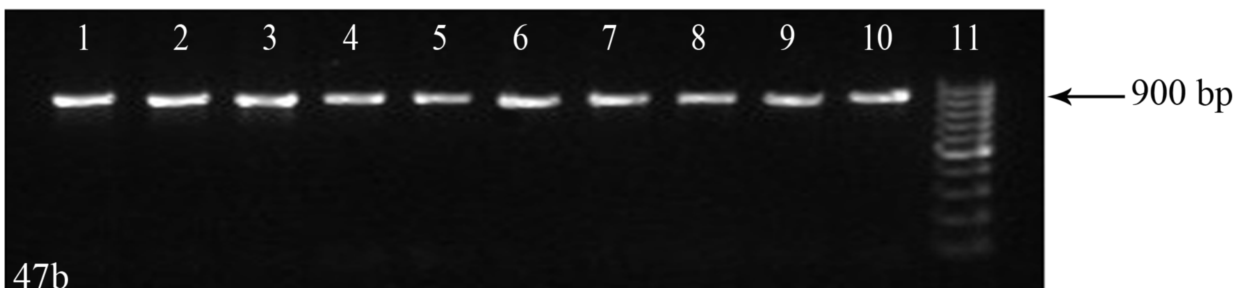
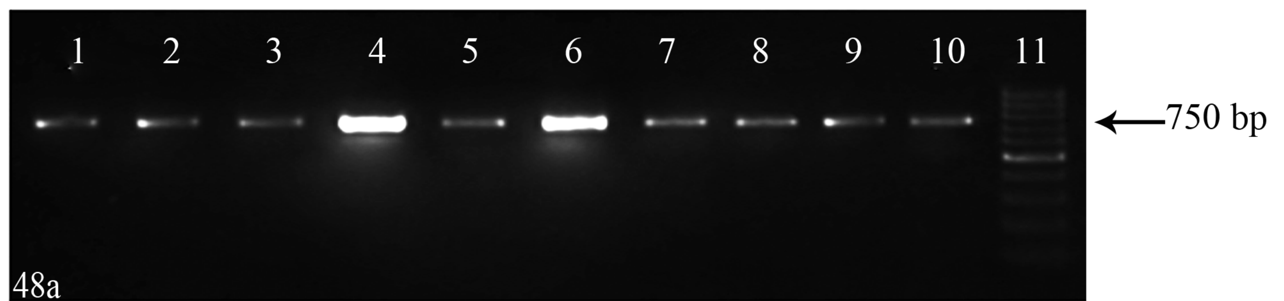
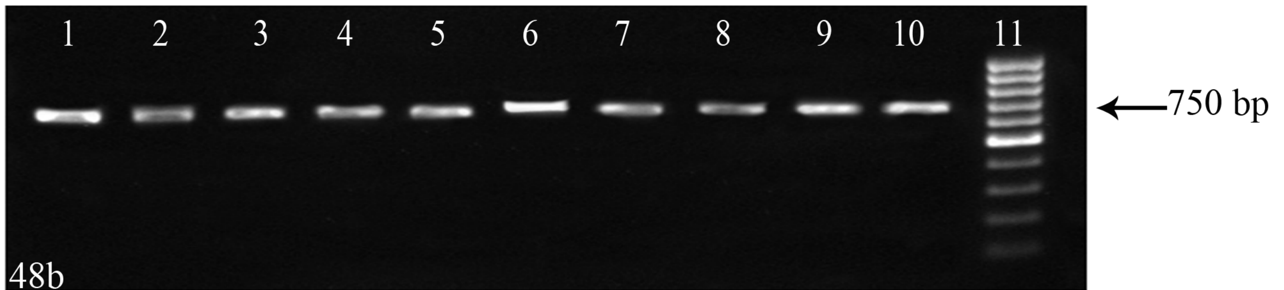
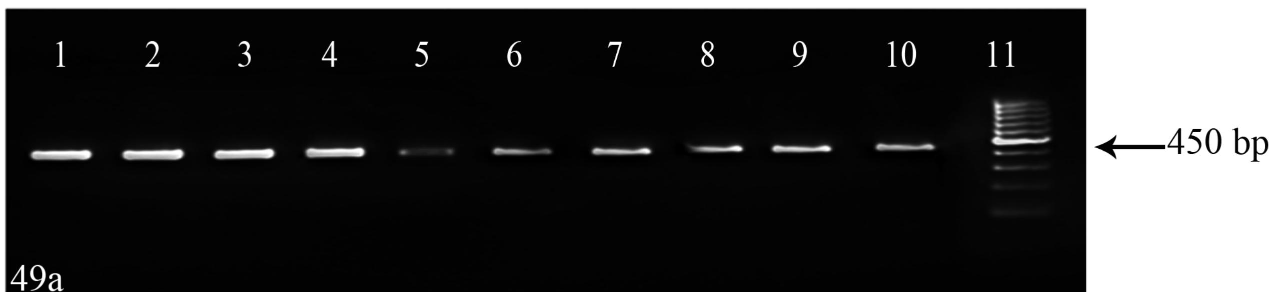


Figure 46a. Amplification of *rbcL* locus in *Myristica* species. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100bp ladder (Fermentas).
Figure 46b. Amplification of *rbcL* locus in *Myristica*, *Knema* and *Gymnocranthera* species. Lanes 1-2 – *M. andamanica*, lanes 3-4 – *M. fatua*, lane 5 – *M. amygdalina*, lanes 6-8 – *M. beddomei*, lane 9 – *K. attenuata*, lane 10 – *G. farquhariana*, lane 11 - 100bp ladder (Fermentas).
Figure 47a. Amplification of *matK* locus in *Myristica* species. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100bp ladder (Fermentas).
Figure 47b. Amplification of *matK* locus in *Myristica, Knema* and *Gymnocranthera* species. Lanes 1-2 – *M. andamanica*, lanes 3-4 – *M. fatua*, lane 5 – *M. amygdalina*, lanes 6-8 – *M. beddomei*, lane 9 – *K. attenuata*, lane 10 – *G. farquhariana*, lane 11 - 100bp ladder (Fermentas).







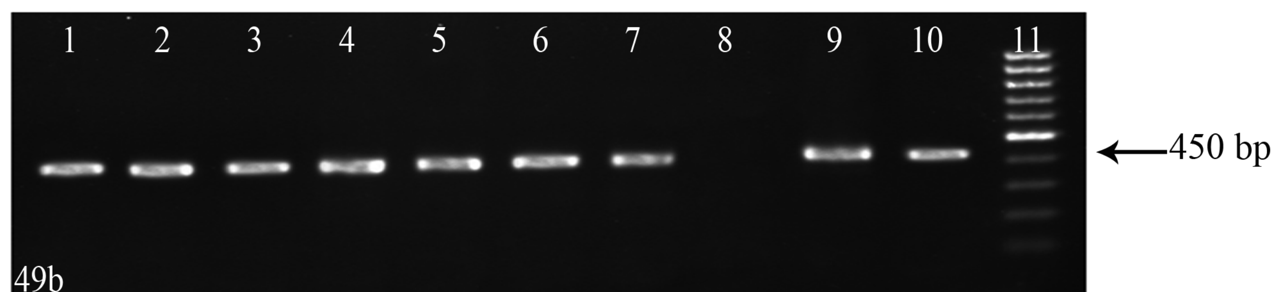


Figure 48a. Amplification of ITS locus in *Myristica* species. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100bp ladder (Fermentas).
Figure 48b. Amplification of ITS locus in *Myristica*,*Knema* and *Gymnocranthera* species. Lanes 1-2 – *M. andamanica*, lanes 3-4 – *M. fatua*, lane 5 – *M. amygdalina*, lanes 6-8 – *M. beddomei*, lane 9 – *K. attenuata*, lane 10 – *G. farquhariana*, lane 11 - 100bp ladder (Fermentas).
Figure 49a. Amplification of *psbA-trnH* locus in *Myristica* species. Lanes 1-5 – *M. fragrans*, lanes 6-10 – *M. malabarica*, lane 11- 100bp ladder (Fermentas).
Figure 49b. Amplification of *psbA-trnH* locus. Lanes 1-2 – *M. andamanica*, lanes 3-4 – *M. fatua*, lane 5 – *M. amygdalina*, lanes 6-8 – *M. beddomei*, lane 9 – *K. attenuata*, lane 10 – *G. farquhariana*, lane 11 - 100bp ladder (Fermentas).

Sequencing success was 100% for *rbcL*, *matK* and *psbA-trnH* while in case of ITS the forward and reverse reads could not be assembled in to contigs due to poor sequence quality. The sequence length range obtained for *rbcL, matK* and *psbA-trnH* was 510 bp-579 bp, 695 bp-782 bp and 299 bp-413 bp respectively. BLAST analysis of the contig sequences obtained for *rbcL, matK* and *psbA-trnH* exhibited maximum similarity to sequences of the respective locus of genus *Myristica* and other genus of family Myristicaceae (Tables 35-37). The generated sequences were deposited in the GenBank database of National Centre for Biotechnology Information (NCBI) (Table 38).

Table 35. BLAST analysis of *rbcL* sequences of *Myristica* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E value
M. fragrans 1	*M. chapelieri*	AF197594	99	99	0
M. fragrans 2	*M. chapelieri*	AF197594	100	99	0
M. fragrans 3	*M. chapelieri*	AF197594	100	99	0
M. fragrans 4	*M. chapelieri*	AF197594	98	99	0
M. fragrans 5	*M. chapelieri*	AF197594	98	99	0
*M. malabarica*1	*H. amygdalina*	KR529437	96	99	0
M. malabarica 2	*M. chapelieri*	AF197594	98	99	0
M. malabarica 3	*M. chapelieri*	AF197594	97	98	0
M. malabarica 4	*M. chapelieri*	AF197594	99	100	0
M. malabarica 5	*M. chapelieri*	AF197594	100	99	0
M. andamanica 1	*Horsfieldia bassifissa*	JF738900	100	99	0
M. andamanica 2	*M. chapelieri*	AF197594	98	99	0
M.fatua 1	*H. amygdalina*	KR529437	100	99	0
M. fatua 2	*M. chapelieri*	AF197594	100	100	0
M. amygdalina	*Horsfieldia bassifissa*	JF738900	99	99	0
M. beddomei 1	*Myristica elliptica*	KU853183	100	99	0
M. beddomei 2	*M. chapelieri*	AF197594	98	99	0
M. beddomei 3	*M. chapelieri*	AF197594	98	99	0
K. attenuata	*M. chapelieri*	AF197594	99	100	0
G. farquhariana	*M. chapelieri*	AF197594	100	99	0

Table 36. BLAST analysis of *matK* sequences of *Myristica* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E value
M. fragrans 1	*Myristica maingayi*	DQ401374	100	99	0
M. fragrans 2	*M. maingayi*	DQ401374	100	99	0
M. fragrans 3	*M. maingayi*	DQ401374	100	99	0
M. fragrans 4	*M. maingayi*	DQ401374	99	99	0
M. fragrans 5	*M. maingayi*	DQ401374	99	100	0
*M. malabarica*1	*M. maingayi*	DQ401374	100	100	0
M. malabarica 2	*M. maingayi*	DQ401374	99	100	0
M. malabarica 3	*M. maingayi*	DQ401374	100	99	0
M. malabarica 4	*M. maingayi*	DQ401374	100	99	0
M. malabarica 5	*M. maingayi*	DQ401374	99	99	0
M. andamanica 1	*M. maingayi*	DQ401374	100	99	0
M. andamanica 2	*M. maingayi*	DQ401374	100	99	0
M. fatua 1	*M. maingayi*	DQ401374	100	99	0
M. fatua 2	*M. maingayi*	DQ401374	100	100	0
M. amygdalina	*M. maingayi*	DQ401374	100	99	0
M. beddomei 1	*M. maingayi*	DQ401374	100	99	0
M. beddomei 2	*M. maingayi*	DQ401374	99	99	0
M. beddomei 3	*M. maingayi*	DQ401374	99	100	0
K. attenuata	*Knema laurina*	AY220450	99	99	0
G. farquhariana	*G. farquhariana*	AY220446	99	99	0

Table 37. BLAST analysis of *psbA-trnH* sequences of *Myristica* spp.

Species	Top Hit Plant from GenBank	Accession Number	Query coverage	% Identity	E value
M. fragrans 1	*M. fragrans*	KT758178	99	99	3e-154
M. fragrans 2	*M. fragrans*	KT758178	86	98	7e-161
M. fragrans 3	*M. fragrans*	KT758178	99	99	1e-162
M. fragrans 4	*M. fragrans*	KT758178	100	99	1e-168
M. fragrans 5	*M. fragrans*	KT758178	99	99	4e-158
M. malabarica 1	*C. preussi*	KC688811	10	94	7e-151
M. malabarica 2	*M. fragrans*	KT758178	82	86	6e-92
M. malabarica 3	*M. fragrans*	KT758178	98	95	1e-143
M. malabarica 4	*C. preussi*	KC688811	98	94	1e-153
M. malabarica 5	*C. preussi*	KC688811	98	94	2e-156
M. andamanica 1	*C. preussi*	KC688811	99	95	9e-176
M. andamanica 2	*C. preussi*	KC688793	100	96	0
M. fatua 1	*Staudtia kamerunensis*	KC688706	100	93	9e-150
M. fatua 2	*M. fragrans*	KT758178	99	94	2e-136
M. amygdalina	*S. kamerunensis*	KC688706	100	94	1e-168
M. beddomei 1	*M. fragrans*	KT758178	100	95	1e-137
M. beddomei 3	*M. fragrans*	KT758178	99	95	1e-143
K. attenuata	*C. preussi*	KC688811	99	95	2e-161
G. farquhariana	*C. preussi*	KC688811	100	85	2e-161

Table 38. GenBank numbers for *Myristica* spp.

Species	Accession Number		
	rbcL	*psbA-trnH*	*matK*
M. fragrans 1	KT367808	KY966347	KT367809
M. fragrans 2	KT380141	KY966348	KT380142
M. fragrans 3	KT445277	KY966349	KT445278
M. fragrans 4	KY945257	KY966350	MF547523
M. fragrans 5	KY945258	KY966351	MF547524
M. malabarica 1	KY945259	KY966342	MF547530
M. malabarica 2	KY945260	KY966343	MF547531
M. malabarica 3	KY945261	KY966344	MF547532
M. malabarica 4	KY945262	KY966345	MF547533
M. malabarica 5	KY945263	KY966346	MF547534
M. andamanica 1	MF158638	MF086596	MF547528
M. andamanica 2	MF158639	MF086597	MF547529
M. fatua 1	MF186596	MF086592	MF547525
M. fatua 2	MF186597	MF086593	MF547526
M. amygdalina	MF417801	MF086598	MF547527
M. beddomei 1	MF186599	MF086594	MF547536
M. beddomei 2	MF186600	-	MF547537
M. beddomei 3	MF186601	MF086595	MF547538
K. attenuata	MF547520	MF086599	MF547535
G. farquhariana	MF547519	MF086600	MF547539

4.2.2.3. Sequence characteristics

Sequences of individual loci viz. *rbcL, matK, psbA-trnH* and concatenated sequences of multilocus combinations viz. *rbcL+matK, rbcL+psbA-trnH*, *matK+psbA-trnH* and *rbcL+matK+psbA-trnH* were aligned and trimmed to a uniform size. Sequences downloaded from GenBank database of NCBI were also included in the phylogenetic analysis of individual loci. *rbcL* sequences taken from GenBank are *M. fragrans* (AF206798), *M. fragrans* (AY298839), *M. fatua* (GQ248653), *Myristica cf. sepicana* (FJ976152), *Myristica globosa* (KF496610), *Staudtia kamerunensis* (KC628429), *S. kamerunensis* (KC628405), *Compsoneura mexicana* (EU090522) *H. amygdalina* (KR529436) and *H. amygdalina* (KR529434). *matK* sequences taken from GenBank are *M. fragrans* (AJ966803), *M. fragrans* (EU669472), *M. fatua* (GQ248165), *Myristica maingayi* (DQ401374), *M. maingayi* (AY220452)*, Myristica yunnanensis* (KR531264), *M. yunnanensis* (KR531265), *Myristica cinnamomea* (KJ709010), *M. cinnamomea* (KJ709009), *C. preussii* (AY743475), *C. preussii* (KC685082), *Knema globularia* (AB924720) and *K. globularia* (AB924868). *psbA-trnH* sequences taken from GenBank are *M. fragrans* (KT758178), *M. fatua* (GQ248350), *M. globosa* (GQ248351), *M. yunnanensis* (KR533281), *M. yunnanensis* (KR533282), *H. amygdalina* (KR533273), *H. amygdalina* (KR533274) *S. kamerunensis* (KC667945), *C. preussii* (KC688793) and *C. preussii* (KC688811). Multilocus combinations were done using those species that generated sequences for all the loci. The characteristics of the aligned sequences that are used in phylogenetic analysis are given in Table 39.

Table 39. Sequence characteristics of the loci for *Myristica* spp.

Sequence Characteristics	*rbcL*	*matK*	*psbA-trnH*	*rbcL+ matK*	*rbcL+ psbA-trnH*	*matK+ psbA-trnH*	*rbcL+matK+ psbA-trnH*
Number of sequences	31	34	30	21	20	20	20
Aligned Length (bp)	474	705	352	1413	905	1302	1810
Number of variable sites	16	400	173	303	16	407	423
Number of Parsimony Informative sites	5	382	137	13	5	137	107
Number of indels	0	41	112	265	431	422	456

4.2.2.3. Phylogenetic Analysis

Phylograms were constructed using single locus and multilocus combinations. The phylogenetic relationship between *Myristica* species was studied using the individual (*rbcL, matK* and *psbA-trnH*) and concatenated (*rbcL+matK*, *rbcL+psbA-trnH*, *matK+psbA-trnH*, *rbcL+matK+psbA-trnH*) sequences with an outgroup using Bayesian inference (BI) methods. Only those species that generated sequences for all the loci were taken for multilocus combinations. The phylogeny inferred from the BI tree for each individual locus and their combination with bootstrap (BS) support is given below. Bootstrap values shown on the trees indicate the number of times the node of the tree is repeated in the replicates.

4.2.2.3.1. Phylogenetic analysis based on *rbcL* sequences

Phylogenetic analysis was conducted using the *rbcL* sequences of *Myristica* species along with sequences taken from GenBank viz. *M. fragrans* (AF206798), *M. fragrans* (AY298839), *M. fatua* (GQ248653), *Myristica cf. sepicana* (FJ976152), *Myristica globosa* (KF496610), *Staudtia kamerunensis* (KC628429), *S. kamerunensis* (KC628405), *Compsoneura Mexicana* (EU090522), *H. amygdalina* (KR529436), *H. amygdalina* (KR529434) and *Magnolia acuminata* (HQ235604) as the outgroup with overlaid bootstrap support. The phylogram was drawn using a final alignment of 474 bp (Figure 50).

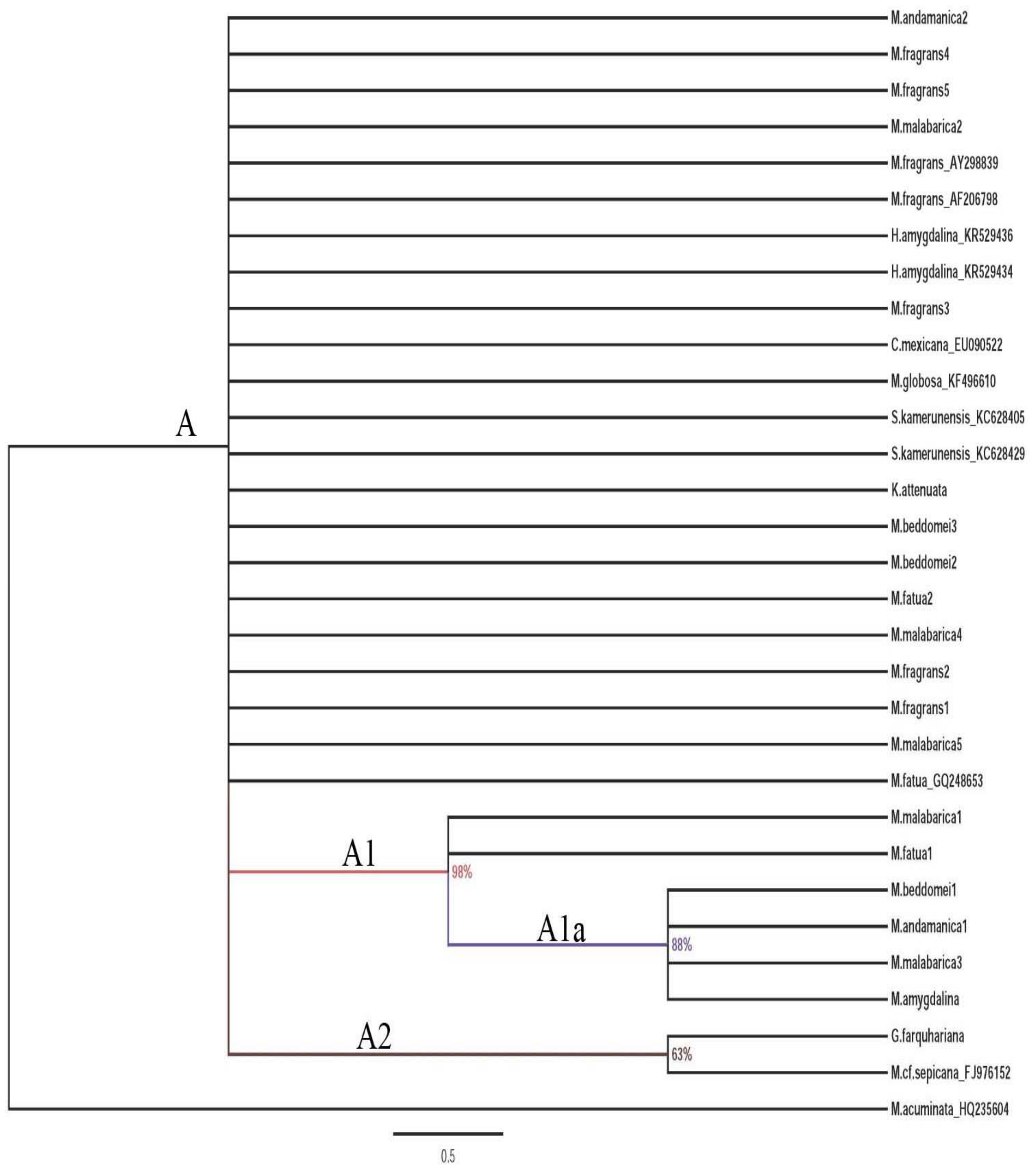


Figure 50. Bayesian phylogram for *rbcL* sequences in *Myristica* spp.

The phylogram obtained was rooted with *M. acuminata,* separated from the inner group. This phylogram was not able to provide any information regarding the phylogeny of *Myristica* spp. The tree exhibited polytomy for the ingroup section. In all the three clades different species grouped together rather than accessions of a species forming a single group. Discrimination of species of different genera was not possible based on the phylogram. In clade A, species of different genera viz. *Myristica, Horsefieldia, Staudtia, Compsoneura* and *Knema* clustered together. Two subclades A1 and A2 were found in the tree. In A1, *M.*

beddomei, M. andamanica, M. malabarica and *M. amygdalina* formed a subclade A1a with *M. malabarica* and *M. fatua* positioned sister to it. The bootstrap value of subclade A was 98% while that of A1a was 88%. *G. farquhariana* and *M. cf. sepicana* formed subclade A2 with a bootstrap support of 63%.

4.2.2.3.2. Phylogenetic analysis based on *matK* sequences

Phylogenetic analysis was conducted using the *matK* sequences of *Myristica* species along with sequences taken from GenBank viz. *M. fragrans* (AJ966803), *M. fragrans* (EU669472), *M. fatua* (GQ248165), *Myristica maingayi* (DQ401374), *M. maingayi* (AY220452), *Myristica yunnanensis* (KR531264), *M. yunnanensis* (KR531265), *Myristica cinnamomea* (KJ709010), *M. cinnamomea* (KJ709009), *C. preussii*(AY743475), *C. preussii* (KC685082), *Knema globularia* (AB924720), *K. globularia* (AB924868) and *M. acuminata* (HQ235322) as the outgroup. The phylogenetic tree was drawn based on *matK* sequences with a final alignment of 688 bp and was comprised of two clades (Figure 51).

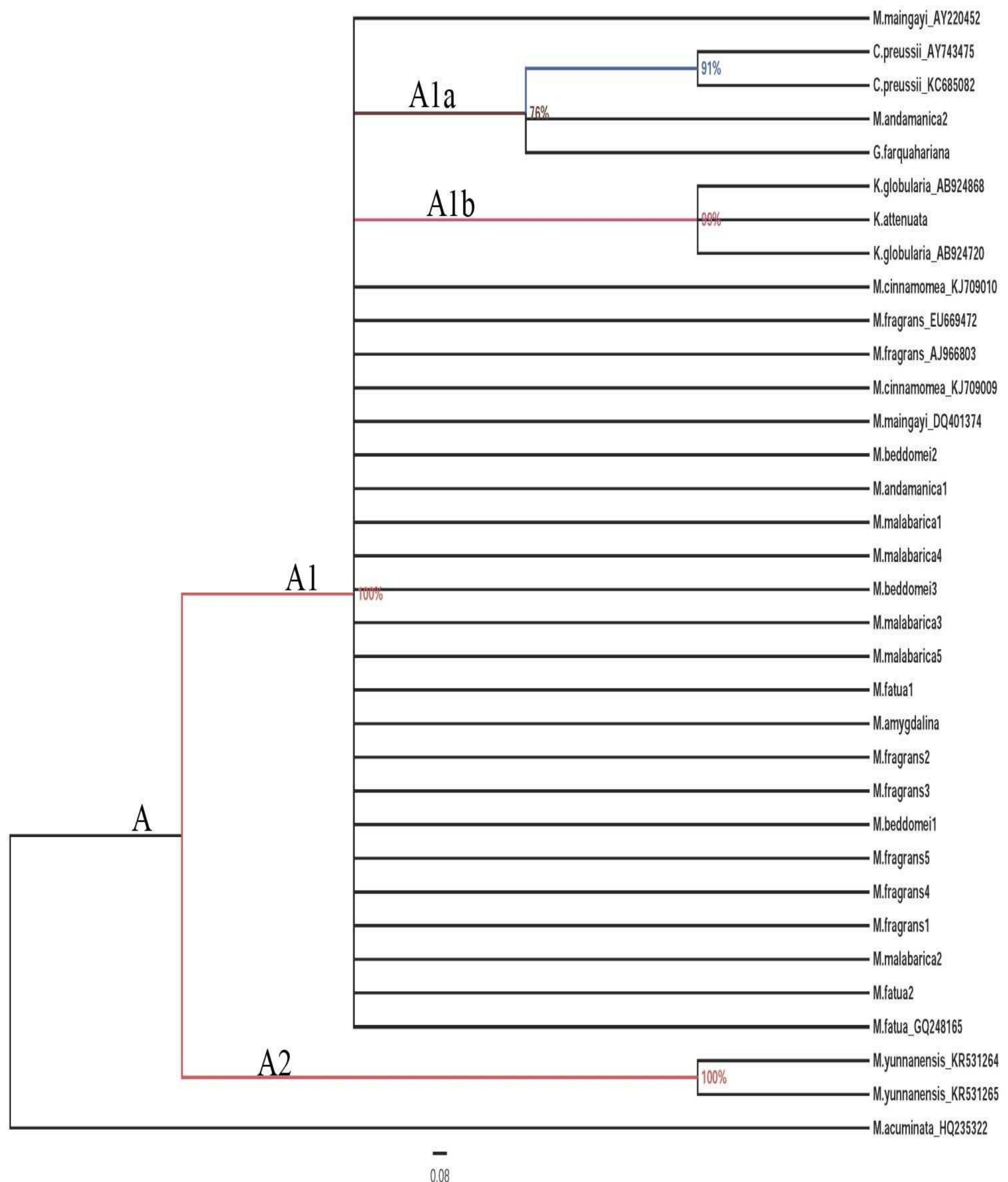


Figure 51. Bayesian phylogram for *matK* sequences in *Myristica* spp.

The *matK* phylogram was rooted with *M. acuminata* that branched separately from the inner group. Differentiation of *Myristica* species was not possible using this locus. Polytomy was exhibited in the ingroup section. Accessions of the different *Myristica* species clustered together. The tree supported with moderate to high boot strap support was composed of two main clades A1 and A2. Within clade A1, two subclades (A1a and A1b) were formed. In A1a, *C. preussii* sequences downloaded from GenBank formed a monophyletic clade (91% BS) with *G. farquhariana* and *M andamanica* positioned sister to it with a bootstrap support

of 76%. *K. attenuata* together with two species of *K. globularia* formed subclade A2 with 90% BS. Clade B was a monophyletic clade of *M. yunnanensis* downloaded from GenBank.

4.2.2.3.3. Phylogenetic analysis based on *psbA-trnH* sequences

Phylogenetic analysis was conducted using *psbA-trnH* sequences of *Myristica* species along with sequences taken from GenBank viz. *M. fragrans* (KT758178), *M. fatua* (GQ248350), *M. globosa* (GQ248351), *M. yunnanensis* (KR533281), *M. yunnanensis* (KR533282), *H. amygdalina* (KR533273), *H. amygdalina* (KR533274) *S. kamerunensis* (KC667945), *C. preussii* (KC688793), *C. preussii* (KC688811) and *M. acuminata* (AY727183) as the outgroup. The phylogenetic tree was drawn using *psbA-trnH* sequences with a final alignment of 281 bp (Figure 52).

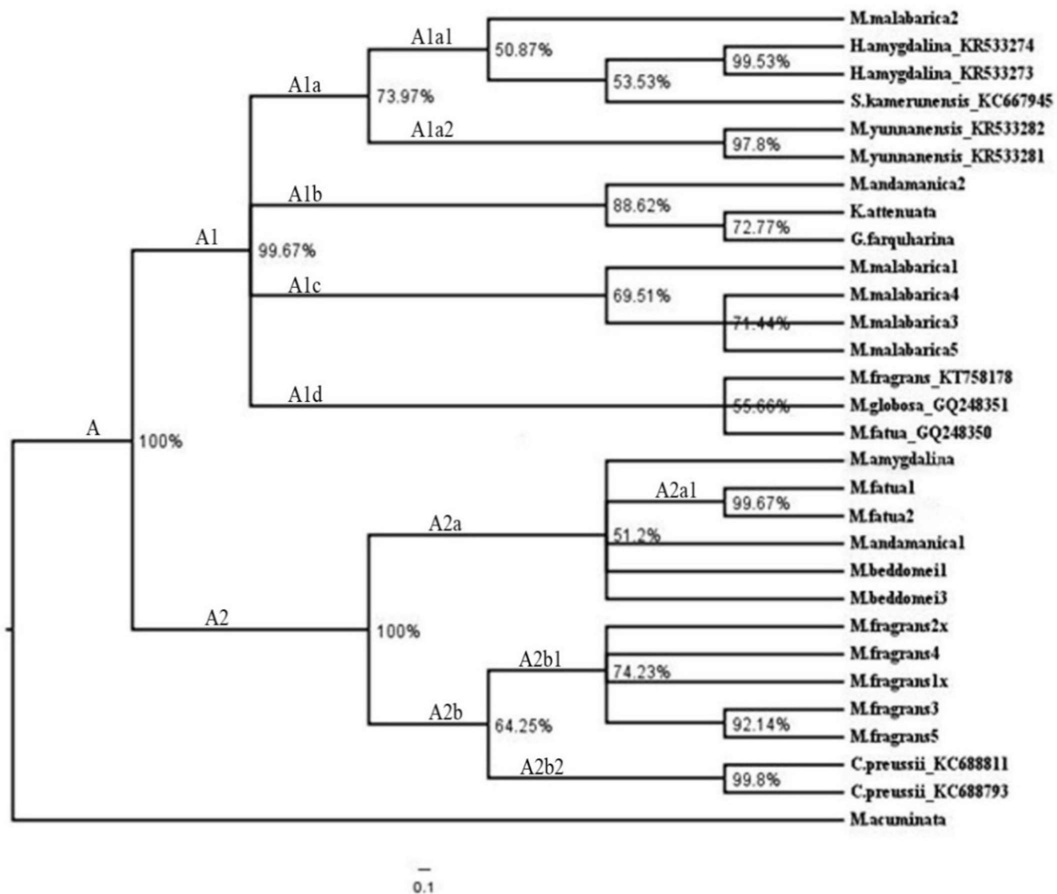


Figure 52. Bayesian phylogram for *psbA-trnH* sequences in *Myristica* spp.

The phylogram constructed had the outgroup *M. acuminata* branched separately from the main inner clade A which was divided in to two subclades A1 and A2 with 100% BS. Subclade A1 was further divided in to subclades A1a, A1b, A1c and A1d with weak to high bootstrap support. A1a with a BS support of 73.97% was further grouped to subclades A1a1 and A1a2. In subclade A1a1, *M. malabarica* occupied a sister position with weak bootstrap

support (50.87% BS) to a cluster comprised of *S. kamerunensis* (53.53% BS) positioned sister to a monophyletic group of *H. amygdalina* (99.53% BS). A1a2 was monophyletic subclade of *M. yunnanensis* that appeared sister to subclade A1a1.

A1b (88.62% BS) was a paraphyletic group of *K. attenuata* and *G. farquhariana* (72.77% BS) to which *M. andamanica* occupied a sister position. A1c was a monophyletic clade of *M. malabarica* sequences while A1d was yet another paraphyletic group containing sequences of *M. fragrans, M. fatua* and *M. globosa* downloaded from GenBank.

Subclade A2 with 100% BS was subdivided to two subclades A2a and A2b. A2a was a paraphyletic group with *M. amygdalina, M. beddomei* and *M. andamanica* clustered together. Within this clade, a monophyletic subclade of *M. fatua* (A2a1) was formed with 99.67% bootstrap support. A2b was composed of monophyletic subclades of *M. fragrans* (A2b1) and *C. preussii* (A2b2) with moderate to high bootstrap support.

4.2.2.3.4. Phylogenetic analysis based on multilocus approach of *rbcL+matK*

The *rbcL+matK* phylogram was drawn using the concatenated *rbcL* and *matK* sequences. The dataset contained 20 sequences trimmed to a final alignment of 1413 bp that was used to generate a phylogenetic tree (Figure 53)

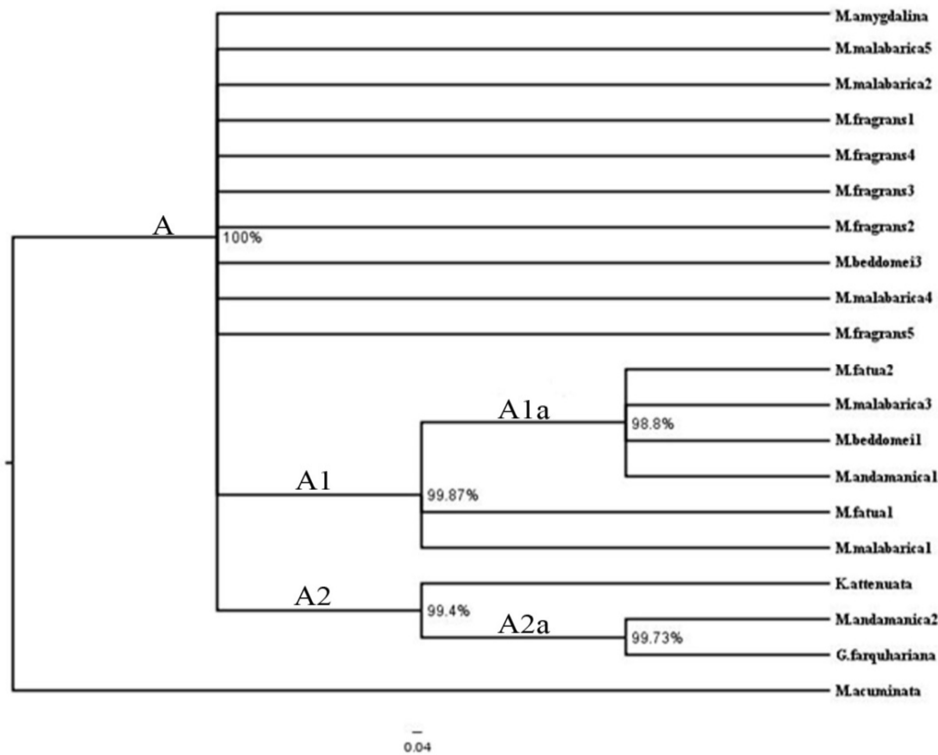


Figure 53. Bayesian phylogram for the combined *rbcL+matK* sequences in *Myristica* spp.

The *rbcL-matK* phylogram was rooted with *M. acuminata* that branched separately from the inner group. High bootstrap support was obtained for the nodes but different accessions of the same species were seen in different clades. Relationship between *M. fragrans, M. malabarica* and *M. amygdalina* remained unresolved. Two subclades A1 and A2 were formed. A1 contained a paraphyletic subclade A1a with 98.8% boot strap value containing *M. malabarica, M. amygdalina, M. andamanica* and *M. beddomei* clustered together. *M. fatua* and *M. malabarica* appeared sister to A1a with 99.4% boot strap support. A2 was comprised of a paraphyletic subclade A2a containing *M. andamanica*2 and *G. farquhariana. K. attenuata* was seen as a sister group to A2a within subclade A2.

4.2.2.3.5. Phylogenetic analysis based on multilocus approach of *rbcL+psbA-trnH*

The *rbcL+psbA-trnH* phylogenetic tree was drawn using the concatenated dataset of *rbcL* and *psbA-trnH* sequences aligned to a length of 905 bp (Figure 54).

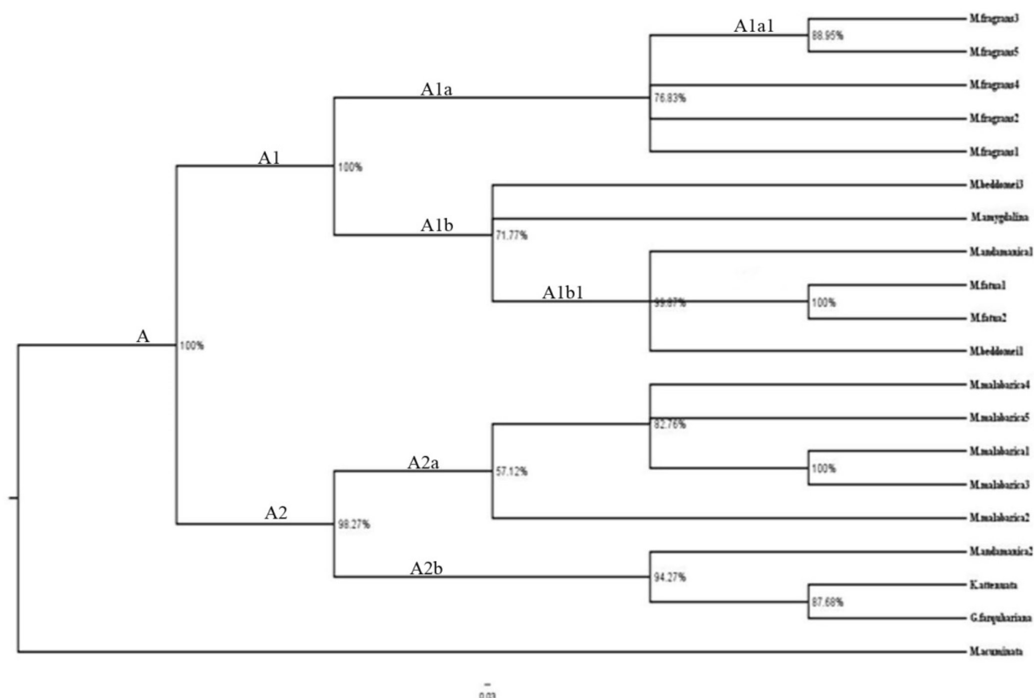


Figure 54. Bayesian phylogram for the combined *rbcL+psbA-trnH* sequences in *Myristica* spp.

A strict *rbcL+psbA-trnH* phylogram was drawn with the outgroup *M. acuminata* branched separately from the inner clade A with moderate to high bootstrap support. The phylogram was divided in to two subclades A1 and A2. A1 was divided in to subclades A1a and A1b.

Subclade A1a was a monophyletic group of *M. fragrans* (77.77% BS) and subclade A1b was a paraphyletic group consisting of *M. fatua, M. beddomei, M. andamanica* and *M. amygdalina.* Within subclade A1b**,** *M. fatua, M. andamanica* and *M. beddomei* formed a subclade A1b1 in which *M. fatua* formed a monophyletic group with 100% bootstrap support. Subclade A2 was divided in to two subclades A2a and A2b. A2a was a monophyletic group of *M. malabarica* sequences while *M. andamanica, G. farquhariana* and *K. attenuata* (71.32% BS) formed A2b.

4.2.2.3.6. Phylogenetic analysis based on multilocus approach of *matK+psbA-trnH*

The *matK+psbA-trnH* phylogenetic tree was drawn using the concatenated dataset of *matK* and *psbA-trnH* sequences aligned to a length of 1302 bp (Figure 55).

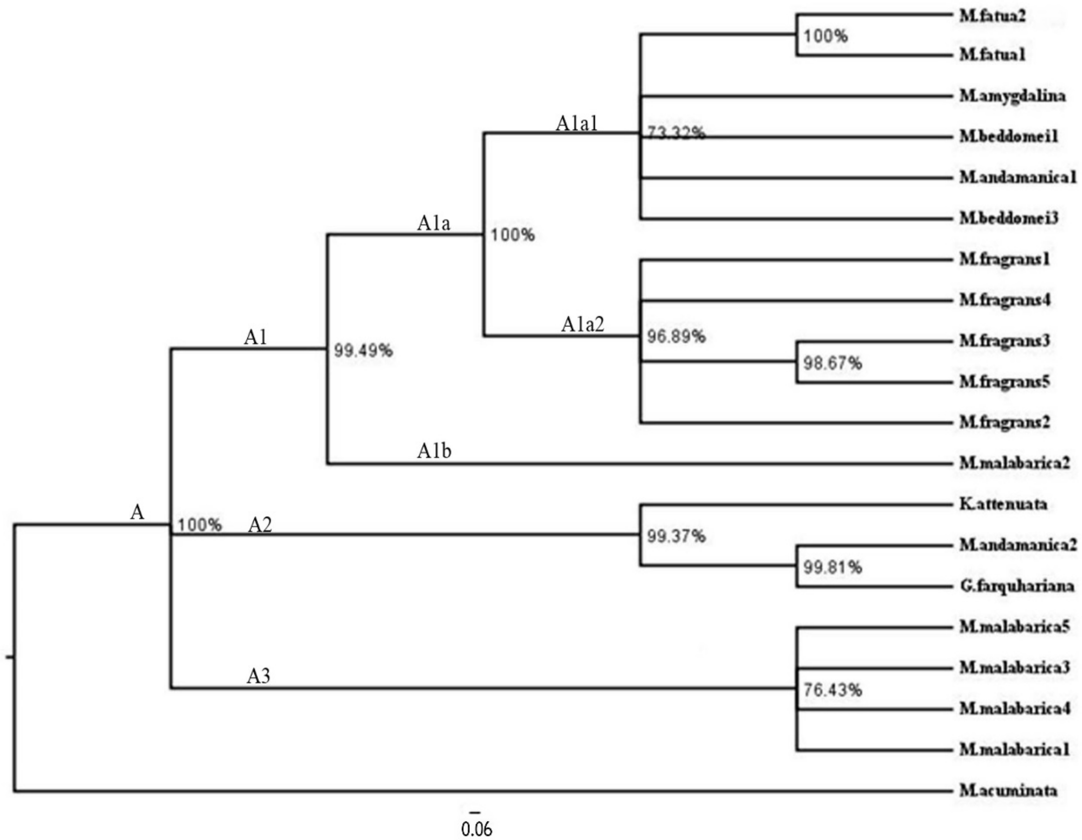


Figure 55. Bayesian phylogram for the combined *matK+psbA-trnH* sequences in *Myristica* spp.

A *matK+psbA-trnH* phylogram was drawn with the outgroup *M. acuminata* branched separately from the inner clade A with high bootstrap support consisting of three subclades. Subclade A1 (99.49%) was further subdivided into subclades A1a and A1b. Subclade A1a

(100% BS) consisted of 2 subclades A1a1 and A1a2. A1a1 (73.32% BS) consisted of *M. beddomei, M. amygdalina, M. andamanica* and a monophyletic group of *M. fatua* (100% BS). Subclade A1a2 was a monophyletic group of *M. fragrans* formed with a bootstrap support of 96.89%. *M. malabarica* 2 was positioned in A1b. *K. attenuata, M. andamanica* and *G. farquhariana* clustered together in A2 (100% BS); *K. attenuata* (99.37% BS) was positioned sister to the paraphyletic group (99.81% BS) consisting of *M. andamanica* and *G. farquhariana.* Four samples of *M. malabarica* grouped together to form the subclade A3 (76.43% BS).

4.2.2.3.7. Phylogenetic analysis based on multilocus approach of *rbcL+matK+psbA-trnH*

The *rbcL+matK+psbA-trnH* phylogenetic tree was drawn using the concatenated dataset of *rbcL*, *matK* and *psbA-trnH* containing 20 sequences with a final alignment of 1810 bp (Figure 56).

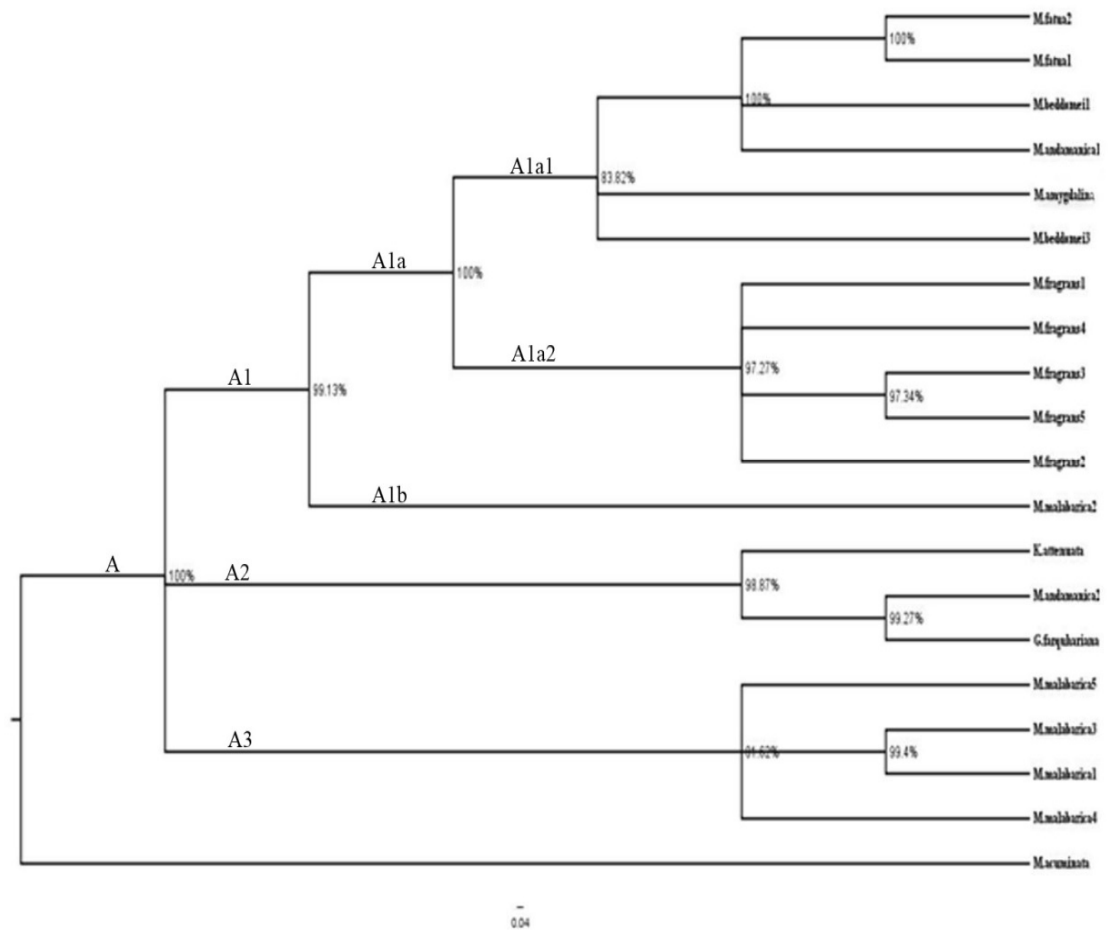


Figure 56. Bayesian phylogram for the combined *rbcL+matK+psbA-trnH* sequences in *Myristica* spp.

The phylogram was drawn using the concatenated sequences of *rbcL, matK* and *psbA-trnH* with the outgroup *M. acuminata* branched separately from the inner clade A. The topology of the tree was similar to the *matK+psbA-trnH* tree with high bootstrap support. Three subclades A1, A2 and A3 were formed with subclade A1 further divided in to A1a and A1b with 100% BS. A1a contained *M. amygdalina* and *M. beddomei* positioned sister to a paraphyletic subclade A1a1 that contained a monophyletic group of *M. fatua* (100% BS). *M. beddomei* and *M. andamanica*1(100%) occupied a sister position to *M. fatua* group. *M. fragrans* accessions grouped together to form the monophyletic subclade A1a2. *M. malabarica* 2 was positioned in subclade A1b. *K. attenuata, M. andamanica* and *G. farquhariana* clustered together in A2 (100% BS); *K. attenuata* (98.87% BS) was positioned sister to the paraphyletic group (99.27% BS) consisting of *M. andamanica*2 and *G. farquhariana.* Four accessions of *M. malabarica* grouped together to form subclade A3. (81.62% BS).

Chapter 5

Discussion

Spices are agricultural products that occupy a pivotal position in the international trade. They are traded in the form of whole or powdered spices, essential oils, oleoresins, dehydrated pastes and extractives (Sasikumar et al., 2016). All these products assume economic significance owing to their application in food, medical, cosmetic and perfumery industries. Fraudulent adulteration of spices by default or design to obtain economical gain has been reported. Adulteration of spices with inferior substances or closely related inferior plant species may have a disastrous effect on public health besides eroding the biological efficiency of the commodity.

Adulteration detection or food product authentication is a matter of primary concern for food processors and consumers in order to meet food quality and safety requirements (Man et al., 2005). The trade of spices are under the scrutiny of international organisations like the International Organisation for Standardisation (ISO), American Spice Trade Association (ASTA), The Food Safety and Standards Authority, India (FSSAI) etc. that takes measures to ensure that the products meet the quality specifications put forward by them to ensure food quality and safety.

Cinnamon (*Cinnamomum verum*) and nutmeg (*Myristica fragrans*) are two important aromatic tree spices that are vital in food, medical and cosmetic industries that are often substituted by their inferior counterparts.

C. verum or Ceylon cinnamon, with its distinctive aroma and taste is the true cinnamon that is widely used as a flavorant. It is mostly substituted by its inferior counterpart *C. cassia* that is found to contain 5% coumarin, a hepatotoxic substance that cause health problems to sensitive individuals if consumed above the tolerable limits (Lungarini et al., 2008). It is also adulterated with *C. malabatrum* grown in homestead gardens of India and Sri Lanka (Swetha et al., 2014).

According to FAO statistics of 2014 (http://www.fao.org/faostat/en/#data/QC) the annual production of true cinnamon was found to be 51,963 tonnes that falls short to meet the global demand. This shortage has become an incentive for the fraudulent adulteration of *C. verum* with Cassia cinnamon obtained from *C. cassia* in China, *C. loureirii* from Vietnam and *C.*

burmannii from Indonesia. It has been found that since the past few years the average productivity of *C. verum* remained constant at 500 kg/ha while that of Cassia cinnamon from China and Indonesia has depicted an average productivity of 1350 kg/ha and 1000 kg/ha, respectively (Piyasiri and Wijeratne, 2016). *C. verum* has declined 1% in its global trade while an increasing trend has been observed in the export of cassia products (Piyasiri, 2014).

The global import of cinnamon and cinnamon flowers in 2016 was estimated to be 1,53,213 tonnes, worth 470.19 million US $ which were mainly exported from Indonesia (48,900 tonnes worth 94.15 million US $), China (42,417 tonnes worth 91.51 million US $), Vietnam (29,981 tonnes worth 71.75 million US $) and Sri Lanka (16,133 tonnes worth 158.69 million US $) (http://www.intracen.org/itc/market-info-tools/statistics-export-product - country/).

ITC trade statistics showed that the import of cinnamon by India in 2016 was 27,393 tonnes worth 44.5 million US $, mainly from Vietnam (19,910 tonnes worth 41.04 million US $) and China (4577 tonnes worth 4.71 million US $). Only 474 tonnes of cinnamon worth 2.50 million US $) were imported from Sri Lanka (http://www.intracen.org/ itc/ market - info-tools/statistics-export-product-country/). This trade data sheds light on the fact that the Indian markets are dominated by inferior cinnamon that has been banned by the food regulatory agencies like FSSAI. This aspect has also been reported by the print media. It is really difficult to differentiate between the genuine and counterfeit cinnamon by mere look.

M. fragrans is found to be mostly adulterated with its closely related *M. malabarica.* It is possible to distinguish between the two in fresh form based on the colour of their mace. Mace of *M. fragrans* is red while that of *M. malabarica* is yellow. But seasoned mace of these two species is of the same colour (yellow) that makes their sensory discrimination difficult. This aspect made the substitution or adulteration of *M. fragrans* mace with *M. malabarica* quite easy (Swetha et al., 2017).

Authentication of spices and herbs is mainly done using physical, chemical and molecular methods (Sasikumar et al., 2016). Physical methods include differentiation based on their density, bulk solubility (Dhanya and Sasikumar, 2010), macroscopic characters (Torey et al., 2010) and microscopic characters (Tremlova, 2001; Zu and Zhao, 2014). Analytical methods employed involve Thin layer chromatography (TLC) (Darr et al., 2013; Mohammad et al., 2015), High performance liquid chromatography (HPLC) (Lage and Cantrell, 2009; Blahov and Svobodov, 2012; Parvathy et al., 2014), Capillary electrophoresis (Zougagh et

al., 2005), UV-visible spectroscopy (di Anibal et al., 2014; Zalacain et al., 2015), Raman spectroscopy (di Anibal, 2010), Nuclear magnetic resonance spectroscopy (NMR) (Yilmaz et al., 2010; di Anibal et al., 2011; Petrakis et al., 2015) and Mass Spectroscopy (Silvis et al., 2015). The need for skilled experts and time consuming process of physical methods and the influence of environmental conditions on the chemical fingerprint and demand for expensive standards for the analytical methods restricts their use in authentication (Swetha et al., 2016). DNA based methods finds more application in authentication of spices especially to trace out plant based adulterants due to their specificity and sensitivity (Mafra et al., 2008; Dhanya, 2009).

Among the molecular methods, DNA barcoding, a technique based on the amplification of short nucleotide regions called barcodes conserved within a species, is now widely used for authentication of food commodities including spices (Galimberti et al., 2014; Scarano and Rao, 2014; Parvathy et al., 2014; Swetha et al., 2014; Parvathy et al., 2015; Soffritti et al., 2016; Swetha et al., 2016). Preservation of the barcode regions in the processed foods facilitates its use in quality control, guaranteeing food safety and minimizing food piracy (Barcaccia et al., 2016). Reliable identification of a commodity by barcoding approach depends on the recovery of a barcode sequence from a sample, representation of relevant representative species in reference database and sufficient nucleotide variability in the sequence to differentiate between closely related species (Stoeckle et al., 2011).

5.1. Commodity Authentication using Barcoding

5.1.1. Sampling

Comparison of a barcode sequence of an organism with the reference database containing sequences of its closely related species is necessary for its identification (Singh et al., 2012). Thus reference database is very important in barcoding. The number of specimens to be included in a reference database for species identification has been a point of concern in barcoding (Zhang et al., 2010). Excessive number of individuals in a reference database is always waste of effort and increases the expenditure (Liu et al., 2012). DNA barcoding database has suggested the use of 5-10 individuals per species (http://www.barcodinglife.org/ views/login.php)

In this study we have taken leaves from 5 samples of *C. verum,* 5 samples of *C. aromaticum* and 6 samples of *C. malabatrum* as reference and 10 samples of traded cinnamon barks. In

nutmeg group, 5 samples each of *M. fragrans* and *M. malabarica* were taken as reference. Five samples of traded nutmeg mace were tested for the presence of adulterants.

The replicate samples of the reference species were included to determine the intraspecific divergence (Xin et al., 2013). Use of multiple individuals per species is also required as it allows comparison to be made between the sequences (de Vere et al., 2012). The effect of geographical isolation on intraspecific variation in barcoding can also be studied by using multiple individuals of a species (Pettengil and Neel, 2010; Zhang et al., 2014b; Bhagwat et al., 2015).

Samples of 2-5 accessions per species were taken for the authentication of *Illicium verum* from its adulterants (Meizi et al., 2012). Federici et al., (2013) reported sampling of 3-5 individuals per *Thymus* species for distinguishing between *Thymus* species using DNA barcoding. Two to five individuals for each species were taken for a barcoding study on *Roscoea* species (Zhang et al., 2014b). Kumar et al., (2015) sampled 3-4 accessions per species for the authentication of the raw drug 'Bala' sold in the markets. Three to seven accessions per species were taken for the authentication of the traditional Chinese medicines Arisaematis Rhizoma and Pinelliae Tuber (Moon et al., 2016).

5.1.2. Isolation of DNA

Isolation of high quality DNA is prerequisite for any molecular method. But DNA isolation from plant tissues is often problematic due to the presence of polysaccharides, proteins and polyphenols contained in them (Pirttila et al., 2001). The problems that are mainly encountered are endonuclease degradation of DNA and co-isolation of the highly viscous polysaccharides and inhibitors like polyphenols (Khanuja et al., 1999). So an effective DNA isolation protocol can be perfected only through trial and error methods (Syamkumar et al., 2003; Remya et al., 2004).

5.1.2.1. Group I – Cinnamon

5.1.2.1.1 Isolation of DNA from *Cinnamomum* species

DNA was isolated from the leaves of *Cinnamomum* species using the Qiagen DNeasy kit. Isolation of high quality DNA from leaves of different plants have been done using Qiagen kit (Lee et al., 2016)

5.1.2.1.2. Isolation of DNA from cinnamon barks

Barks/wood are recalcitrant tissues that are rich in sap, polysaccharides, polyphenols, proteins, lignins, tannins, secondary metabolites and pigments that hinder DNA isolation (de Filipis and Magel, 1998; Finkeldey et al., 2010). Polysaccharides form complexes with nucleic acids forming a gelatinous mass that hinder the activity of DNA modifying enzymes like polymerases, ligases and restriction enzymes (Sharma et al., 2002; Karaca et al., 2005; Singh and Kumar, 2012). Co-precipitation of polysaccharides along with DNA make it viscous causing difficulty while loading in the gel (Sablok et al., 2009). Polyphenolic compounds are oxidised on cell disruption and irreversibly react with protein and nucleic acid fractions thereby hindering further downstream applications of DNA (Loomis, 1974; Deshmukh et al., 2007).

Several protocols, established for isolating DNA from recalcitrant tissues like rhizomes of turmeric and ginger (Syamkumar et al., 2003), dried capsules of cardamom (Syamkumar et al., 2005), processed wood of *Gonystylus bacanus* (Asif and Cannon, 2005), dried berries of black pepper (Dhanya et al., 2007) and stem bark of leguminosae trees (Novaes et al., 2009) were tried to isolate DNA from cinnamon barks. But all these protocols failed to yield DNA. Though several modifications to all these protocols were also tried, only the modified Asif and Cannon (2005) method yielded DNA (Swetha et al., 2014). The modifications done in Asif and Cannon method included the use of 10 ml of homogenisation buffer for 1g of powdered bark, increased concentration of CTAB (5%) and sodium chloride (3M) in extraction buffer, addition of PVP in extraction buffer, addition of sodium acetate during chloroform-isoamylalcohol extraction and precipitation of DNA using isopropanol (Swetha et al., 2014).

Increased concentration of CTAB (detergent) and sodium chloride has been found effective in the removal of polysaccharides in different plant tissues (Lele, 2011; Syamkumar et al., 2005). The use of sodium chloride concentration greater than 0.5 M along with CTAB has

been found to efficiently remove polysaccharides (Sahu et al., 2012). In the presence of high concentration of sodium chloride, CTAB form complexes with polysaccharides and proteins that can be removed during chloroform extraction (Maliyakal, 1992; Surzycki, 2000; Michiels et al., 2003; Jitu and Konwar, 2008; Maltas et al., 2011). Inclusion of PVP has been found to be effective in removal of phenolic compounds (Lele, 2011; Shankar et al., 2011). PVP forms complex hydrogen bonds with phenolic compounds, co-precipitates with cell debris on lysis and can be removed during chloroform extraction (Maliyakal et al., 1992). Though Syamkumar et al., (2005) reported ethanol precipitation superior over isopropanol to get better DNA yield, it was not consistent with our results. Isopropanol precipitation was found better in our case. DNA precipitation has been done from different plant tissues like leaves, stem bark and seeds using isopropanol (Kim et al., 1997; Deshmukh et al., 2007; Novaes et al., 2009; Arif et al., 2010; Lele, 2011; Singh and Kumar, 2012).

Another problem associated with DNA isolation from bark was that the DNA yield may be low due to partial degradation of DNA (Rachmayanti et al., 2009; Tang et al., 2011; Jiao et al., 2012). Degradation of DNA may be due to the mechanical treatment employed during bark harvesting process or microbial decomposition of wood. Drying of bark/wood may also cause cell death following which DNA may be fragmented in to small pieces (Finkedeley et al., 2007). Hence successful amplification of DNA fragments by PCR is considered to be more appropriate than the spectrophotometric estimation of quality and quantity of DNA (Finkeldey et al., 2010).

5.1.2.2. Group II – Nutmeg

5.1.2.2.1. Isolation of DNA from *Myristica* species.

High quality DNA was isolated using the protocol of Sheeja et al., (2008) developed for isolating DNA from nutmeg leaves.

5.1.2.2.2. Isolation of DNA from nutmeg mace

Isolation of DNA from recalcitrant tissues is difficult due to the presence of polysaccharides, polyphenols, secondary metabolites and degradation by endonucleases (Varma et al., 2007; Amani et al., 2011; Anuradha et al., 2013). Mace being a recalcitrant tissue, different methods were tried to isolate DNA from it viz., the isolation protocols used for nutmeg leaves (Sheeja et al., 2008), nutmeg seeds (Lele, 2011), okra (Singh and Kumar, 2012), hibiscus (Reddy, 2009), and recalcitrant dried berries of black pepper (Dhanya et al., 2007). Successful DNA isolation was accomplished by modifying the protocol of Dhanya et al.,

(2007) by overnight soaking of the mace in water, replacing CTAB with sodium dodecyl sulphate (SDS), incorporating 2% PVP and 1% β-mercaptoethanol in extraction buffer and increasing the concentration of potassium acetate to 6 M. (Swetha et al., 2017)

Overnight soaking of the samples softened the tissues and helped to remove the phenolics and skin pigments in them. Water soaking has been reported to be useful in isolating DNA from recalcitrant materials (Zhao et al., 1997; Wu et al., 2006; Chen et al., 2009). Though CTAB is widely used in DNA isolation and was used in nutmeg leaf and traded nutmeg seed DNA isolation, SDS was found to be a better option for cell lysis and releasing DNA in this study. Echevarria-Machado et al., (2005) has also reported that CTAB extraction buffer cannot guarantee the elimination of some polysaccharides and lipids. Increasing the concentration of sodium chloride from 1.5 M to 2 M helped the efficient removal of polysaccharides. Removal of polysaccharides in the presence of high salt concentration has been previously reported (Aljanabi et al., 1999; Shankar et al., 2011). Incorporation of PVP and β-mercaptoethanol has helped to remove the secondary metabolites and phenolics. The presence of PVP and β-mercaptoethanol prevents oxidation of secondary metabolites in the disrupted plant cell material (Khanuja et al., 1999; Muge et al., 2009; Pirttila et al., 2001). β-mercaptoethanol also denatures the sulphate linkage between proteins and makes it easy to remove them from nucleic acids (Maltas et al., 2011). PVP forms hydrogen bonds with the phenolics and co-precipitate with cell debris upon lysis during chloroform-isoamylalcohol extraction (Ibrahim, 2011). Potassium acetate contributes to the efficient removal of secondary metabolites and polysaccharides thereby increasing the quality of DNA (Dhanya et al., 2007; Lele, 2011). Polyethylene glycol was used as the DNA precipitating agent. The superiority of PEG over isopropanol / ethanol precipitation in yielding DNA preparation free from contaminants is already reported (Dhanya et al., 2007). The ability of PEG to remove coloured pigments from the DNA preparation has also been stressed (Syamkumar et al., 2005).

5.1.3. Standardisation of PCR conditions

A standardised PCR reaction for a barcode locus should yield a single clear band with minimal sub bands when observed on agarose gels, which is achieved by optimising the cycling conditions and altering the concentration of PCR reagents (Hajibabaei et al., 2005). The efficiency of PCR amplification is dependent on the concentration of magnesium, primer, nucleotides, template DNA and *Taq* polymerase used (Ellswoth et al., 1993). Here

amplification of the four loci viz. *rbcL, matK, psbA-trnH* and ITS was done using universal primers and the concentration range for each component was tested to determine the optimum level needed to accomplish a successful PCR amplification. This method was successfully employed by Dhanya (2009) to standardise the composition of PCR reaction mixture.

Amplification of the loci is mainly dependent on the annealing temperature (T_a) of the primer as low T_a results in non-specific PCR products while high T_a produces an amplicon with low yield and purity (Rychlik et al., 1990). Here the annealing temperatures for each locus for samples in cinnamon and nutmeg group were determined using gradient PCR, a technique that allows to determine the optimum annealing temperature for a PCR reaction in the least number of steps (Prezioso and Jahns, 2000). The optimum annealing temperature for both spices was thus determined by testing a gradient temperature of 50°C - 60°C.

5.1.4. Identification of ideal barcode loci

An ideal barcode locus should be easy to amplify and sequence, the PCR product size should not exceed 1 Kb and should possess higher interspecific divergence than intraspecific divergence (Wong et al., 2013). The important criteria for using short sequences reside in the need to amplify DNA from degraded samples (Kress et al., 2005). The Consortium for the Barcode of Life (CBOL) has proposed *matK* and *rbcL* as core barcode regions with *psbA-trnH* and ITS as supplementary barcodes for application in plant barcoding (CBOL Plant Working Group, 2009). So in this study the effectiveness of the above four loci were tested in both group I and group II.

5.1.4.1. Group I - Cinnamon

5.1.4.1.1. PCR amplification and sequencing success

Amplification and sequencing success are the primary criteria of a barcode region (CBOL Plant Working Group, 2009). The PCR amplification and sequencing success rates were different for different barcodes. In the present study 100% amplification success was obtained for *rbcL* and *psbA-trnH* loci. This is consistent with the results obtained by other workers who have reported an amplification success rate greater than 90% for *rbcL* (Kress et al., 2005; Fazekas et al., 2008; Parvathy et al., 2014; Parvathy et al., 2015) and *psbA-trnH* (Li et al., 2012; Raterta et al., 2014; Parvathy et al., 2014).

In the case of *matK* and ITS, two sets of primers were used. First set of primers failed to amplify any samples though we attempted different conditions. The second set of primers ITS5, ITS4 and *matK*3F, *matK*1R gave an amplification rate of 61.5% for these two loci. The amplification success rate of *matK* has been found to vary widely in literature; it was reported to be 24% by Sass et al., (2007), 30% by Kool et al., (2012), 68% by Gonzalez et al., (2009) and 69% by Kress et al., (2009). Liu et al., (2012) have reported the poor PCR amplification efficiency of ITS in Lauraceae plants. Low amplification success (50%) of ITS locus was also reported by Raterta et al., (2014). The difficulty in amplification of ITS in some taxa could be due to incomplete concerted evolution (Li et al., 2011).

Both these loci amplified the reference species but they could not amplify the traded samples. The same trend was found in the case of authentication of *Sida cordifolia* herbal products. Amplification of reference *Sida* species was carried out but problems in amplifying ITS and *matK* loci in traded herbal products of *Sida cordifolia* were observed (Kumar et al., 2015). Difficulty in amplification of *matK* has also been previously reported in spices like mint, thyme and rosemary (de Mattia et al., 2011). Difficulty of *matK* amplification in traded samples may be due to the interaction between the *matK* primer specificity and material type as suggested by de Vere et al., (2012). They reported that *matK* amplified the freshly isolated material than the herbarium specimens. As these loci failed to amplify in traded samples they were excluded from further analysis as done by other workers like Sass et al., (2007) and Piredda et al., (2011).

rbcL and *psbA-trnH* exhibited 100% sequencing success for all the samples. High sequencing efficiency of these loci were reported by de Mattia et al., (2011), Parvathy et al., (2014), Aziz et al., (2015), Kumar et al., (2015) and Tang et al., (2016).The length of the barcodes was 510 bp - 579 bp and 402 bp - 501 bp for *rbcL* and *psbA-trnH,* respectively. Ran et al., (2010) and von Cräutlein et al., (2011) have reported similar sequence length for *rbcL* locus while Wong et al., (2013) have reported similar range for *psbA-trnH*.

5.1.4.1.2. Species identification using BLAST analysis

The performance of the barcode was also assessed using BLAST analysis, an algorithm that measures the efficiency of species identification against a global sequence database like GenBank or BOLD (Munch et al., 2008; Mankga et al., 2013). Correct identification was said to be achieved when the highest BLAST % identity was shown to the same species or species belonging to the same genera. When the highest identity was shown to multiple

genera of the same family, identification was said to be ambiguous and when the highest identity was shown to sequences belonging to different species, genera or family identification was considered as incorrect (Zahra et al., 2016). Identification efficiency was determined by an identity value closer to 100% and E-value closer to zero (Mankga al., 2013).

Species identification was successful for *rbcL* and *psbA-trnH* loci as all the sequences showed maximum identity to the respective loci of *Cinnamomum* species with identity greater than 97% and zero E-value.

5.1.4.1.3. Analysis of intraspecific and interspecific divergence

Higher sequence variation between species (interspecific divergence) than within a species (intraspecific divergence) is an important criteria for a barcode (Lahaye et al., 2008).Two parameters were assessed for calculation of intraspecific divergence (average intraspecific distance and maximum intraspecific distance) and interspecific divergence (average interspecific distance and minimum interspecific distance) (Cabelin and Alejandro, 2016).

The average interspecific distance of *rbcL* was 0.004±0.002 and the average intraspecific distance value was zero. Maximum intraspecific distance value and minimum interspecific distance value was also zero. In case of *rbcL* though maximum intraspecific and minimum interspecific values were zero, the average interspecific distance was higher than their intraspecific distance values.

The average interspecific distance of *psbA-trnH* was 0.025±0.008 and the average intraspecific distance value was 0.013±0.005. Maximum intraspecific distance value was 0.042±0.012 and minimum interspecific distance value was zero.

Here the interspecific value of *psbA-trnH* was found to be greater than *rbcL* loci. But the intraspecific parameter, maximum intraspecific distance value of *psbA-trnH* was found to be greater than its minimum interspecific value. Thus the locus did not meet the ideal locus criterion. For *rbcL* locus the average interspecific distance was greater than its intraspecific distances and it thus satisfied the requirements of a barcode.

The Wilcoxon two sample test calculates the significant differences between the inter- and intra-specific divergences of the candidate DNA regions (Cabelin and Alejandro, 2016) and here it was found that the interspecific distances of *rbcL* was significantly greater than its

intraspecific distances ($p<0.05$) indicating the potential of the locus to discriminate the species. Earlier works have highlighted this fact that discrimination between closely related species is possible only when the interspecific divergence is significantly greater than the intraspecific divergence of a barcode (Hebert et al., 2004b; Savolainen et al., 2005; Lahaye et al., 2008; Mankga et al., 2013).

5.1.4.1.4. Sequence analysis and species discrimination.

Species specific substitutions and indels are marker nucleotides that help to authenticate individual species (Kim et al., 2016). SNP identification has helped to distinguish *Artemisia argyi* from its closely related species and counterfeits (Mei et al., 2016).

In this study *rbcL* locus exhibited three nucleotide substitutions or single nucleotide polymorphisms specific to *C. aromaticum* at positions 53, 54 and 301. These *C. aromaticum* specific substitutions, found in seven of the ten traded samples confirmed the extent of *C. aromaticum* adulteration in commercial samples of true cinnamon. These SNP sites can be exploited to design *C. aromaticum* specific primers enabling kit development for easy detection of adulterants at the band level thereby bypassing the cost of sequencing. Thus *rbcL* was found as the potential barcode for authentication of cinnamon with cassia (*C.aromaticum*). However, there were no informative sites to differentiate between *C. verum* and *C. malabatrum.*

The efficiency of *rbcL* as an ideal barcode has already been established by Kress and Erickson (2007) and CBOL Plant Working Group (2009). Zhao et al., (2007) reported the presence of 19 polymorphic sites in *rbcL* locus that differentiated the medicine "*Mianma guanzhong"* derived from the fern *Dryopteris crassirhizoma* from its adulterants. *rbcL* in combination with *matK* was used in the molecular authentication of the ethno-medicinal plant *Sabia parviflora* (Sui et al., 2011). The polymorphic sites in *rbcL* revealed that the traded samples of *Elephantopus scaber*, *Pandanus tectorius, Abrus precatorius* sold were genuine while *Rubus reflexus* was substituted (Li et al., 2012). Nair et al., (2013) have also suggested the utility of this locus in the molecular distinction of two morinda (*Morinda reticulata* and *Morinda umbellate*) species on the basis of SNPs. Authentication of plant drugs used in traditional Chinese medicine was also done using *rbcL* locus (Herrmann and Wink, 2014). Species specific substitutions in *rbcL* locus were observed at two positions in *Paeonia lactiflora*, one position in *Paeonia japonica*, two positions in *Paeonia veitchii* and four positions in *Paeonia suffruticosa* which helped in their identification (Kim et al., 2016).

Vassou et al., (2016) revealed that of the raw Ayurvedic drugs sold in market 79% were authentic and the remaining were adulterated using *rbcL* based DNA barcoding.

5.1.4.1.5. Neighbour joining (NJ) tree identification

Neighbour joining tree identification is done in many barcoding studies in which each species are separated into monophyletic clades. In this study *rbcL* differentiated *C. verum* and *C. aromaticum* in to two different clades. All the genuine market samples clustered within *C. verum* - *C. malabatrum* clade while the adulterated samples clustered with *C. aromaticum.* The clustering of adulterated samples with the reference sequence of the adulterant has been previously reported (Srirama et al., 2010; Yu et al., 2016).

All these results showed *rbcL* as the ideal barcode for identifying *C. aromaticum* adulteration in traded cinnamon.

5.1.4.2. Group II – Nutmeg

5.1.4.2.1. PCR amplification and sequencing success

Amplification and sequencing success are primary requirements of a DNA barcode (CBOL Plant Working Group, 2009). Here *rbcL, psbA-trnH* and ITS exhibited 100% amplification success while *matK* had 66.6% amplification success. Though *matK* amplified the reference samples, it failed to amplify the traded samples which resulted in its exclusion from further analysis.

High amplification rate of *rbcL, psbA-trnH* and ITS have been reported in various literature (Li et al., 2012; Jian et al., 2014; Zhang et al., 2015). Likewise the inconsistent amplification of *matK* has also been reported (Ford et al., 2009; Pettengill and Neel, 2010; de Mattia et al., 2011). Poor PCR recovery of *matK* has handicapped its potential as a barcode and resulted in its exclusion from further analysis in other studies also (Sass et al., 2007; Kress et al., 2009; Piredda et al., 2011; Kool et al., 2012). Difficulties in amplification of *matK* locus in traded samples of spices have also been stressed by de Mattia et al., (2011) and Swetha et al., (2014). Difficulty of *matK* amplification in traded samples may be due to the interaction between the *matK* primer specificity and material type as suggested by de Vere et al., (2012). They reported that *matK* amplified the freshly isolated material rather than the herbarium specimens. As these loci failed to amplify in traded samples they were excluded from further analysis as done by other workers (Sass et al., 2007; Piredda et al., 2011).

Sequencing success was found to be 100% for *rbcL* and *psbA-trnH.* High sequencing efficiency of these loci was reported by de Mattia et al., (2011), Parvathy et al., (2014), Aziz et al., (2015), Kumar et al., (2015) and Tang et al., (2016). The length of *rbcL* and *psbA-trnH* sequences obtained in this study was in the range of 516-585 bp and 204-397 bp, respectively. Other workers have reported *rbcL* sequence length to be 507 bp in *Picea* (Ran et al., 2010), 520bp in *Salix* (von Cräutlein et al., 2011) and 589 bp in *Primula* (Yan et al., 2011).The length of sequences obtained for *psbA-trnH* was in the range of 228-296 bp in *Aconitum* species (He et al., 2010), 200-400 bp in *Scutellaria* species (Guo et al., 2011) and 332-357 bp in *Lonicera* species (Sun et al., 2011).

ITS locus has been reported as an efficient barcode for authentication in medicinal plants (Pang et al., 2013; Chen et al., 2010). But in the present study the contig sequences could not be assembled from the forward and reverse reads rendering it of no relevance. Also majority of the samples gave mixed sequence data. The messy sequence data of ITS is attributed to the presence of the gene in multiple copies due to incomplete concerted evolution and simultaneous sequencing of these multiple variants resulted in sequence data that cannot be further analysed thereby limiting its application as a potential barcode candidate (Hollingsworth, 2011). ITS is also reported to be a problematic barcode due to its characteristics like gene duplication, pseudogenes and presence of paralogs (King and Roalson, 2008; Starr et al., 2009). The multicopy nature of ITS also results in high level of within species and within individual sequence differentiation that also restricts its use as a barcode (Kress and Erickson, 2007). Low sequencing efficiency of ITS has also been reported in different plants (Liu et al., 2012; Bhagwat et al., 2015).

5.1.4.2.2. Species discrimination using BLAST analysis

BLAST analysis of *rbcL* sequences gave ambiguous identification as the top hit was a sequence belonging to different genera of the same family. *psbA-trnH* sequences of *M. fragrans* showed maximum identity to *psbA-trnH* sequences of the same species. But in the case of *psbA-trnH* sequences of *M. malabarica,* maximum identity was shown to another genus of the same family pointing to ambiguous identification. This may be due to the absence of adequate *Myristica* sequences in GenBank. Absence of adequate reference sequences resulting in ambiguous identification has been reported earlier (Zahra et al., 2016).

5.1.4.2.3. Analysis of intraspecific and interspecific divergence

Interspecific divergence greater than intraspecific divergence is an essential criteria for a DNA barcode (Lahaye et al., 2008). The average interspecific distance of *rbcL* was 0.004±0.002 and the average intraspecific distance value was 0.003±0.002. Maximum intraspecific distance value was 0.011±0.004 and minimum interspecific distance value was zero.

The average interspecific distance of *psbA-trnH* was 0.771±0.098 and the average intraspecific distance value was 0.057±0.011. Maximum intraspecific distance value was 0.166±0.024 and minimum interspecific distance value was 0.748±0.092.

By comparison of the interspecific divergences of the two loci, *psbA-trnH* was found to have higher interspecific divergence. Also the interspecific divergence was greater than its intraspecific divergence. On the other hand *rbcL* had a higher intraspecific divergence than interspecific divergence which was contradictory to the criteria of an ideal barcode. Olivar et al., (2014) has also reported that *rbcL* exhibited intraspecific divergence greater than interspecific divergence in *Ficus* spp. Intraspecific variation of *rbcL* was also reported by Little and Jeanson (2013).

The Wilcoxon two sample test calculates the significant differences between the inter- and intra-specific divergences of the candidate DNA regions (Cabelin and Alejandro, 2016) and here it was found that the interspecific distances of *psbA-trnH* was significantly greater than its intraspecific distances ($p<0.05$) implying the potential of the locus to discriminate the species. Several workers have highlighted this fact that discrimination between closely related species is possible only when the interspecific divergence is significantly greater than the intraspecific divergence of a barcode (Hebert et al., 2004b; Savolainen et al., 2005; Lahaye et al., 2008; Mankga et al., 2013).

Though *rbcL* had high amplification and sequencing success, it was not suitable for authentication. The low species resolution of *rbcL* locus is widely reported in a number of species like *Composonura* (Newmaster et al., 2007) *Salvia* (Wang et al., 2013), *Gentiana* (Wong et al., 2013), *Roscoea* (Zhang et al., 2014b), *Dalbergia* (Bhagwat et al., 2015) and family Lauraceae (Liu et al., 2012).

5.1.4.2.4. Sequence analysis and species discrimination.

Authentication of a species is made possible by species specific substitutions and indels (Kim et al., 2016). A wide range of literature exists where the species specific substitutions help in authentication of genuine species from adulterant ones (Sui et al., 2011; Sun et al., 2011; Little and Jeanson, 2013; Chen et al., 2014; Moon et al., 2016).

In this study 60 polymorphic sites and 9 indels were identified in the *psbA-trnH* sequence alignment that discriminated between *M. fragrans* and *M. malabarica.* These polymorphic sites point to the high sequence variation of this locus. Though indels in this locus were said to be non-informative for species discrimination as their occurrence does not correlate with a species (Zhang et al., 2015), the indels observed here were specific either to *M. fragrans* or *M. malabarica.*

The polymorphic sites in *psbA-trnH* locus can be employed to develop species specific primers for *M. malabarica* in order to facilitate its detection bypassing sequencing expense. These primers can also be used by food safety agencies to screen samples to check for its authenticity.

The high amplification, sequencing success, interspecific divergence and the presence of species specific substitutions made *psbA-trnH*, an ideal locus to authenticate *M. fragrans.* This is in accordance to the result by Newmaster et al., (2007) that *psbA-trnH* is the ideal locus for barcoding *Composonura* genus of Myristicaceae family. *psbA-trnH* is considered as the most promising locus for a DNA barcode in land plants due to the PCR priming sites within highly conserved flanking sequences, high sequence divergence and the diagnostic insertion/deletion mutations in it (Kress and Erickson, 2007). A relatively short length and the high frequency of nucleotide substitutions are added advantages that help in species level discrimination (Kress et al., 2005). The indel polymorphisms of this locus aid in species discrimination under the character based identification (Zhang et al., 2015). The high frequency of variations in this region is credited to its faster evolutionary rate (Meizi et al., 2012).

The efficiency of *psbA-trnH* as an ideal barcode in authentication of medicinal plants and other commodities has been widely reported. *Stemona tuberosa*, a medicinal plant of Thailand could be discriminated from its closely related species based on the sequence differentiation in *psbA-trnH* (Vongsak et al., 2008). Eighteen species of the Polygonaceae

family, important in Chinese Pharmacopoeia, could be differentiated based on *psbA-trnH* locus (Song et al., 2009). He et al., (2010) proposed *psbA-trnH* as a marker for the authentication of medicinal *Aconiticum* species. Authentication of the medicinal plant *Taxillus chinensis* could be done using *psbA-trnH* locus (Lil et al., 2010). Discrimination of *Scutellaria baicalensis* from its adulterants was made possible by the sequence variations in *psbA-trnH* locus (Guo et al., 2011). Species admixture in traded samples of *Phyllanthus amarus* was detected using *psbA-trnH* based barcoding, 24% of traded samples were found to be admixtures of species like *Phyllanthus debilis, Phyllanthus urinaria, Phyllanthus maderaspatensis,* and *Phyllanthus kozhikodianus* (Srirama et al., 2010). Traceability of spices in family Lamiaceae was accomplished using non-coding *psbA-trnH* spacer (de Mattia et al., 2011). Canola oil and saffron oil contaminations in olive oil were detected using *psbA-trnH* barcodes (Kumar et al., 2011). Sun et al., (2011) proposed this locus as a barcode to differentiate *Lonicera japonica* from its closely related species based on the species specific substitutions. Identification of *Valeriana jatamansi,* an adulterant of medicinal *Paris polyphylla* could be detected based on the length polymorphism of this locus in the two species (Yang et al., 2011). Differentiation of *Ilicium verum* from its congeneric adulterants like *Ilicium micranthum, Ilicium simonsii, Ilicium modestum, Ilicium jgadifengpi, Ilicium henryi,* and *Ilicium dunnianum* var. *latifolium* was done on the basis of the indels and variable sites in *psbA-trnH* (Meizi et al., 2012).

Gao et al., (2013) proposed *psbA-trnH* as an ideal barcode for the authentication of medicinal plants of Fabaceae because of its universality and species discrimination power. Ma et al., (2013) have also proposed *psbA-trnH* as a suitable marker for the identification of Pteridophytes. *psbA-trnH* was found to be the most discriminant marker to identify the plant species in honey (Bruni et al., 2015). Christina and Anamalai (2014) have stressed the applicability of *psbA-trnH* in differentiating *Ocimum* species. The incidence of chilli adulteration in traded samples of black pepper powder was detected using *psbA-trnH* based barcoding (Parvathy et al., 2014). Identification of medicinal plants sold in the markets of Manila could be done by *psbA-trnH* locus (Raterta et al., 2014). *psbA-trnH* was proposed as the best barcode for the species level identification of traditional medicines used in Malaysia (Aziz et al., 2015). Authentication of drugs sold as "Bala" in the South Indian markets was successfully done using *psbA-trnH* (Kumar et al., 2015; Vassou et al., 2015). Dissanayake et al., (2016) also identified the presence of chilli and papaya seed adulteration in black pepper powder using length polymorphisms of *psbA-trnH* locus. Discrimination of *Piper*

kadsura, a medicinal plant used in Chinese medicine from its closely related adulterant species was also accomplished by *psbA-trnH* based barcoding (Yu et al., 2016).

5.1.4.2.5. Neighbour joining tree identification

In a NJ tree, sequences that belong to a particular species often cluster together to form monophyletic clades. Reference sequences of the genuine sample are clustered separately from the adulterant sequences. In this study NJ tree drawn based on *psbA-trnH* sequences distributed *M. fragrans* and *M. malabarica* sequences in to two separate clades. The genuine market samples clustered with *M. fragrans* while the adulterated samples clustered with *M. malabarica.* Identification of adulteration based on NJ tree clustering has been previously reported by Srirama et al., (2010) and Yu et al., (2016).

5.1.5. Validation studies.

DNA barcode primers may amplify DNA from excipients and give a false negative for the species in question. This bias in PCR can be overcome by cloning the specific amplicon in suitable vectors and sequencing them (Parveen et al., 2016). In this study, cloning was performed for both groups, cinnamon and nutmeg, to make sure the sequence results were not false negative. All the polymorphic sites obtained on direct sequencing of the PCR products were retrieved on sequencing of the plasmids obtained from the cloned cells for (i) *rbcL* locus in reference *Cinnamomum* species and traded cinnamon barks and (ii) reference *Myristica* species and traded nutmeg mace.

5.1.6. Generation of barcodes.

All the barcode sequences generated in this study were deposited in the GenBank database of NCBI. Submission of sequences in database will contribute to increase the sequence volume of the database so that it can be utilized by others for authentication of cinnamon and nutmeg. The presence of the sequences of the genuine and adulterant entities in the database will make their identification easier.

5.1.7. Significance of DNA barcoding in commodity authentication

DNA barcoding is a rapid, easy and reliable tool for tracking the origin of raw materials and identifying the poisonous and allergenic components present in food matrices (Galimberti et al., 2013). Availability of freely accessible reference databases and well equipped laboratories to perform the analysis has increased the feasibility of this method (Ferri et al., 2015). In the present scenario where adulteration has become a common practice, DNA barcoding serves as an ideal method for food safety as DNA can be retrieved from whole,

processed or powdered food products as it can withstand the complex sample processing methods employed in industries (Ferri et al., 2015).

The present study confirms the commodity authentication power of DNA barcoding. The technique could successfully detect the plant based adulterant *C. aromaticum* in seven traded samples of cinnamon bark and *M. malabarica* in three traded nutmeg mace samples. DNA barcoding can thus be adopted by the National and International Food Regulatory Agencies to check for possible adulterations and substitutions in food commodities including spices to safeguard the interests of the consumers.

5.2. Phylogenetic Analysis

Phylogenetic analysis gives an idea regarding the species origin and evolution through construction of rooted or unrooted trees that group closely related species together. Rooted trees have an added advantage of implying the direction of evolutionary change and depicts the species relationship with respect to an outgroup (Michu, 2007). Plant phylogenetic studies are done mostly based on chloroplast DNA (Patwardhan et al., 2014). Here we have attempted to study the evolutionary relationship of *Cinnamomum* and *Myristica* species by construction of rooted trees using individual chloroplast genes and their combinations. Multilocus approach may help to resolve species in cases where single locus approach fails. It involves the combination of phylogenetically conserved locus like *rbcL*, that help in assigning the individuals into genera or groups within genera with variable genes like *matK*, *psbA-trnH* or ITS, that will differentiate species within these higher group (Kress and Erickson, 2007; Kress et al., 2009). Combination of two or more loci can generate phylogenies that are more robust than single locus (Chase et al., 1997; Cunningham, 1997; Davis et al., 1998).

5.2.1. Phylogenetic analysis of *Cinnamomum* spp.

The genus *Cinnamomum* belonging to family Lauraceae is comprised of about 300 species of evergreen trees and shrubs (Robi et al., 2014). It belongs to sub tribe Cinnamominea that belongs to tribe Cinnamomea which is a part of the Lauroidea subfamily of family Lauraceae (Kostermans, 1957).

Cinnamomum genus is characterised by plants that exhibit uniformity in their floral features and wide variability in their morphological characters that makes this genus taxonomically challenging (Shylaja, 1984).Though considerable morphological variations exist to study *Cinnamomum* phylogeny the relationship within and between species could not be deciphered from the morphological variations alone as the environmental factors influence

the plant morphology (Abeysinghe et al., 2014; Abeysinghe et al., 2009). Liu et al., (2012) has also commented on the complex relationship among species of *Cinnamomum* genus.

Several workers have reported the use of molecular markers like RAPD, SRAP, ISSR, and ITS for resolving the phylogeny of *Cinnamomum* species (Joy and Maridass, 2008; Abeysinghe et al., 2009; Ho and Hung, 2011; Sandigawad and Patil, 2011; Abeysinghe et al., 2014; Huang et al., 2016).

Here we have attempted to study the relationship between 12 *Cinnamomum* species using chloroplast genes *rbcL, matK, psbA-trnH* and ITS.

5.2.1.1. Amplification and sequencing success

All the loci taken in this study exhibited 100% amplification success. This was in accordance with the high amplification rate reported by Kress et al., (2005), Li et al., (2012), Zhang et al., (2015) and Kumar and Yusuf (2016) for these loci. Sequencing success was high for *rbcL, matK* and *psbA-trnH.* But in case of ITS the forward and reverse reads obtained on sequencing could not be assembled to contigs due to the low sequence quality and mixed sequence data. The low sequence recovery rate of ITS in Lauraceae was previously reported by Liu et al., (2017).

5.2.1.2. Phylogenetic analysis of *Cinnamomum* species

Previous workers utilized ITS locus to study the phylogenetic relationships between *Cinnamomum* species in Sri Lanka, Taiwan and Korea (Abeysinghe et al., 2009; Lee et al., 2010; Ho and Hung, 2011; Doh et al., 2017). But the primers used by them were not uniform. In the present study two sets of ITS primers were used but contig generation was not possible due to messy sequence data. The messy sequence data of ITS is attributed to the presence of the gene in multiple copies due to incomplete concerted evolution and simultaneous sequencing of these multiple variants generate sequence data that cannot be further analysed (Hollingsworth, 2011).

Phylogenetic trees were constructed using both single and multilocus combinations of *rbcL, matK* and *psbA-trnH* with overlaid bootstrap support, in the present study. In all the trees the outgroup was clearly separated from the inner group containing *Cinnamomum.* Both single and multilocus combinations failed to give a clear picture regarding its phylogeny. The clustering pattern of the species differed in the different trees.

rbcL phylogram could not give any information regarding the phylogeny of *Cinnamomum.* Majority of the species remained unresolved in this tree as they shared the same sequence haplotype. But *C. aromaticum* samples along with a sequence taken from GenBank formed a separate monophyletic group with high support. It was found to share close relation with *C. camphora, C. glaucescens* and *C. burmanni.* C. *riparium* and *C. travancoricum* also formed a separate clade. The clustering of these two species together may be due to the same geographical location from which the samples were collected and also as these two species are endemic to Western Ghats (http://indiabiodiversity.org/species/show/9259; Kumary et al., 2013). Species viz., *C. verum, C. malabatrum*, *C. tamala*, *C. sulphuratum*, *C. citriodorum*, *C. heyneanum* and *C. loureiroi* could not be resolved using *rbcL* locus. Liu et al., (2012) has reported about the low sequence divergence of *rbcL* locus in plants of Lauraceae which hinders species resolution. Although the locus could not resolve phylogenetic relationships between *Cinnamomum* species, it could differentiate *C. verum* from its adulterant entities viz., *C. aromaticum*, *C. burmanni* and *C. loureiri.*

In the *matK* phylogram, the species clustered in to two clades. Clade A comprised of *C. camphora, C. glaucescens, C. aromaticum, C. brensii, C. polyadelphum, C. bejolghota* and *C. burmannii*. Within these species *C. camphora* and *C. glaucescens* clustered together in to a subclade depicting the close relationship between them. In clade B, *C. verum, C. malabatrum, C. alexei, C. heyneanum, C. riparium, C. travancoricum, C. sulphuratum, C. tamala, C. citriodorum* and *C.loureiroi* were found. Relationship between these species also could not be clearly understood as most of the species shared the same haplotype. *C.riparium, C. travancoricum* and *C. malabatrum* clustered together in a single clade which may be due to the same geographical location from where the samples were collected. These three species were also endemic to Western Ghats (http://indiabiodiversity.org/ species/ show/9259;http://indiabiodiversity.org/species/show/9304; Kumary et al., 2013). Different *Aquilaria* species of the same geographical origin were found to cluster together in a *matK* based tree (Lee et al., 2016).

Thus as in case of the *rbcL* phylogram, the resolution of *Cinnamomum* species was not possible in the *matK* phylogram too. Rohwer (2000) and Liu et al., (2012) have also reported about the low sequence divergence of *matK* in Lauracea family.

In *psbA-trnH* phylogram sequences of different accessions of the same species clustered in separate clades. It showed incongruence in the clustering of species with respect to the other

two loci. *C. verum*4 was seen clustered together with *C. aromaticum*. *C. aromaticum*5 was seen unresolved in the clade B. *C. malabatrum* sequences were distributed in subclades B1 and B2. According to this tree, *C. camphora* sequences showed close relation with *C. sulphuratum* while in the above two trees *C. camphora* depicted relation with *C. glaucescens*. *C. burmanni* appeared to have a sister relation with *C. camphora* and *C. sulphuratum* while two sequences of *C. tamala* formed a monophyletic clade.

matK+ *rbcL* depicted a sister relationship between *C. aromaticum* and *C. camphora* while *C. glaucescens* was found to be closely related to *C. camphora.* These relationships were supported by high bootstrap value. *C. aromaticum* and *C. camphora* were found to be distant from other sequences that were placed in a separate clade. The close relationship between *C. camphora* and *C. glaucescens* was also suggested by Kumar (2015) when he evaluated the inter relationship between *Cinnamomum* species using RAPD markers. Joy and Maridass (2008) have also shown the close relationship shared between *C. camphora* and *C. glaucescens* using RAPD markers. The close association between these species may be due to their Himalayan origin. Deciphering the relationship between other species like *C.malabatrum, C. verum, C. tamala* and *C. heyneanum* was not possible here as they shared the same haplotype. *C. malabatrum* 6*, C. alexei, C. riparium* and *C. travancoricum* were found to be closely related in this tree. The topology of *rbcL+psbA-trnH, matK+psbA-trnH* and *rbcL+matK+psbA-trnH* trees were similar to that of the *psbA-trnH* tree.

In most of the phylograms, *C. malabatrum* was found in more than one clade due to its intraspecific variability. Cluster analysis done by Shylaja (1984) on *Cinnamomum* species occurring in Kerala also showed the positioning of *C. malabatrum* in different clusters due to its intraspecific variability. The reason attributed was distribution of *C. malabatrum* in different ecological conditions resulting in the formation of small isolated populations that underwent divergence due to gene exchange or segregation thereby resulting in the intraspecific variability of *C. malabatrum* (Shylaja 1984).

Species resolution was not possible here as different species shared the same chloroplast genotype. The absence of unique plastid sequences has been attributed by various factors like low mutation rate, wide interspecific hybridisation, incomplete lineage sorting, sharing of ancestral polymorphism and reticulation (Rieseberg and Soltis, 1991; Okuyama et al., 2005; Hardig et al., 2010; Simeone et al., 2013). Rieseberg and Soltis (1991) have reported that interspecific hybridisation may result in cytoplasmic gene flow or chloroplast capture

which may lead to the replacement of the native cytoplasm with foreign one. The difficulty in resolving/discriminating species due to interspecies hybridisation has been previously reported in species like *Curcuma* (Zaveska et al., 2012), *Salix* (Hardig et al., 2010), *Quercus* (Simeone et al., 2013) and *Solanum* sect *Petota* (Spooner, 2009), though such a phenomenon/reasoning in *Cinnamomum* species is doubtful.

The limitation of chloroplast markers in resolving the phylogenetic relationships in Lauraceae have been previously reported (Chanderbali et al., 2001; Fijridiyanto and Murakami, 2009; Li et al., 2011). Here, none of the phylograms drawn could give a clear understanding regarding the phylogeny of *Cinnamomum* genus as most of the species remained unresolved due to low sequence divergence. The low sequence divergence in chloroplast DNA based phylogenies have been reported in other genera of Lauraceae like *Neocinnamomum* (Wang et al., 2010), *Persea* (Li et al., 2011), *Endiandra* (Rohwer et al., 2014), *Machilus* (Song et al., 2015) and *Cryptocarya* (van der Merwe et al., 2016). Slow rate of plastid mutation and speciation events are considered as the factors for the low chloroplast sequence divergence in Lauraceae family (van der Merwe et al., 2016).

Thus lack of sufficient nucleotide variation acted as the limiting factor for the plastid genes in resolving the phylogenetic relationships. Maybe an approach involving nuclear genes that have undergone different evolutionary dynamics than the chloroplast DNA may be able to shed some light on *Cinnamomum* phylogeny.

5.2.2. Phylogenetic analysis of *Myristica* spp.

Myristica is the largest genus of the primitive family Myristicaceae with New Guinea as its centre of origin and distribution (http://www.fao.org/docrep/x5047E/x5047E04.htm). About 18 genera are found in Myristicaceae family of which the four Asian genera *Myristica, Knema, Gymnocranthera* and *Horsfieldia* are found in India (Sheeja et al., 2013). Phylogenetic reconstruction in Myristicaceae was reported to be complicated due to lack of molecular variation (Sauquet et al., 2003). Previous phylogenetic works on *Myristica* spp. were done using RAPD, ISSR markers and 18S rDNA region. Here an attempt to study the relationships within *Myristica* species and between *Myristica, Gymnocranthera* and *Knema* genera was done using chloroplast regions and nuclear ITS region.

5.2.2.1. Amplification and sequencing success

rbcL, matK and ITS exhibited 100% amplification success while *psbA-trnH* showed 95% amplification success. High amplification success of these loci was previously reported (Kress et al., 2005; Li et al., 2012; Zhang et al., 2015; Kumar and Yusuf, 2016). Sequencing success was high for *rbcL, matK* and *psbA-trnH.* But in case of ITS, contig assembly could not be done due to low sequence quality and mixed sequence data. Hollingsworth (2011) has reported that the presence of ITS in multiple copies due to incomplete concerted evolution and simultaneous sequencing of these multiple variants result in messy sequence data that cannot be further processed. Sequencing failure of ITS has also been reported in various works (Liu et al., 2012; Li et al., 2014; Bhagwat et al., 2015).

5.2.2.2. Phylogenetic analysis

The phylograms were drawn for all single and multilocus combinations of *Myristica, Knema* and *Gymnocranthera* species with high bootstrap support. In all the trees the outgroup was placed separately from the inner group containing the species under study. Among the individual loci tested only *psbA-trnH* could resolve the species.

rbcL locus failed to resolve the phylogeny of *Myristica* species due to its low discrimination power. Species of different genera remained unresolved and clustered together in this phylogram. Different accessions of the same species were also clustered together. Difficulty of *rbcL* locus in resolving closely related species was previously reported by Gielly and Taberlet (1994).

matK phylogram also failed to resolve the *Myristica* species. But they could differentiate between the different genera. *G. farquhariana, Composonura preussi* and *Knema* species formed separate clades. The limitation of *matK* locus in discriminating closely related *Myristica* species was inferred by Tallei et al., (2015) though she also reported the utility of *matK* in differentiating the different genera in Myristicaceae family.

In *psbA-trnH* phylogram, *M. andamanica* 2, *K. attenuata* and *G. farquhariana* were found to be closely related. *M. fragrans* and *M. malabarica* appeared in separate clusters pointing to the distant relationship between them. *M. beddomei, M. amygdalina, M. fatua* and *M. andamanica* 1 were found to have close association with each other. Sequences of other genera formed monophyletic groups and appeared to be sister groups to *Myristica* spp. The

utility of *psbA-trnH* in discriminating plant species of different tree genera like *Alnus* (Ren et al., 2010) and *Quercus* (Simeone et al., 2013) have been previously reported.

rbcL+matK phylogram showed close relation between *M. beddomei, M. fatua* and *M. andamanica*1. It also showed the close association of *M. andamanica*2, *K. attenuata* and *G. farquhariana.* But this tree could not resolve *M. fragrans, M. amygdalina* and *M. malabarica.* Though *rbcL+matK* combination was proposed as ideal for plant species discrimination by CBOL Plant Working Group, it failed to resolve *Myristica* spp. The failure of this combination in phylogenetic analysis has been previously reported by Li et al., (2014) in *Calligonum* species.

Separate monophyletic groups of *M. fragrans* and *M. malabarica* were obtained by the *rbcL+psbA-trnH* combination in the phylogram, depicting their distant relationship. *M. amygdalina, M. beddomei, M. fatua* and *M. andamanica* 1 were grouped together indicating their closeness. *M. fragrans* was found to be in a sister relation with this cluster. *M. andamanica*2, *K. attenuata* and *G. farquhariana* clustered together and were found to share close relationship with each other. *M. malabarica* was found to be closely related with this cluster. *rbcL+psbA-trnH* was proposed by Kress and Erickson (2007) to be superior over *rbcL+matK* in discriminating closely related species.

The topology of *psbA-trnH, matK+psbA-trnH* and *rbcL+matK+psbA-trnH* trees were similar to *rbcL+psbA-trnH* tree except for a single sample of *M. malabarica* positioned outside the *M. malabarica* cluster. That distinct specimen of *M. malabarica* was collected from Nagercoil, Tamil Nadu while rest of the samples were collected from Kerala. Geographical separation may be the reason for its positioning outside *M. malabarica* cluster. In these trees, *M. andamanica*1 collected from Andamans and *M. andamanica*2 collected from Kerala were also placed in different clades and showed close relation with different species which may also be due to geographical separation. Schneider et al., (2015) has revealed that genetic differences between species may be caused due to geographical separation. The accumulation of genetic differences by geographical isolation may have arisen in the process of adapting to different environmental conditions (Souframanien and Gopalakrishnan, 2004). A close relation was observed between *M. beddomei, M. amygdalina, M. andamanica*1 and *M. fatua* in the present study. This is in accordance with the previous phylogenetic study conducted by Sheeja et al., (2014) on Myristicaceae; the phylogenetic analysis done using 18S rDNA sequences that depicted the close relationship between *M.*

beddomei, M. amygdalina and *M. andamanica* and their closeness was explained on the basis of the strong similarity in their mace and seed characters. *M. fragrans* was found to be closely related to *M. beddomei, M. amygdalina, M. andamanica*1 and *M. fatua* and distinct from *M. malabarica.* The close association between *M. fragrans* and the other species could be perhaps indicative of their common ancestry. *M. fragrans* and *M. malabarica* were clustered in two distinct clades due to the sequence divergence in *psbA-trnH* between these two species. *Gymnocranthera* and *Knema* species formed a sister clade within *Myristica* cluster. The sister relationship between *Knema* and *Myristica* was reported by Sauquet et al., (2003). *Gymnocranthera, Knema* and *Myristica* are Asian genera of Myristicaceae family. Clustering of *Gymnocranthera* and *Knema* along with *Myristica* may be due to the monophyly of the Asian genera of Myristicaceae (Sauquet et al., 2003).

The present study gave an insight on the genetic relationship among the members of *Myristica* genus. The locus *psbA-trnH* with its high sequence divergence provided information regarding the interspecific relation between species within the genus *Myristica*. Among the studied barcoding loci, the locus *psbA-trnH* may have potential to elucidate the relationships between other genera of Myristicaceae family.

Chapter 6

Summary and Conclusion

Spices are high value compounds traded in the form of whole spices, powders, essential oils or their extractives that assume importance in the international trade and are extensively used in food, medical and cosmetic industries both within the country and outside. The high cost coupled with the low volume of spices have resulted in its adulteration with closely related inferior plant based adulterants that may pose health hazards to consumers in addition to the various synthetic adulterants. Identification of genuine commodity from the adulterant becomes difficult once the commodity loses its morphological diagnostic features on powdering, drying and storage. Hence adulteration detection has now become a matter of primary concern. Testing for the presence of plant based adulterants were initially carried out using physical and chemical approaches that were later replaced by molecular marker techniques like DNA barcoding owing to their sensitivity and specificity.

Cinnamon (*C. verum*) and nutmeg (*M. fragrans*) belongs to genus *Cinnamomum* and *Myristica,* respectively, which are characterised by more than 100 species. They are two important aromatic tree spices that have high economic significance. *C. verum* is reported to be adulterated with its closely related species, *C. aromaticum* and *C. malabatrum* while *M. fragrans* is reported to be adulterated with *M. malabarica.*

The present study was an attempt to detect the presence of adulterants in traded samples of cinnamon bark and nutmeg mace using the barcoding genes viz. *rbcL, matK, psbA-trnH* and ITS. Attempts were also made to elucidate the relationship of *C. verum* and *M. fragrans* with other species of genus *Cinnamomum* and *Myristica,* respectively.

6.1. Commodity Authentication

6.1.1. Cinnamon

High quality genomic DNA was isolated from the leaves of 5 samples of *C. verum,* 5 samples of *C. aromaticum* and 6 samples of *C. malabatrum* taken as reference species and 10 samples of traded cinnamon bark. DNA was isolated from the leaves of the reference species using Qiagen DNeasy kit with a yield of 132-145 $\mu g g^{-1}$ and purity ratio in the range of 1.8-1.9. A protocol was developed for isolating DNA from cinnamon bark by modifying the protocol

used to isolate DNA from processed wood of *Gonystylus bacanus*. Modifications done included the use of 10 ml of extraction buffer for 1g of bark sample, increase in the concentration of CTAB (5%) and sodium chloride (3M) in extraction buffer, addition of PVP in extraction buffer, addition of sodium acetate during chloroform-isoamylalcohol extraction and overnight precipitation of DNA using isopropanol at -40°C. High quality DNA with a yield of 150-350 $\mu g g^{-1}$ and purity ratio in the range of 1.8-1.9 was obtained from the traded barks using the newly perfected protocol.

The PCR success rate, sequencing efficiency, intra and inter specific divergence and occurrence of single nucleotide polymorphisms (SNPs) were utilized to assess the potential of each barcode loci to authenticate *C. verum* from its related adulterants. Amplification of genomic DNA from reference and market samples were carried out using the universal primers for *rbcL, matK, psbA-trnH* and ITS. The reaction mixture and temperature profile for each locus was standardised. *rbcL* and *psbA-trnH* loci exhibited 100% amplification success while *matK* and ITS could not be amplified in market samples. *matK,* ITS, *rbcL* and *psbA-trnH* loci generated amplicons of length 900 bp, 750 bp, 600 bp and 550 bp, respectively. The failure of ITS and *matK* to amplify the traded samples resulted in its exclusion from further study. *rbcL* and *psbA-trnH* amplicons were purified and sequenced at Scigenom labs and the contig sequences were generated from the forward and reverse reads. BLAST analysis of the sequences showed that they gave maximum identity to the sequences of the respective loci of *Cinnamomum* species and the generated sequences (16 *rbcL,* 16 *matK* and 16 *psbA-trnH*) were submitted in GenBank database of NCBI.

psbA-trnH locus exhibited higher intraspecific divergence than interspecific divergence which was against the ideal barcode criteria. *rbcL* locus with its higher interspecific divergence than its intraspecific divergence fulfilled the ideal barcode criteria. Wilcoxon two sample test showed that the interspecific divergence of *rbcL* was significantly greater than its intraspecific divergence.

rbcL and *psbA-trnH* sequences of the reference and market samples were aligned and trimmed to 508 bp and 307 bp, respectively. Sequence analysis of *rbcL* locus showed the presence of three single nucleotide polymorphisms specific to *C. aromaticum* at positions 54, 55 and 304 in seven out of the ten market samples studied. However, no SNPs were found to differentiate between *C. verum* and *C. malabatrum.* Though *psbA-trnH* showed polymorphic sites they were not informative enough to differentiate between the three

species. The SNPs detected in *rbcL* locus were further validated by cloning. NJ tree drawn based on *rbcL* sequences clustered the adulterated samples with *C. aromaticum* while the remaining market samples clustered within *C. verum - C. malabatrum* clade. Thus *rbcL* was found to be the ideal locus to detect the presence of adulterants in traded cinnamon bark samples with its high amplification, sequencing efficiency, high interspecific divergence and presence of polymorphic sites. Out of the ten market samples studied seven tested positive for *C. aromaticum.*

Sixteen sequences each of *rbcL, matK* and *psbA-trnH* corresponding to *C. verum, C. aromaticum* and *C. malabatrum* were submitted to GenBank database of NCBI. The addition of these sequences enriched the number of *Cinnamomum* sequences in the Genbank database.

6.1.2. Nutmeg

High quality genomic DNA was isolated from the leaves of 5 samples of *M. fragrans* and *M. malabarica* taken as reference species and 5 samples of traded nutmeg mace. DNA was isolated from the leaves of reference species using the protocol of nutmeg leaf DNA isolation with a yield of 135-170 $\mu g g^{-1}$and purity ratio in the range of 1.8-1.9. A protocol was developed for isolating DNA from nutmeg mace by modifying the protocol developed to isolate DNA from recalcitrant dried berries of black pepper. Modifications done were overnight soaking of the mace in water, replacing CTAB with SDS, incorporating 2% PVP and 1% β-mercaptoethanol in extraction buffer and increasing the concentration of potassium acetate to 6 M. High quality DNA with yield of 231.4-306.8 $\mu g g^{-1}$and purity ratio in the range of 1.79-1.83 was obtained from the traded nutmeg mace samples using the developed protocol.

The PCR success rate, sequencing efficiency, intra and inter specific divergence and occurrence of single nucleotide polymorphisms (SNPs) were utilized to assess the potential of each barcode loci to authenticate *M. fragrans* from its related adulterants. Amplification of genomic DNA from reference and market samples was carried out using the universal primers for *rbcL, matK, psbA-trnH* and ITS. The reaction mixture and temperature profile for each locus were standardised. *rbcL, psbA-trnH* and ITS loci exhibited 100% amplification success while *matK* could not be amplified in market samples. *matK,* ITS, *rbcL* and *psbA-trnH* loci generated amplicons of length 900 bp, 750 bp, 600 bp and 450 bp, respectively. The failure of *matK* to amplify the traded samples resulted in its exclusion from

further analysis. ITS, *rbcL* and *psbA-trnH* amplicons were purified and sequenced at Scigenom labs and the contig sequences were generated from the forward and reverse reads. *rbcL* and *psbA-trnH* exhibited 100% sequencing success while contigs could not be assembled from ITS due to messy sequence data. BLAST analysis of the sequences showed that they gave maximum identity to the sequences of the respective loci of *Myristica* and other species belonging to family Myristicaceae and the generated sequences (16 *rbcL,* 16 *matK* and 16 *psbA-trnH*) were submitted in GenBank database of NCBI.

rbcL locus exhibited higher intraspecific divergence than interspecific divergence which was against the ideal barcode criteria while *psbA-trnH* locus with its higher interspecific divergence than intraspecific divergence fulfilled the ideal barcode criteria. Wilcoxon two sample test showed that the interspecific divergence of *psbA-trnH* was significantly greater than its intraspecific divergence.

rbcL and *psbA-trnH* sequences of the reference and market samples were aligned and trimmed to a final length of 425 bp and 217 bp, respectively. *rbcL* locus did not exhibit much sequence variation between *M. fragrans* and *M. malabarica*. Sixty polymorphic sites and 9 indels (positions 25-37, 44-47, 59-61, 101-103, 115-118, 171-174, 186-188, 192 and 210) were identified in the *psbA-trnH* locus between these two species. Out of the five market samples analyzed, three showed SNPs and indels similar to *M. malabarica* thus pointing to a possible substitution of *M. fragrans* with *M. malabarica* samples. NJ tree drawn based on *psbA-trnH* sequences distributed *M. fragrans* and *M. malabarica* sequences in to two separate clades. The genuine market samples clustered with *M. fragrans* while the adulterated samples clustered with *M. malabarica.*
Thus *psbA-trnH* locus was found to be the ideal locus to detect the presence of adulterants in traded nutmeg mace with its high amplification rate, sequencing efficiency, high interspecific divergence and presence of polymorphic sites. Out of the five market samples studied three tested positive for *M. malabarica.*

Ten sequences each of *rbcL, matK* and *psbA-trnH* corresponding to *M. fragrans* and *M. malabarica* were submitted to GenBank database of NCBI. The addition of these sequences enriched the number of *Myristica* sequences in the Genbank database.

6.2. Phylogenetic Analysis

6.2.1. *Cinnamomum*

The evolutionary relationship between 12 species of *Cinnamomum* viz. *C. alexei, C. aromaticum, C. camphora, C. citriodorum, C. glaucescens, C. heyneanum, C. malabatrum, C. riparium, C. sulphuratum, C. tamala, C. travancoricum* and *C. verum* was assessed using chloroplast regions *rbcL, matK, psbA-trnH* and nuclear ITS region. High quality genomic DNA was isolated from the leaves of the above species using Qiagen DNeasy kit with a yield of 132-185 $\mu g g^{-1}$ and absorbance ratio in the range of 1.8-1.9 indicating its purity.

Amplification of *rbcL, matK, psbA-trnH* and ITS was carried out using universal primers and the reaction mixture composition and the temperature profile were standardised. All the loci exhibited 100% amplification success with amplicons of size 600 bp, 900 bp, 550 bp and 750 bp for *rbcL, matK, psbA-trnH* and ITS loci, respectively. The amplicons were purified and sequences obtained were assembled to contigs for *rbcL, matK* and *psbA-trnH.* Contig assembly was not possible for ITS due to mixed sequence data. The sequences were checked by BLAST analysis and submitted to GenBank database of NCBI (28 sequences each of *rbcL, matK* and *psbA-trnH*).

Sequences obtained were aligned, trimmed and phylogenetic trees were constructed using Bayesian inference method for both single locus and multilocus combinations. But none of the phylograms generated could give a clear understanding of the *Cinnamomum* phylogeny. Incongruence was observed in the clustering of species by different loci. Different accessions of the same species were found to cluster in different clades and species resolution was not possible due to low sequence divergence in the chloroplast regions. But *rbcL* locus could differentiate *C. verum* from other adulterant species like *C. aromaticum, C. burmanni* and *C. loureiri.*

6.2.2. *Myristica*

The evolutionary relationship between 6 species of *Myristica* viz. *M. andamanica, M. amygdalina, M. beddomei, M. fatua, M. fragrans, M. malabarica, Knema attenuata* and *Gymnocranthera farquhariana* was assessed using chloroplast regions *rbcL, matK, psbA-trnH* and nuclear ITS region. High quality genomic DNA was isolated from the leaves of the above species using protocol of nutmeg leaf DNA isolation with a yield of 135-271 $\mu g g^{-1}$ and absorbance ratio in the range of 1.8-1.9 indicating its purity.

Amplification of *rbcL, matK, psbA-trnH* and ITS was carried out using universal primers and the reaction mixture composition and the temperature profile were standardised. *rbcL, matK* and ITS loci exhibited 100% amplification success with amplicons of size 600 bp, 900 bp, and 750 bp, respectively. *psbA-trnH* locus showed 95% amplification success with an amplicon size of 450 bp. The amplicons were purified and the sequences obtained were assembled to contigs for *rbcL, matK* and *psbA-trnH.* Contig assembly was not possible for ITS due to mixed sequence data. The sequences were checked by BLAST analysis and submitted to GenBank database of NCBI (20 sequences of *rbcL*, 20 sequences of *matK* and 19 sequences of *psbA-trnH*).

The sequences were aligned, trimmed and phylogenetic trees were constructed using Bayesian inference method for both single locus and multilocus combinations. Phylograms drawn based on *rbcL* and *matK* locus singly and in combination could not give any information regarding *Myristica* phylogeny. The phylograms drawn using the other loci could resolve the species. The topology of *psbA-trnH, matK+psbA-trnH* and *rbcL+matK+psbA-trnH* trees was similar to *rbcL+psbA-trnH* tree except for a single sample of *M. malabarica* positioned outside the *M. malabarica* cluster. A close relation was observed between *M. beddomei, M. amygdalina, M. andamanica*1 and *M. fatua* in the present study. *M. fragrans* was found to be closely related to *M. beddomei, M. amygdalina, M. andamanica*1 and *M. fatua* and was found distinct from *M. malabarica. Gymnocranthera* and *Knema* species were found to share sister relation with other *Myristica* spp.

The following conclusions could be drawn from the present study:-

- DNA barcoding could be used as a successful tool for the authentication of traded spices like cinnamon and nutmeg. *rbcL* locus detected the presence of *C. aromaticum* adulteration in seven out of the ten traded bark samples while *psbA-trnH* locus detected *M. malabarica* adulteration in three out of the five market samples analysed thereby demonstrating the extent of adulteration in traded cinnamon bark and nutmeg mace. The polymorphic sites in *rbcL* and *psbA-trnH* barcodes may be exploited in designing *C. aromaticum* and *M. malabarica* specific primers, respectively thereby enabling kit development for easy detection of adulterants at band level itself bypassing the cost of sequencing. The adoption of DNA barcoding as an authentication tool by the food safety agencies can help to trace these adulterants in

traded cinnamon and nutmeg products and thereby protect the interests of the consumers and exporters.

- Chloroplast regions *rbcL, matK* and *psbA-trnH* could not provide information regarding the phylogeny of *Cinnamomum* genus due to the low sequence divergence in these regions. An attempt involving more divergent nuclear regions may give some insight on the *Cinnamomum* phylogeny. In case of *Myristica* species *psbA-trnH* and combinations involving *psbA-trnH* locus gave information regarding the relationship of species in *Myristica* genus.
- A total of 84 *Cinnamomum* and 59 *Myristica* sequences are deposited in the GenBank database of NCBI.
- GenBank database of NCBI was enriched by the addition of the new sequences of *Cinnamomum* and *Myristica.*

Chapter 7

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Annexure

STOCK SOLUTIONS/REAGENTS/BUFFERS/CULTURE MEDIA

I.DNA ISOLATION

1M Tris (pH-8)

121.1gm of Tris base (Sigma) was dissolved in 800ml of distilled water. pH of the solution was adjusted to 8.0 using concentrated hydrochloric acid (HCl). The volume of the solution was made up to 1000 ml with distilled water. Autoclaved and stored at room temperature.

0.5 M Ethylene Diamine Tetra Acetate 2H_2O (EDTA) (pH-8)

186.1gm of EDTA 2H_2O (Sigma) was dissolved in 800 ml of water. pH of the solution was adjusted to 8.0 using sodium hydroxide (NaOH) pellets with constant stirring using a magnetic stirrer to dissolve the salt completely. The solution was made up to 1000 ml using distilled water and autoclaved. Stored at room temperature.

5 M sodium chloride (NaCl)

292.2 gm of NaCl (Sigma) was dissolved in 800 ml of distilled water, final volume of solution was made up to 1000 ml and autoclaved. Stored at room temperature.

10% Cetyl trimethylammonium bromide (CTAB)

10 gm of CTAB (Sigma) was dissolved in 80 ml distilled water, final volume made up to 100 ml, autoclaved and stored at room temperature.

10% Sodium dodecyl sulphate (SDS)

10 gm of SDS (Himedia) was dissolved in 80 ml distilled water, final volume made up to 100 ml, autoclaved and stored at room temperature.

6 M Potasssium acetate solution

59.04 gm of potassium acetate (Himedia) was dissolved in distlled water and autoclaved. Stored at room temperature.

3 M sodium actetae solution (pH-5.2)

24.61 gm of sodium acetate (Sigma) was dissolved in 70 ml of distilled water. pH of the solution was adjusted to 5.2 using glacial acetic acid, final volume of the solution made upto 100 ml and autoclaved. Stored at room temperature.

Chloroform : isoamylalcohol (24:1)

96 ml of Chloroform (Merck) and 4 ml of Isoamylalcohol (Merck) were mixed and stored at room temperature in dark coloured bottles.

Phenol: Chloroform : Isoamylalcohol (25:24:1)

Equal volume of Tris saturated phenol (pH > 6.8, Sigma) and chloroform : isoamylalcohol (24:1) was mixed and stored in dark bottles at 4°C.

30% polyethylene glycol (PEG)

30 g of PEG 8000 (Sigma) was dissolved in 80 ml distilled water and final volume of solution was made up to 100 ml with distilled water.

70% ethanol

30 ml of distilled water was added to 70 ml of ethanol and stored at room temperature.

II. AGAROSE GEL ELECTROPHORESIS

10 X Tris Borate EDTA (TBE) Buffer

Tris base (Sigma) – 108 gm

Boric acid (Himedia) – 55 gm

0.5 M EDTA (Sigma) – 40ml

Dissolved the above components in 800ml of sterile distilled water and then made up the volume to 1000 ml using the same.

1 X TBE

100 ml of 10 X TBE was mixed with 900 ml of sterile distilled water to make 1 litre of 1 X TBE.

0.8% agarose

0.8 gm of agarose dissolved in 100 ml of 1 X TBE.

1% agarose

1 gm of agarose dissolved in 100 ml of 1 X TBE.

Ethidium bromide (10 mg ml^{-1})

1 gm of ethidium bromide (Himedia) was added to 100 ml of distilled water and stirred on a magnetic stirrer to dissolve the dye. The solution was stored at room temperature in dark coloured bottles.

Gel loading dye

25 mg of bromophenol blue (Himedia) and xylene cyanol (Himedia) was dissolved in 74 ml sterile distilled water. It was mixed with 26 ml of 87% glycerol (Himedia) using a magnetic stirrer and the solution was stored at 4°C.

III. Cloning

Luria Bertani (LB) Agar

4 gm of LB Agar, Miller (Himedia) was dissolved in 100 ml distilled water. It was heated to dissolve the medium completely and sterilized by autoclaving.

Luria Bertani (LB) Broth

2.5 gm of LB broth, Miller (Himedia) was dissolved in 100 ml of water and sterilized by autoclaving.

Ampicillin (50mg ml^{-1})

250 mg of ampicillin was dissolved in 5 ml of sterile nuclease free water. The solution was filter sterilized using micron membrane (0.22 μ) and stored at -20°C.

X-gal (5-bromo-4-chloro-3-indolyl- β-D-thiogalactopyranoside)

20 mg X-gal was dissolved in 1ml of N, N-dimethylformamide and stored at -20 °C in the dark.

IPTG (isopropyl- β-D-thiogalactopyranoside)

1.2 gm of IPTG was dissolved in 50 ml sterile nuclease free water. It was filter sterilized and stored at 4°C.

Preparation of selective LB agar plates

100 ml of LB agar was melted and allowed to cool. 100 µl of ampicillin was added to LB agar, mixed by swirling and poured to plates. 40 µl of X-gal and IPTG was spread on the plates using a L-rod. The plates were then kept for prewarming at 37°C for 30 minutes.