Detection of plant based adulterants in selected market samples of spices using DNA barcoding technique

THESIS

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By

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Under the Supervision of
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Dedicated to my parents

DECLARATION

I hereby declare that the thesis entitled **"Detection of plant based adulterants in selected market samples of spices using DNA barcoding technique"** submitted by me for the award of the degree of Doctor of Philosophy in Biotechnology, University of Calicut contains the results of bonafide research work done by me at the ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, under the guidance of Dr. B. Sasikumar, Head & Principal Scientist, Crop Improvement and Biotechnology Division, Indian Institute of Spices Research, Kozhikode, Kerala. This thesis or part of it has not been submitted to any other university for the award of any other degree or diploma previously. All sources of help received by me during the course of this study have been duly acknowledged.

Kozhikode **PARVATHY VISWANATH A**

Date

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abbreviations

α	Alpha
β	Beta
%	Percentage
~	Approximately
µg	Micro gram
µl	Micro liter
AFLP	Amplified fragment length polymorphism
AP PCR	Arbitrarily primed polymerase chain reaction
ARMS	Amplified refractory mutation system
ASTA	American spice trade association
Bee-BOL	Bee barcode of life initiative
BLAST	Basic local alignment search tool
BOLD	Barcode of life data system
bp	Base pair
CBOL	Consortium for the barcode of life
GBIF	Global biodiversity information facility
CBSA	Canada border services agency
CE-MS	Capillary electrophoresis-mass spectroscopy
cDNA	Complimentary DNA
CDS	Coding DNA sequences
CO1	Cytochrome *c* oxidase
CTAB	Cetyl trimethyl ammonium bromide
DAF	DNA amplification fingerprinting

DAMD	Directed amplification of minisatellite region DNA
DDBJ	DNA data bank of Japan
DNA	Deoxy ribo nucleic acid
dNTP	Dinucleotide triphosphate
EC	European commission
ECBOL	European consortium for barcode of life
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immuno sorbent assay
EMBL	European molecular biology laboratory
ESA	European spice association
ESI	Electrospray ionization
EU	European union
FAO	Food and agricultural organisation
FISSR	Fluorescent inter simple sequence repeats
FISH-BOL	Fish barcode of life champaign
FSSAI	Food safety and standards authority, India
g	gram
GAP	Good agricultural practices
GC	Gas chromatography
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
iBOL	International barcode of Life
IPTG	Isopropyl β-D- thiogalactopyranoside
IR	infra red
ISO	International organization for standardization

ISSR	Inter simple sequence repeats
ITC	International trade centre
ITS	Internal transcribed spacer
Kb	Kilobase
Kg	kilogram
Kg^{-1}	Per kilogram
K2P	Kimura 2 parameter
LB broth	Luria bertani broth
LC	Liquid chromatography
NCDEX	National commodity & derivatives exchange
N-J tree	Neighbour joining tree
M	Molar
MarBol	Marine barcode of life
matK	Maturase kinase
mg	Milli gram
MgO	Magnesium oxide
ml	Milli liter
ml^{-1}	Per milli liter
mM	Milli molar
MMDBD	Medicinal materials DNA barcode database
MB	Mosquito barcode initiative
MEGA	Molecular evolutionary genetics analysis
MS	Mass spectroscopy
MUSCLE	Multiple sequence comparison by log- expectation
NCBI	National centre for biotechnology information

ng	Nano gram
nM	Nano meter
OD	Optical density
ORFs	Open reading frames
NMR	Nuclear magnetic resonance
ºC	Degree Celsius
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDO	Protected designation of origin
PEG	Poly ethylene glycol
PFA	Prevention of food adulteration
PGI	Protected geographical indication
Polar-BOL	Polar barcode of life
ProWG	Protest working group
PSI	Preshipment inspection
PVP	Poly vinyl pyrrolidone
PVPP	Poly vinyl poly pyrrolidone
QBOL	Quarantine barcode of life
RAPD	Random amplified polymorphic DNA
rbcL	Ribulose 1, 5 bisphosphate carboxylase/oxgenase
RFLP	Restriction fragment length polymorphism
RNA	Ribo nucleic acid
rpoB	RNA polymerase β subunit
SCAR	Sequence characterized amplified regions
SDS	Sodium dodecyl sulphate

SFE	Super critical fluid extraction
Shark BOL	Shark barcode of life
SNP	Single nucleotide polymorphism
SPS	Sanitary and phyto sanitary
Sponge BOL	Sponge barcoding project
SSCP	Single strand conformation polymorphisms
SSR	Simple sequence repeats
Ta	Annealing temperature
TAE	Tris acetic acid EDTA
TBE	Tris borate EDTA
TBI	Tephritid barcode initiative
TCM	Traditional chinese medicine
TLC	Thin layer chromatography
Trichoptera BOL	Trichoptera barcode of life
TSG	Traditional specialties guaranteed
U	Unit
UAE	United Arab Emirates
UK	United Kingdom
USA	United States of America
USP	United states pharmacopeia convention
UV	Ultra violet
WTO	World trade organization
WHO	Wold health organisation
Xgal	5-bromo-4-chloro-3-Indoyl- β-D- galactopyranoside

INTRODUCTION

A spice is a seed, fruit, root, bark, berry, bud or any other part except leaves which are of vegetable origin. Leaves are mainly categorised as herbs. However, the International Organisation for Standardisation (ISO) has categorised 112 plant species as spices and herbs, of which 38 are leafy spices (ISO, 1995). Appealing aroma, rich flavour and medicinal properties of spices makes them a high value commodity. Spices are used for variety of purposes like additives for processed foods, as flavourings, cosmetics, dyes, medicinal, perfumery and culinary purposes. Their uses are also well renowned in religious practices and in traditional systems of medicine. Spices are traded in varied forms as whole fresh/ dried spice and value- added products. Value added forms of spices are crushed or powdered forms or fluid/ pastes, dried dehydrated forms, spice oils, oleoresins and extractives and other value added products (Dhanya and Sasikumar, 2010).

European Union (EU) and United States of America (USA) are the two largest markets of spices, purchasing over half of the world spices export. Singapore, Japan, Malaysia, Mexico, Saudi Arabia and Canada are the other major destinations in the global spices trade. The world market for the spices and herbs trade is large, valued at around 4 billion US dollars. The developing countries earn a substantial amount through the trade of spices like black pepper, ginger, paprika, coriander, cumin, cinnamon, turmeric, etc (http: //www. intracen.org/ itc/ sectors/ spices/). India is known as the home of spices and cherishes a long history of trade and houses the most sought-after spices globally, given their exquisite aroma, texture and taste. Out of the different varieties of spices, the country produces more than 52 of them and also is one of the largest domestic markets for spices in the world (http: //www. indianspices. com/ spices

development/ spice-catalogue). India is the largest producer, consumer and exporter of spices accounting for nearly half of the global trading in spices. Indian spice trade accounts for 48% of volume and 43% in terms of value of the global spice trade. The country exports more than 170 spices and value added products to nearly 145 countries A total of 8, 43,255 tons of spices and spice products valued Rs. 16,238.23 crore (2482.83 Million US $) have been exported from India during the year 2015-16 (http://www.indianspices.com/export/major-itemwise-export).

Among the major spices exported in the powdered form, black pepper, turmeric and chilli occupy the major share (http://dasd.gov.in/index.php/statistics.html). Chilli accounts for 39% whereas turmeric occupies 10 % of the total volume of spice trade. In terms of value, chilli contributed 24%, black pepper 8% and turmeric 5% as per 2014-15 statistics (http://www.indianspices.com/statistics). As per the latest data available in print, India exported 21,450 tonnes of black pepper powder, 86,000 tonnes of turmeric powder and 3,47,000 tonnes of chilli powder in the year 2014-15 (http://dasd.gov.in/index.php/latest-news/962-indian-spices-exports-moved-up-2014-15.html).

The international trade scenario of spices is provided by International Trade Centre (ITC) UNCTAD/WTO (UN Commission on Trade and Development /World Trade Organisation). The total export of spices during the year 2014- 15 was 28,69,370 tonnes worth 97,18,008 US $. The total export during the year 2014-15 for the genus *Piper* (dried or crushed or ground fruits of genus Capsicum or of genus Pimento) was reported to be 10,44,163 tonnes in quantity valued 4758588 US $. The export of *Curcuma* was 1,21,298 tonnes valued 1,93,423 US $. The import figures for the year 2014-15 for genus *Piper* (dried or crushed or ground fruits of genus Capsicum or of genus Pimento) were 10,8 8,994 tonnes worth 47,00,358 US $. *Curcuma* trade was 113689 tonnes of volume valued at 192315 US $.

Being a high value, low volume commodity, spices and its value added products are reported to be adulterated with other inferior products. The high cost coupled with low volume and the increasing demand of spices is an inductive to spice adulteration (Singhal *et al*., 1997). Adulteration can be defined as the deliberate substitution, addition, tampering or misinterpretation of the food and its ingredients or may be false / misleading statements about the product (http://foodfraud.msu.edu/wp-content/uploads/2014/07/food-fraud-ffg-backgrounder-v11-Final.pdf). The major motive of adulteration is economic gain or it may be unintentional due to absence of proper evaluation, storage methods and other clerical errors. Adulteration is also defined by United States Pharmacopeia Convention (USP) as "the fraudulent addition of non-authentic substances or removal/ replacement of genuine substances without the knowledge of consumer/ purchaser for economic gain".

Adulteration in spices varies from earthly materials, synthetic/chemical components to plant based adulterants. The presence of earthly materials is due to the lack of proper hygienic harvesting and processing strategies and mostly unintentional. The chemical/ synthetic adulterants are usually added intentionally for economic gain and causes serious health issues. The plant based adulterants of spices like black pepper, chilli, turmeric etc include papaya seeds, 'choti ber', almond shell dust, dried red beet pulp, hazel nuts, cassava starch, fillers, related wild species of the spice plants, etc. The percentage of adulteration or admixing is more in powdered or processed spices and its value added products when compared to whole commodity. This may be due to the advantage of the foreign substance being visually going undetected due to the change of morphological characteristics during processing (Chakrabarthi and Roy, 2003).

Black pepper powder is reported to be adulterated with plant based adulterants like ground papaya seed, ground berries of wild *Piper* spp. or stained rice flour, chilli etc. (Tremlova,

2001; Paradkar *et al*., 2001; Paramita *et al*., 2003; Parvathy *et al*., 2014). Adulterants of turmeric powder ranged from artificially coloured cassava starch, rice flour to ground rhizomes of wild *Curcuma* spp. (Purseglove *et al*., 1981; Sasikumar *et al*., 2004). Chilli powder contained almond shell dust, red beet pulp, dried and powdered fruits of choti ber (*Ziziphus nummularia*) (Berke and Shieh, 2001; Dhanya *et al*., 2008). The extra amounts of bleached pericarp, seeds, calyx and peduncle of chilli added in chilli are also considered as adulterants (Govindarajan, 1986). The plant based adulterants can pose health hazards and allergic reactions to the consumers.

To ensure the standards of the traded spices, detection of adulteration and determination of the quality of spices and their value added products are of paramount importance. The FAO (Food and Agriculture Organisation) and WHO (World Health Organisation) jointly in 1962 have established Codex Alimentarius to ensure the quality and standards of internationally accepted food to protect health and economic interests of consumers as well as to ensure fair practices in the trade of food commodities. Other major organisations like American Spice Trade Association (ASTA), International Organization for Standardization (ISO), European Commission (EC) etc. guide the spice industry concerned in trade at international levels (Pruthi, 2003). To ensure the purity and the standards of spices, the American Spice Trade Association (ASTA) and the European Spice Association (ESA) have laid down stringent specifications and guidelines. ASTA rejects consignments like turmeric powder containing as little as 0.5% of foreign matter (Plotto, 2004).

In case of spices, geographical origin also needs to be taken into consideration for authentication. In European legislation, additional attention is paid to correct labelling of the geographical origin of specific food products. This growing awareness and international regulations on the food safety and adulterant free products is useful in mitigating the health

impact of allergenic reactions, intolerances and other outbreaks (e.g. the recently adopted Reg. (EU) No1169/2011; EC No, 1169/2011). With globalization of trade, especially after the WTO (World Trade Organisation) agreement, the non tariff agreements like Sanitary and Phytosanitary (SPS) and Pre Shipment Inspection (PSI) agreements insist on the standards and conformity assessments of the traded products (Rao, 2001). In the post WTO era, to maintain the legendary fame of Indian spices, it is essential to certify the products to be safe, free of adulterants and having desired quality. To prevent adulteration, Govt. of India, under the provisions of Prevention of Food Adulteration Act, 1954 [Act No. 37 of 1954, Govt. of India] have made adulteration a punishable offense.

Rejection of consignments on the basis of adulteration and quality issues still continues in midst of all these technological advances and regulations. The Economic Times dated 21st August 2013 reported the order of Kerala Food Safety Commissioner of National Commodity & Derivatives Exchange (NCDEX) to annihilate 900 tonnes of mineral oil adulterated black pepper. The Hindu Business line dated December 24, 2012, also highlighted the contamination of black pepper with mineral oil, which caused six warehouses in Kerala to be sealed due to complaints on the quality. The addition of mineral oil may be done to suppress the growth of fungus/ moulds and give more weight and dark shining appearance to black pepper. Chilli powder adulterated with Sudan dye by a leading Spices Export Firm based in Kerala was recently confisticated and was widely reported in the local press. Approximately 1200 kg of chilli power to be exported to the USA was seized and destroyed. Analysis proved the presence of 14 mg of Sudan dye for every 100 g of the branded chilli powder. Presence of starch powder in the commodity has resulted on imposing ban on some brands of chilli, coriander and turmeric powder.

Food Safety and Standards Authority of India (FSSAI) has registered cases against some major brands of spices including turmeric and chilli for claiming the product to be “pure”

while containing undeclared components (http://www.ibtimes.co.in/kerala-food-safety-department-bans-nirapara-powdered-spices-645543). There have been other reports of adulteration of curry powders and masala powders with biological or non biological carcinogenic adulterants (http://timesofindia.indiatimes.com/city/bhopal/4-out-of-6-spice-samples-found-adulterated/articleshow/12312151.cms). The Food Fraud databases of United States Pharmacopeia Convention (USP) records the cases of food adulteration. The food products most frequently associated with adulteration are milk, vegetable oils, spices, seafood, clouding agents and lemon juice (www.foodfraud.org). The reports on the ways and levels of adulteration in traded spices are alarming and pose serious health hazards to the consumers.

Globalization of food trade, which gives importance on the traceability of origin to guarantee quality and authenticity of commercially available food products has encouraged the search for efficient methods to detect adulteration (Barbuto et al., 2010). Tracing the origin of food products is a proactive action to prevent fraudulent or deceptive labeling and to certify the origin and quality of products (European Commission, 2002). To authenticate the quality, geographical provenance of food and spices and to prevent commercial frauds of adulteration cases, a reliable, reproducible and cost effective technique is very essential. The validation of the authenticity relies mostly on the analysis of chemical compounds, proteins and/or DNA sequences (Galimberti et al., 2013). Earthy/ synthetic adulterants in spices could be successfully traced using physical and analytical techniques like microscopic, spectroscopic, chromatographic, electronic nose and immunological methods. But detection of plant based adulterants often proved to be difficult. In such situations biotechnological methods become handy and more effective.

The DNA sequences have the advantage as they are highly stable to processing and least affected by age and other physiological conditions and is well established within a species.

Successful identification of biological species using DNA-based techniques has triggered the development of a DNA marker-based platform for authentication of plant materials. RAPD-SCAR (Random Amplified Polymorphic DNA- Sequence Characterized Amplified Region) and other PCR (Polymerase Chain Reaction) based techniques have been successfully used for authentication due to their sensitivity and specificity. The advent of new techniques for the detection of adulterants, in food and spices, have led to the existence of several patents for adulterant detection in agricultural food commodities like tea (US Patent 6942976, US Patent 6541624), basmati rice (Wipo Patent WO 2007116414, US patent 2006269941), concentrated fruit juice (US Patent 5128243), meat (US Patent 2007009910) etc. PCR based molecular marker (random primer derived SCAR markers) for the detection of adulterants in herbal and medicinal materials has been patented (US Patent No. 6803215).

Technical advancement to check routine samples is core solution to restrict fraudulent or unscrupulous trade practices. Lack of efficient tools for detection provides incentive for adulteration /substitution. DNA barcoding, is a robust technique, proposed to identify herbal ingredients and to detect adulteration. DNA barcoding discriminates the biological entities by analyzing the variability in a single or in a few standard molecular marker(s) (Herbert et al., 2003). The technique has been used to detect adulteration in different commodities like medicinal plants (Newmaster *et al*., 2013), tea (Stoeckle *et al*., 2011), olive oil (Kumar *et al*., 2011), spices like saffron (Gismondi *et al*., 2013), cinnamon (Swetha *et al*., 2014), nutmeg (Swetha *et al*., 2016), star anise (Meizil *et al*., 2012) and members of family Lamiaceae (de Mattia *et al*., 2011).

The present study, taken up under this situation, is an attempt to detect plant based adulterants in selected market samples of traded spices using DNA barcoding technique. The

selected spices include black pepper (group I), turmeric (group II) and chilli (group III). The major objectives of the study include:

1. To standardize an ideal barcode loci for spices viz., black pepper, turmeric, chilli and their plant based adulterants.
    a. DNA isolation from genuine and probable adulterants.
    b. Primer selection, PCR amplification and standardization using barcoding loci.
    c. Sequencing and data analysis.
    d. Selection of the ideal locus depending on the PCR and sequencing success.
2. To trace out the plant based adulterants in traded/ commercial spice samples of black pepper, turmeric and chilli powders using DNA barcoding technique.
    a. Sequence alignment of the market samples with the genuine and probable adulterant sequences and SNP detection.
    b. NCBI BLAST and N-J tree construction.
    c. Validation studies using simulation and cloning.
3. To construct a reference library for the spices and its related adulterants using barcoding loci and barcode generation.
    a. Contig generation of the sequences from forward and reverse sequences from the barcoding loci tested.
    b. Sequence alignment and editing using bioinformatics software.
    c. Proof reading and data uploading in the NCBI Genbank databases.

REVIEW OF LITERATURE

2.1 General Introduction

Spices are aromatic vegetable products used as flavourings or condiments. Apart from their use as flavourings, they are used in cosmetics, perfumery, medicine, food and beverages as well. Spices are also valued for their nutritional, antioxidant, antimicrobial, insect repellent and medicinal properties (http://www.indianspices.com/spices-development/spice-catalogue). The antioxidant properties enable them to prevent lipid degradation and food rancidity. Spices used as flavourants in foods serve as sources of natural food colours. β carotene, cryptoxanthin, lutein and crocin are some of the colour components present in them. Spices are rich source of proteins, vitamins and minerals like calcium, sodium etc. The chemical constituents present in the essential oils and oleoresins of the spices are responsible for its flavour, pungency and medicinal properties. Any change in standards of these may alter its functioning in the respective areas.

India is known as the “Land of Spices” – a treasure house of spices, mainly due its vast diversity of spice cultivation. The Spices Board of India lists about 52 spices produced and exported from India, clearly stating the fact of India’s rich spice diversity. Indian spices enjoy premium status in the world trade due to its elite qualities. Among the many spices India houses, black pepper, turmeric and chilli are the three major spices of trade importance.

2.2 Spice-Economy

The world markets for the trade of spices and culinary herbs occupies a major share around 4 billion US dollars. According to the International Trade Centre UNCTAD/WTO (ITC), the total export of spices during the year 2014- 15 was 2869370 tonnes worth 9718008 US $. The

export of spices has increased substantially when compared to the previous year data of 2013-14 which was 2600496 tonnes worth 9539857 US $. The trade has increased both in quantity and value (http://www.intracen.org/itc/market-insider/spices/)

The spice trade in India accounts significantly to the world trade both in quantity and quality. Indian spices have reported a substantial growth in the past five years, with an average growth rate of 14% in terms of rupees and 5 % in terms of dollar value. The varying climatic conditions of the Indian landscape provide ample scope for the cultivation of a variety of spices with almost all Indian states producing spices from a total land area of 3.15 million hectares of spice cultivation. A total of 8, 43,255 tons of spices and spice products valued Rs. 16,238.23 crore (2482.83 Million US $) have been exported from India during the year 2015-16. The total exports increased by 9% in terms of volume and 7% in terms of value (US$) when compared to the previous year statistics (http://www.indianspices.com/export/major-itemwise-export).

A significantly high amount of Rs.1,910.90 crore from 11,475 tonnes of value added spice products like spice oils and oleoresins was obtained, registering a growth of 1% in volume and 10 % in value when compared with previous year data (Arecanut and Spices Database, 2007). The quantity wise export registered a hike of 104% in terms of volume and 116% in rupee and 110% dollar terms (http://www.indianspices.com/) when compared with the previous year's trade. This figure highlights the importance of spice trade in Indian economy. Major share, approximately 11.65% of the export of spices from India is to USA, a major importer of Indian spices with an annual growth of 2 % in the imports yearly. Exports to the USA stood at 2,89,325 lakhs followed by China at 1,05,523 lakhs as per 2015-16 estimates (http://indianspices.com/sites/default/files/cou16web.pdf). Among the major trade

contributors, black pepper, chilli and turmeric play a significant role (http://www.indianspices.com/sites/default/files/Annual%20Report%202014-15.pdf).

As per the annual report (2014-15) Spices Board of India, Cochin, chilli contributes to the major share among the exported spices followed by cumin. Chilli accounts for 347,000 tonnes worth Rs.3,517 crore in 2014-15. The export grew by 11.04 percent in quantity and 29.20 percent in value as compared to 2013-14. Black pepper significantly contributes to the spice trade bringing 1,208.42 crore from an export volume of 21,450 tonnes in 2014-15. Black pepper marked an increase of 29% in terms of value when compared to the 2013-14 trade of Rs.940.02 crore for 21,250 tonnes. The turmeric exports fetched earnings of Rs.744.35 crore from 86,000 tonnes in 2014-15 as compared to 77,500 tonnes and Rs. 666.76 crore during 2013-14 (http: //www. indianspices. com/ sites/ default/ files/ Annual% 20Report% 202014-15.pdf). The percentage share contributed by the three major spices and the spice oils and oleoresins was chilli (24%), turmeric (5%), black pepper (8%), spice oils and oleoresins (13%) etc. (http: //dasd.gov.in/ index.php/ statistics. html).

2.2.1 Black pepper

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae, cultivated for its fruit which is black in colour and is native to India. The genus *Piper* includes >1000 species making it one of the largest in the family *Piperaceae*. Out of these 1000 species 110 are of Indian origin (Purseglove *et al*, 1981). *Piper nigrum* (black pepper of commerce), *P. galetaum, P. attenuatum* etc. shares a common habitat and are the more common entities found in the centre of origin of *P. nigrum*, namely Western Ghats of India and are distinct morphologically.

Black pepper is known as “The King of Spices” and as “Black Gold”, revealing its importance and uses. Black pepper has a strong impact on the influence on human culture by

its role in the trade and civilisations. It was for this spice the major trade relations occurred between the Malabar Coast and the rest of the world. The spice is credited with medicinal properties such as antimicrobial, antioxidant, antiinflammatory and antitoxic properties (Vijayan and Thampuran, 2000).

The "black gold", berries of the tropical vine, *P. nigrum* L, occupies a formidable position among the spices. It is mostly traded in the form of whole dried berries or processed forms like powdered black pepper or may be as value added products like white pepper, ground pepper/ black pepper powder, dehydrated green pepper, freeze dried green pepper, pepper oil and oleoresin (Spices Statistics, 2004). Black pepper contains about 3% essential oil, whose aroma is dominated (max. 80%) by monoterpenes hydrocarbons (https: //www.mdidea.com/ products/ new/ new06809.html). The pungency of black pepper is mainly due to the presence of piperin and resin and the spicy smell is contributed by the essential oil present in it (Tremlova, 2001). Powdered black pepper is the most common and preferred item due to its highly process friendly nature and utility in culinary preparations.

In terms of quality and quantity, black pepper occupies a significant position in the world trade. Among the major share of spice exports from India during 2015-16, black pepper occupies 3% in quantity and 11% value in terms of rupee of the total export spice commodity (http://www.indianspices.com/sites/default/files/MajorItemwiseExport2016.pdf).India,Brazil, Indonesia, Malaysia, Sri Lanka, Vietnam, China, Thailand, Madagascar, Ecuador, and Cambodia are the major producers and exporters of black pepper in the world trade (Arecanut and Spices Database, 2007). The total world production of black pepper is about 338380 metric tonnes from a total area of 476514 hectors of cultivation (http://www.ipcnet.org/n/statpdf/pdf/1.06.pdf).

A total of 265254 metric tonnes of black pepper is being exported from the black pepper producing countries as of 2010-11 data with Vietnam, Indonesia and Brazil leading the export market of black pepper (http://www.ipcnet.org/n/statpdf/pdf/1.06.pdfn). The contribution of black pepper to the world spices trade is estimated to be 327592 metric tonnes valued 1188689 US $. The United States remains the largest importer of black pepper followed by Europe, Singapore, Germany, Netherlands, United Arab Emirates (UAE), Japan and Canada (http://www.intracen.org/).

The total value of black pepper exported from India during 2015-16 is 28,100 tonnes of quantity worth Rs. 1730.415crore (http://www.indianspices.com/export/major-itemwise-export). Major export markets of Indian pepper are USA, UK, Germany, Netherlands and Italy. The total area coverage of cultivation in India during 2013-14 is 122400 hectors with a production of 37000 tonnes (http://www.indianspices.com/). According to Spice Board of India, black pepper is mainly grown in the states of Kerala, Karnataka and Tamil Nadu, where Kerala enjoys the major share in production and cultivation contributing 16000 tonnes of black pepper from an area of 27955 hectors (http: //indianspices.com/ sites/ default/f iles/ Major-spice-state-wise-area-production-web-2015. pdf).

2.2.2. Turmeric

Turmeric, *Curcuma longa* L., belongs to the family Zingiberaceae, is a well known spice for its use in the cosmetic, culinary and medicinal properties. The spice is well characterized with its medicinal properties like anti-inflammatory, anticancerous, anti-oxidant, antimicrobial, and antiviral properties; as an antiseptic; and in the treatment of diabetes and Alzheimer's disease (Sasikumar, 2005). Its attributed biological property is mainly due to the presence of curcumin, the yellow pigment, apart from oleoresins, oils and secondary metabolites. Its vibrant yellow color has given its name as "The golden Spice/ Indian saffron". This spice

forms an integral part of the Hindu rituals (Sasikumar, 2012). Turmeric is mostly traded in the form of dried and/or fresh rhizomes, powder, oleoresin and turmeric oils (Sasikumar, 2001).

Iran, USA, Malaysia, UAE, Japan and UK serve as the major market for Indian turmeric. India, China, Pakistan, Bangladesh, Vietnam, Thailand, Philippines, Japan, Korea, Sri Lanka, Nepal, South Pacific Islands, East and West Africa, Malaysia, Carribbean Islands and Central America are the major countries producing turmeric (Sasikumar, 2005). Among the major share of spice exports during 2015-16, turmeric occupies 10% in quantity and 5% value in terms of rupee of the total export spice commodity. The total export of *Curcuma* increased from 109189 tonnes, value of 160851 US $ to 121298 tonnes of value 193423 US $ in the year 2014 and 2015, respectively (http://www.intracen.org/itc/market-info-tools/statistics-export-product-country/).

India stands as the leading producer and exporter of turmeric in the world with an annual production of around 1092628 tonnes from an area of 207570 hectors (http://www.indianspices.com/statistics). The total value of turmeric exported from India during 2015-16 is 88500 tonnes of quantity worth Rs. 921.65 crore. There is a substantial increase in the export of turmeric as compared to the previous year (2014-15). India exported 86000 metric tonnes of turmeric worth Rs.744.35 crore during the year 2014-15(http://www.indianspices.com/export/major-itemwise-export). The major share of Indian turmeric is contributed by Tamilnadu amounting about 461990 tonnes of turmeric from 76983 hectors of area followed by other states like Telengana, Andhra Pradesh, Karnataka, Gujarat, West Bengal, Kerala and other North eastern regions states (http: //indianspices.com/ sites/ default/ files/ Major-spice-state-wise-area-production-web-015.pdf .

2.2.3 Chilli

Chilli (*Capsicum annuum* L), belongs to the family Solanaceae, a spice of commercial importance. It is mainly used as a culinary spice and also in medicine. The colour and the pungency of the spice are attributed to the presence of capsaicin. Chilli is used mainly in curry powders, masala preparations, pickles and also for seasoning eggs, fish and meat preparation, sauces, chutneys, frankfutters, sausages etc. (Susheela, 2000). Chillies are best used natural colorants. Apart from its culinary purposes, it is found to be of great medicinal purposes. The medicinal property of chilli includes digestive, thermogenic, carminative, stimulant, cardiotonic, antipyretic, serdorific, rubefacient & sialagogue.

Chillies are rich source of Vitamin C, potent water soluble antioxidant and capsaicin oil obtained from chillies are effective for pain relieving (Srinivasan, 2005; http:// www. indianspices.com/ spices-development/ properties/ medicianal-other-values-spices). India, China, Pakistan, Korea, Mexico and Bangladesh are the prominent producers of chilli (Thampi, 2003). Among the major share of spice exports during 2015-16, the total value of chilli exported from India is 347500 tonnes of quantity worth Rs. 3931.70 crore (http://www.indianspices.com/export/major-itemwise-export).

The data of the last two consecutive years show a consistent increase in the export of the chilli by 11% in quantity and 29% in value. The total area of chilli cultivation during 2013-14 was estimated to be 791930 hectors giving a production value of approximately Rs.13764 crore. Andhra Pradesh ranks first in India followed by Karnataka, West Bengal, Maharashtra for chilli production. Chilli is mainly exported as dry whole fruits, powder or as value added products. Chilli is the most important ground spice exported from India and it enjoys the major share in the spice trade (Pruthi, 2003). United States of America, United Arab

Emirates, United Kingdom, Canada, Saudi Arabia, South Africa, Pakistan, Singapore and Bangladesh are the main markets of Indian chilli powder.

2.3 Adulteration in spices

Spices, being a high value commodity are frequently subjected to adulteration. International trade of spices occupies a major share of trade in the global economy and the demand is increasing in both developed and developing nations, necessitating better availability of the resources at a higher pace and comfort. Low volume, high value and increasing demand of spices are incentive to adulteration of the commodity leading to erosion of the perceived biological value. International organizations like International Standards Organization (ISO) defines spice and condiments as 'vegetable products or mixtures thereof, devoid of extraneous matter which are used for flavouring, seasoning and imparting aroma in food (ISO, 1995). Quality of the spice is an important criterion for its perceived biological efficiency and any change in its constituents alter its use in medicinal and other pharmaceutical use.

Adulteration may be defined as the intentional or unintentional mixing/substitution of the original/genuine material with inferior, morphologically similar but less quality, spoiled, defective, useless parts of the same or different plants, harmful or chemical/synthetic substances which do not confirm with official standards. A spice is considered adulterated if it contains added poisonous or deleterious substances or filth or unapproved food or colour additives or may contain any substances added to increase the bulk weight or to make it more valuable (Singhal and Kulkarni, 2003). Any omission/ removal or substitution of the valuable contents to conceal the inferior nature may also be considered adulteration (Newmaster *et al*., 2013). The practice of adulteration is either intentional or unintentional. Unintentional

adulteration is mainly due to the absence of proper evaluation, storage methods, clerical errors (Preethi *et al*., 2014).

Deliberate/ intentional adulteration is mainly the addition of inferior, morphologically similar materials of plant origin or synthetic chemicals mainly to increase the bulk weight and for economic gains. Quality of spice is mainly affected by plant origin, cultural practices, harvest date, treatment and storage conditions (Tremlova, 2001). Singhal and Kulkarni, 2003 reports the admixing or substitution of cheaper or inferior substances with traded spices/spice powders. Adulteration detection mainly aims to certify the product to be: 1). Authentic: determine the presence of main ingredient, 2). Contaminated: presence of other substances apart from the genuine sample, 3). Substitution: presence of cheap quality / similar or related species other than the genuine sample, 4). Filler: presence of less quantity of other. Presence of fillers may be due to the low quality in the post harvest and storage conditions substances (Newmaster et al., 2013) or to increase the bulkiness of the item.

Consumption of products containing undeclared constituents may cause intoxication or problems such as allergy in sensitized individuals (Asensio *et al*., 2008). Organisations like the International Organisation for Standardisation (ISO), American Spice Trade Association (ASTA), The Food Safety and Standards Authority, India (FSSAI) etc. are the concerned authorities that impose strict regulations on the quality of spices and herbs imported and exported. Spices are reported to be adulterated with synthetic and plant based adulterants. Synthetic adulterants include dyes, artificial colours, mineral oils, chemicals, chalk powder and other earthy substances. The common chemicals/ synthetic materials present includes Azo dyes, coal tar red, sudan red, para red, vanilyl- n-nonamide, metanil yellow, orange II lead chromate, eugenol, cylon oil, yellow brown dye, mineral oils, synthetic flavourants and dyes like tartrazine, ponceau 2R, sunset yellow, amaranth, orange GG, methyl orange, eosin,

erythrosine, solutions of potassium or ammonium nitrate; sodium sulphate; magnesium sulphate; barium sulphate; borax, oils honey, glycerine, chalk powder; yellow soap stone powder, lime, capsaicin, magnesium salts etc. (Dhanya and Sasikumar 2010). Biological adulteration with microbial and insect filth contamination due to poor storage and package facilities ftp://ftp.fao.org/codex/meetings/ccfh/ccfh46/Report_Spices_Dried_Herbs_Expert%20Meeting.pdf), substitution with morphologically similar/related /unrelated plant parts are also reported (Dhanya and Sasikumar, 2010).

Adulteration with regard to botanical and geographical origin is also a concern in the case of spices. Geographical indications of food products are laid down in Regulation (EC) No. 510/2006. Registration of geographical commodity is mainly of three classes; protected designation of origin (PDO), protected geographical indication (PGI), and traditional specialities guaranteed (TSG). The use of cheap ingredients or mismatched proportions of ingredients, false information about the source of components (geographical origin, botanical, or animal) leads to incorrect labelling of samples. In this context there is an intense need to develop strict regulatory measures to control adulteration. A set of guidelines for the protection of botanical and geographical origin of food products have been proposed. In addition, such products can be labelled with protected designation of origin, protected geographical indication, and traditional speciality guaranteed statements (European Commission, 2006; Majcher *et al*., 2015).

2.3.1Adulterants in Black pepper

Synthetic /artificial adulterants in black pepper include mineral oil, dyes and stones/ earthy materials. Addition of mineral oil may be done to suppress fungus/ moulds and give more weight, dark black colour and shiny appearance to black pepper. Coloured starches from cheaper source were also reported as adulterant in black pepper powder (Archer, 1987; PFA,

2003). Plant based adulterants in black pepper includes dried papaya seed (*Carica papaya* L.) which resembles black pepper berries in colour, texture and size and is one of the main adulterants (Pruthi and Kulkarni, 1969; Bhalla and Punekar, 1975; Wealth of India, 1992). It is cheaply available and a waste product of the papaya industry. The addition of papaya seeds to increases the bulk density of the black pepper is a direct adulteration practice for economic gains. The consumption of papaya seed causes toxicity and anti fertility issues (Sareen *et al*., 1961). Other major adulterants of black pepper are its wild relatives *P. attenuatum* and *P. galeatum.* They are grown in wild habitats and are easily available as Non Timber Forest Produce and can be widely mixed with the genuine black pepper (*P. nigrum*). Dried fruits and berries of *Lantana camara*, *Embelia ribes, Mirabilis jalapa*, and *Schinus molle* which have morphological resemblance to the black pepper are some of the reported minor adulterants (Wealth of India, 1962, 1972; Singhal *et al*., 1997).

Powdered black pepper is adulterated by pigmeal, papaya seeds, seed capsules of various fruits, stones, kernels, flowers, minerals, starch from cheaper source and waste materials of pepper production industry (Dhanya and Sasikumar, 2010). Paradkar *et al*., 2001, reported the adulteration of processed products like oleoresins etc. from adulterated berries. Low quality exhausted black pepper, light berries, stems and chaff of black pepper; normally the waste products of black pepper industry are also added as adulterants (Ravindran and Kalluparackal, 2001). Parvathy *et al*., 2014, recently reported chilli as an adulterant of traded black pepper powder, suggesting the probability of intentional recycling of spent black pepper fortified with chilli.

2.3.2 Adulterants in Turmeric

Turmeric is often sold in the ground form and is frequently adulterated by different means (Govindarajan, 1980; Pruthi, 1980; Purseglove *et al*., 1981; Singhal *et al*., 1997). Common

synthetic adulterants of turmeric ranges from dyes such as Metanil Yellow, Orange II lead chromate to powders like chalk powder, yellow soap stone powder etc (Dhanya and Sasikumar, 2010). Turmeric powder is found to be adulterated with plant based adulterants like foreign starch (tapioca, arrowroot etc.) and with other cheap and low curcumin containing *Curcuma* species (Sasikumar *et al*., 2001). The related species of *Curcuma* mainly used in adulteration are *C. zedoaria* Rosc. or 'yellow shotti' syn. *C. xanthorrhiza* Roxb. ('Manjakua') and *C. malabarica* (Sen *et al*., 1974; Mitra, 1975; Zwaving and Bos, 1992). *C. zedoaria* is reported as toxic in nature and also causes ill health when consumed, apart from its low curcumin content. Though the market samples of turmeric powder contain the prescribed levels of curcumin, they are found to be adulterated using other *Curcuma* species (Sasikumar *et al*., 2005). Parvathy *et al*., 2015 recently reported the adulteration of commercial samples of turmeric powder with *C. zedoaria*, cassava starch and other fillers like wheat, rye, barley etc.

2.3.3 Adulterants in chilli

Chilli is mostly vulnerable to adulteration in its powdered or paste form as the adulterants goes visually undetected (Chakrabarthi and Roy, 2003). Artificial and natural adulterants are reported in chilli. Synthetic or artificial adulterants include coloured dyes like coal tar red, sudan red, para red etc., synthetic pungent compounds, brick powder, talc powder (Mitra *et al*., 1961; Banerjee *et al*., 1974; PFA, 2003). Apart from artificial adulterants, chilli is also reported to be adulterated with natural/plant based products. Plant based adulterants in chilli are mainly dried red beet pulp (*Beta vulgaris* L.) (Schwein and Miller, 1967; Berke and Shieh, 2001) dried and powdered fruits of *Ziziphus nummularia* (Burm.f) Wight & Arn., commonly called "Choti ber" (Dhanya *et al*., 2008) and almond shell dust (Berke and Shieh, 2001). Chilli powder is reported to have the presence of bleached pericarp, seeds, calyx, and peduncle of chilli to increase the bulk weight (Govindarajan, 1986; Chakrabarthi and Roy,

2003). Presence of starches of cheap origin and tomato wastes are also reported in chilli powder (Pruthi, 1980).

2.4 Methods to detect adulteration in food and agricultural commodities

Increased fraudulent means of adulteration had created consumer awareness in food safety and quality control, thereby leading to development of numerous techniques for its detection. Adulteration detection is mainly done by the comparison of the data with a reference standard or control data (Wilhelmsen, 2004). Dhanya and Sasikumar, 2010, recorded adulteration in three different ways; by detecting the presence of a foreign substance in the commodity, by demonstrating a component is deviated from its normal level and by demonstrating that a profile is unlikely to occur in the commodity. The techniques for adulteration detection in food and agricultural commodities includes physical methods, chemical profiling based analytical methods, immunological and the most advanced DNA and protein based biotechnological approaches (Smilie and Khan, 2010).

2.4.1Physical methods

Physical methods involve the macroscopic and microscopic evaluation and other parameters like solubility, bulk density, texture etc. In macroscopic evaluation, morphology of the plant species is taken into consideration for distinguishing from other species. It is also based on the sensory parameters like texture, solubility, bulk density, shape, size, colour, odour, surface characteristics etc. (Choudhary and Sekon, 2011). Traits used for macroscopic analysis range from root and stem features; leaf characteristics like shape size and morphology; inflorescence and floral characters; stamen number and shape; carpels per ovary etc. (Smilie and Khan, 2010). Microscopic analysis is mainly performed when the morphologic characters of the spices are changed eg. powdered/ dried samples. Microscopy is mainly the examination of internal cellular and structural tissues to differentiate between

species (Revathy *et al*., 2012). Examination and comparison of the cell /anatomical structure of the authentic samples with the test samples can reveal admixing / adulteration or abnormality. Microscopy involves the use of instruments like light, electron and phase contrast microscopes for observing the anatomical characteristics, like the presence or absence of hairs (trichomes), starch grains, oil glands, canals, particular cell types, seeds or pollen morphology and vascular traces (Smilie and Khan, 2010). Fritz *et al*., 2008, successfully authenticated Chinese star anise using SEM (Scanning electron microscope).

Detection of adulterants of black pepper has a long time history starting from the attempts of to detect the adulteration of ground black pepper with added pepper shells. The crude fibre, d-glucose, MgO, MgO: d-glucose ratio, and MgO: crude fibre ratio were calculated and was reported as the most valuable criteria for detecting the added pepper shells. Food safety and drug administration, details some quick and effective house hold floating and visual examinations to detect the presence of papaya seeds and light berries. Pruthi and Kulkarni, 1969 traced out papaya adulteration in black pepper using floating test followed by visual and microscopic examination of the floaters.

The test was performed by dipping the black pepper and papaya berries in ethyl alcohol of specific gravity 0.8 to 0.82 at 25/25 degree Celsius. The mature black pepper berries sank while the papaya seeds floated. Different staining methods and microscopic examination was developed for papaya seed adulteration in black pepper (Bhatnagar and Gupta, 1966). Starch concentration could also be used to estimate the amount of light pepper present in black pepper (Mitra *et al.*, 1966). Detection of adulterants in black pepper using microscopy was achieved by Tremlova , 2001. Microscopic analysis of cheaper vegetable sources in turmeric were performed by Pearson, 1976, but was found less useful due to the degradation of starch grains and oleoresin cells by boiling of rhizomes (Purseglove *et al*., 1981). Presence of chalk powder and yellow powders can be easily detected by using simple chemical reactions (PFA,

2003). Adulterants like tomato waste and starch were detected using microscopic examination (Pruthi, 1980). The technique of light microscopy was employed in detecting the adulterants from powdered chilli, black pepper, cumin and mustard (Zu and Zhao, 2014).

Physical or macroscopic evaluation mainly depends on the morphological characters. The adulterants mostly resemble morphologically to the genuine species which creates confusion in distinguishing the genuine and adulterant ones. Also the developmental stages of the plants and the change in the morphologic characters during processing makes the physical evaluation difficult and so the method is not very commonly used in modern times for detection studies. The microscopic examination is tiresome and requires a lot of expertise which made it less feasible in adulteration detection. Even though there are reports of successful authentication of spices using microscopy, the paucity of skilled personal, time, phenological variation, expressivity and penetrance and lack of diagnostic markers etc. limits its utility (Sasikumar *et al*., 2016).

2.4.2 Analytical methods

Analytical/ biochemical techniques includes chromatographic, electrophoretic, spectroscopic chemometric techniques and other hyphenated technologies. Chromatographic techniques include mainly the thin layer chromatography (TLC) gas chromatographic (GC) and high performance liquid chromatography (HPLC). TLC is the simplest and more economic of the chromatographic techniques. The technique is used for getting finger prints of all components of a sample. This is advantageous for the adulteration detection of mixed samples. TLC has proved to be efficient in detecting the presence of Sudan dye in chilli powder (Darr *et al.*, 2013). Volatile and semi volatile compounds present in the specimen form the main detection component of GC. The amino acids, proteins, phenolic acids etc. present in a compound are the focal factors for HPLC analysis. The technique has proved to be very

efficient in the authentication of olive oils from cheaper oils, chilli powder adulteration with para red, saffron adulteration, detection of coumarin content in cinnamon, adulteration of cassia with true cinnamon, presence of sudan dye in hot chilli (Jabeur *et al*., 2014; Blahov and Svobodov, 2012; Riaz *et al*., 2009; Lage and Cantrell, 2009; Sproll *et al*., 2008; Tateo and Bononi, 2004; Ceraci *et al*., 2003, Gamazo-Vazquez *et al*., 2003).

Eletrophoretic methods, mainly capillary electrophoresis are aided by the differences in the electrphoretic and electrosmotic movements of ions in the buffer solution under supplied voltage. The capillary electrophoretic methods include capillary zone electrophoresis, capillary gel electrophoresis, capillary isoelectric focusing, isotachophoresis and micellar electrokinetic capillary chromatography. The technique is used for the quality control, authentication and adulteration detection of many herbs and spices including saffron (Cianchino *et al*., 2007; Zougagh *et al*., 2005). The interaction of electromagnetic radiation with matter is exploited in spectroscopic techniques like UV, visible, infra red (IR), Raman, fluorescence, nuclear magnetic resonance (NMR) and mass spectroscopy (MS) methods. Zalacain *et al*., 2015 were able to detect artificial colorants added to saffron using UV-visible spectroscopy. Chemometrics uses mathematical, statistical and other logical measurements and experiments to provide information by analysing the chemical data from analytical techniques like spectroscopy and chromatography (Massart *et al*., 1988).

The combination of analytical techniques with chemometrics aided in the detection of the banned artificial dye ‘Sudan red’ and potent carcinogens in chilli samples from retail markets (di Anibal, 2009). Variants of MS like direct injection mass spectrometry and proton transfer reaction mass spectrometry are used in the authentication of traded spices like black pepper, chilli paprika, nutmeg, vanilla and saffron in conjunction with chemometrics (Silvis *et al*., 2015, Mohammad *et al*., 2015). Hyphenated techniques are the combination of chromatographic separation technique and spectroscopic detection technology. High

Performance Liquid Chromatography-Mass Spectroscopy (HPLC–MS), Gas Chromatograph-Mass Spectroscopy (GC-MS), Capillary Electrophoresis-Mass Spectroscopy (CE-MS), and Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR) are some of the commonly used hybrid/ hyphen technologies for adulteration detection.

The contributions of hyphenated techniques in adulteration detection of spices are significant. The detection of Sudan red in chilli using LC-MS (Rebane *et al*., 2010), detection of banned dyes like Para Red, Sudan Orange G, Sudan I-IV, Sudan Red 7B and Rhodamine B in spices using HPLC-MS (Botek *et al*., 2007), Oregano adulteration as low as 5 % intentionally blended with typical adulterants including thyme and marjoram with High Performance Liquid Chromatography-Electro Chemical (HPLC-EC) (Hollosi *et al*., 2013) and the detection of aflatoxins in spices (Romagnoli *et al*., 2007) are few of them. Chromatographic and UV based methods to detect papaya adulteration in black pepper powder were successful (Hartman *et al.*, 1973). Thin layer chromatography was employed by Paradkar *et al.*, 2001 and Paramita *et al*., 2003 for the detection of papaya seed adulteration. Super critical fluid extraction (SFE) showed a fluorescent band at 366 nm at R_f 0.172, proving the presence of ground papaya seed adulteration .

Fluorescent characteristics and HPLC finger prints of black pepper along with two market samples were analysed for the adulteration detection of papaya seeds, *Embelia ribes Burn*. and *Lantana camara* L.(Jain *et al*., 2007). *P. nigrum* ether extracts exhibited lemon yellow fluorescence at 365nm in contrast to blue fluorescence exhibited by *C. papaya*. HPLC profiling was done by Parvathy *et al*., 2014 for detecting *C. annuum* adulteration in black pepper market samples. The HPLC profiles of the standards viz., piperin and capsaicin gave distinct peaks and the same was used to compare and detect adulteration of market samples. In turmeric, TLC, spectrophotometric and capillary electrophoresis was used to determine pigment composition of different *Curcuma* species and was useful in distinguishing *C. longa*

from its adulterant *C. xanthorrhiza* (Lechtenberg *et al*., 2004). Essential oil estimation was also tried to distinguish turmeric and its related species (Mitra, 1975, Sen *et al*., 1974, Zwaving and Bos, 1992).

Chromatographic and spectrophotometric techniques were proved to be helpful in detecting different dyes and artificial colours used as adulterants in turmeric powder (Tripathi *et al*., 2007). Laser induced spectroscopic technique for adulteration detection in turmeric powder was performed by Tiwari et al., 2013. The quality of turmeric is mainly attributed to the curcumin content and can be measured using simple spectrophotomeric determination (Jasim and Ali, 1992). A high occurrence of artificial adulterants has resulted in extensive research to develop techniques to detect adulteration in chilli. Microscopic analysis, paper chromatography and spectrophotometric analysis for the detection of dried red beet pulp in capsicums were done by Schwein and Miller in 1967. The presence of low level non volatile ether extract as an indicative to the presence of exhausted capsicums in chilli powder were also reported.

Analytical techniques like solid phase spectrophotometry by Valencia *et al*., 2000, UV spectroscopy by Zhang *et al*., 2005, and mass spectroscopy (MS) by Calbiani *et al*., 2004; Ma *et al*., 2006; Rovellini, 2005; Tateo and Bononi, 2004 and chemiluminescence (Zhang and Sun, 2006) could trace adulteration in chilli. Chromatographic techniques like paper chromatography (Sacchetta, 1960; Mitra *et al*.,1961), thin layer chromatography (Stelzer, 1963; Navarao *et al*., 1965; Marshall, 1977) and high performance liquid chromatography (HPLC) (Ertas *et al*., 2007), were used for the adulteration detection in chilli. Hyphenated techniques like gel permeation chromatography, liquid chromatography, tandem mass spectrometry interfaced with electrospray ionization (GPC LC ESI MS/MS) could successfully detect the adulteration (Sun *et al*., 2007). Capillary electrophoresis (Mejia *et al*., 2007) and polarographic method (De la Cruz Yaguez, 1986) were used to detect artificial

adulterants in chilli powder. The requirement of expensive instruments, high cost of the standards and the non availability of certain standards for analysis restricts its wide application in food authentication. Also the detection of plant based adulterants especially the closely related species is difficult as it may contain overlapping marker compounds. Absence of a marker compound for a particular botanical may also reduces its application (Sasikumar *et al*., 2016).

2.4.3 Immunological Methods

Immunological assays utilize antibodies that are produced in response to an antigen that share a complementary structure to the antibody, and on binding precipitates antigen– antibody complex that allows the identification of the plant material. Enzyme Linked Immunosorbent Assay (ELISA) is the most widely used immunoassay for the authentication of meat, diary, and other products due to its specificity, simplicity and sensitivity (Mackie, 1996). Many immunological methods are used in the authentication and detection of adulteration in medicinal plants (Xue *et al*., 2009). However, the difficulty of distinguishing species from mixed samples, requirement of skilled personnel and well established laboratory are some draw backs of this technique.

2.4.4 DNA based biotechnological methods

PCR based, hybridisation based and sequencing based are the three major DNA based biotechnological methods adopted in authentication and adulteration detection studies (Yip *et al*., 2007). The advantages of this method when compared to other methods are: 1.They are least affected by age, physiological condition and environmental factors. 2. DNA markers are more informative when compared to physical and analytical markers due to the extra resistance showed by DNA to physical, chemical and industrial processes. 3. The detection of adulterants and authentication are possible at lower concentration

and even in the absence of morphological markers, for eg. powdered form, value added products etc.

In non PCR based/ hybridisation methods**,** species specific DNA profiles are generated by hybridising the sample DNA digested with restriction enzymes and further comparing it with labelled probes. Restriction fragment length polymorphism (RFLP), variable number tandem repeat (VNTR), microarray technique etc. are some of the hybridisation techniques used in the authentication of samples. Hybridization using the fluorescent targets and probes spotted on the DNA microarray was useful in identifying the medicinal plants (Niu *et al*., 2011). However, reliability, reproducibility, sensitivity and accuracy limit its application in quality control (Draghici e*t al*., 2005). In PCR (Polymerase chain reaction) and sequencing based molecular methods, PCR has gained much importance in the field of authentication and adulteration detection of agricultural commodities. The method provides more reliable and standardised results on the basis of the nuclear content present.

In PCR based methods, isolation of DNA from samples is crucial and is mostly a limiting factor. In case of agri food products which are of highly processed nature consisting of powdered, dried and recalcitrant tissues, DNA extraction becomes a major component of the technique. High purity DNA is a pre requisite for mainly all PCR based molecular techniques Yau and Nagan, 2002). Sangwan *et al*., 1998 suggests the protocol performed for the DNA extraction differs with different species, the tissue composition and also depending on the composition of secondary metabolites. Pioneer works on development of DNA isolation protocols were done mainly by Dellaporta *et al*., 1983; Doyle and Doyle 1987; Webb and Knapp 1990. Later these protocols were modified depending on the plant material and its composition. In case of commercial powdered spices, the modified protocol of Doyle and Doyle 1987 were used for the DNA isolation from turmeric powder, traded cardamom, dried

black pepper berries and chilli powder (Remya *et al*., 2004; Syamkumar *et al*., 2005; Dhanya *et al*., 2007; Dhanya *et al*., 2008).

Molecular methods based on PCR amplification of the DNA content of the sample are mainly species specific primer amplification (Sasaki *et al*., 2004), rDNA/18S rRNA gene (Cao *et al.*, 2001), DNA fingerprinting methods like random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), 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 characterized amplified regions (SCAR) (Paran and Michelmore, 1993), amplification refractory mutation system (ARMS) (Newton *et al*., 1989), simple sequence repeat (SSR) analysis (Litt and Lutty, 1989), single nucleotide polymorphism (SNP) etc. are being used in the authentication and adulteration detection of agricultural and food commodities.

In species specific primer amplification, specific primers are designed so as to amplify that particular species and not the other. Adulteration/ authentication of the product can be detected by the absence or presence of PCR amplification. rDNA/ 18S rRNA gene amplification are some of the PCR based methods used in adulteration detection studies. RAPD and AP-PCR are most commonly used for the discrimination of closely related species. RAPD is advantageous as it doesn't require prior information of the sequences and no sequencing is required as the detection is at the band level thereby reducing the operating cost. With the advent of molecular techniques, more methods have been used in the adulteration detection and authentication of plants and agricultural commodities (Shinde *et al*., 2007). RAPD markers have immensely contributed in authentication and adulteration detection studies of agri food commodities. Shaw and But, 1995 demonstrated the use of

RAPD in establishing unique bands for *Panax* species and its adulterants. Chinese medicinal plants and herbal products were authenticated using RAPD markers (Cheng *et al*., 1997; Cao *et al*., 1996a and 1996b, Rinaldi *et al*., 2007).

RAPD profiles for species specific identification for closely related and easily confused plants were developed (Hosokawa *et al*., 2000; Zhang *et al*., 2001; Nieri *et al*., 2003).The technique was also used in the quality control and component determination of ayurvedic medicinal products like 'Rasayana Churna' (Shinde *et al*., 2007), *Derris spp.*, herbal drug (Sukrong *et al*., 2006) and pharmaceutically relevant *Echinacea* species (Wolf *et al*., 1999). The plant based adulterant detection studies in powdered plant samples of *Cimicifuga* species (Xu *et al*., 2002), Panax species (Shim *et al*., 2003)were successfully performed. In spices, the plant based adulterants in market samples were effectively detected using RAPD markers by Sasikumar *et al*., 2005, Dhanya *et al*., 2008 and Marieschi *et al*., 2009, in turmeric powder, chilli powder and Mediterranean oregano, respectively .

Prince *et al*., 1995 evaluated the DNA polymorphism within the genus *Capsicum* and the fingerprinting of pepper cultivars to authenticate the samples. Southern analysis and RFLP and RAPD were found useful in discrimination of closely related *C. annuum* genotypes. With the advent of molecular techniques for adulteration detection, ISSR and FISSR PCR markers were used for adulteration detection in chilli. Using these markers Lekha *et al*., 2001, authenticated four disputed chilli seed samples. This report was a step against false marketing of seeds in the name of elite varieties and also a step towards the protection of Plant Breeder's Rights by using reliable and modern DNA technologies. Study of adulteration of turmeric powder in market samples were conducted using RAPD markers to differentiate between *C. longa* and the adulterant *C. zedoaria*. The results proved adulteration of three branded market samples with *C. zedoaria* (Sasikumar *et al*., 2005). Syamkumar *et al*., 2008

used RAPD and ISSR markers in combination with 18s rRNA sequences for identification and authentication studies of Indian *Curcuma* species.

But RAPD method suffers from limitations like variations in amplifying conditions, low reproducibility etc. (Macpherson *et al*. 1993). ISSR and SCAR markers are more suitable in this context. ISSR markers are semi arbitrary markers amplified by PCR using primers longer than RAPD primers and are mainly used in species specific marker development. Authentication of different plant species is achieved using species specific markers of ISSR (Wang, 2011). SCAR markers are evolved from the specific RAPD primers or ISSR primers. Characterized regions of genome are amplified under stringent conditions for the development of SCAR marker and are more reliable and reproducible (Paran and Michelmore, 1993). SCAR markers were developed for the authentication of plant species including *Panax*, Bamboo, *Phyllanthus emblica* L. and *Echinacea* species for the proper authentication from its closely related/ adulterant species (Wang *et al*., 2001; Dnyaneshwar *et al*., 2006; Adinolfi *et al*., 2007; Choi *et al*., 2008).

SCAR markers also played a crucial role in authenticating medicinal plants from herbal mixtures and crude drugs such as for the identification of *Phyllanthus* material in crude drug (dry leaf powder) (Jain *et al*., 2008), authentication of *Embelia ribes*, an important plant used in Indian traditional medicine (Devaiah and Venkatasubramanian 2008a) and traded medicinal plant *Pueraria tuberosa* (Devaiah and Venkatasubramanian, 2008b). SCAR markers have been used for identification of ginger (*Zingiber officinale* Rosc.) from crude drugs and multicomponent formulations besides for the detection of adulteration in black pepper, chilli and turmeric powder (Chavan *et al*., 2008, Dhanya *et al*., 2009, Dhanya *et al*., 2011a,b,). RAPD-SCAR markers were used by Dhanya *et al*., 2009, for the identification of papaya seed adulteration in black pepper. The specific SCAR marker designed was able to detect papaya seed adulteration in one out of five market samples and in simulated samples

also. Dhanya *et al*., 2011a, reported the utility of RAPD -SCAR marker in the adulteration detection of branded powdered turmeric market samples. Two SCAR markers were developed for the detection of *C. zedoaria*/ *C. malabarica* in market samples. Out of six market samples four was found to be adulterated suggesting a high rate of adulteration of turmeric market samples.

Dhanya *et al*., 2011b, reported the detection of dried and powdered fruits of 'Choti ber', dried red beet pulp and almond shell dust, in marketed chilli powders using RAPD-SCAR markers. The SCAR markers were able to detect adulterants at a concentration as low as 10g adulterant per kg of blended sample. The findings reported the presence of 'Choti ber' in one of the commercial chilli powder sample. The conversion of RAPD/ ISSR markers to SCAR markers and the need of verifying more number of primers before authentication of the SCAR primer is technically tiresome. These disadvantageous can be easily overcome by the use of recently evolved DNA barcoding technique which uses barcode regions for amplification and sequencing.

2.5 DNA barcoding

DNA barcoding uses short stretches of DNA sequences that are conserved among species and variable enough to differentiate between species (Herbert *et al*., 2003). It uses standardised and conserved areas within species but with enough sequence diversity, possessing high inter specific divergence to discriminate another species. Species identification based on DNA sequences are highly reproducible, efficient and reliable (Ali *et al*., 2012). DNA barcoding is also defined as the identification of any biological specimen using specific organellar or nuclear DNA (Balachandran *et al.,* 2015). A DNA barcode should be relative short in length for universal use in a wide range of species.

The relative short length favours DNA extraction, amplification and sequencing easy across different groups of plant species (Kress *et al*., 2005). The criteria for an ideal barcode includes (a). Discriminatory power- high species level genetic variability with evolutionary significance (b). Universality- ease of amplification and sequencing, the barcode region should be flanked by conserved regions which can function as the binding site of universal primers and (c). Quality- short nucleotide sequences which facilitate good quality products of amplification and sequencing (Fazekas *et al*., 2009; Cho *et al*., 2004; Kress *et al*., 2005; Hollingsworth *et al*., 2011; Ausubel, 2009). Reliable DNA barcoding technique should posses the recovery of a unique barcode sequences from the sample, representation of these unique sequences in the databases and sufficient variability in nucleotides to distinguish the closely related species (Herbert *et al*., 2003). In 2003, Dr. Paul Hebert came up with this new endeavour of using mitochondrial cytochrome *c* oxidase (CO1) gene as a global identification system for animals.

In his work on lepidopterans, using CO1 gene, he could identify 200 closely allied species thus establishing DNA barcoding to be an efficient cost effective tool for the species identification (Herbert *et al*., 2003). The CO1 gene distinguished over 90% of species in animal kingdom making it an ideal gene for barcoding studies of animal groups such as birds (Kerr *et al*., 2007), amphibians (Smith *et al*., 2008), fishes (Ward *et al*., 2005) and lepidopterans (Hajibabae *et al*., 2006). In plants, due to the slower rate of mitochondrial genome evolution, the CO1 gene was found to be not very effective in distinguishing between species (Mower *et al*., 2007; Cho *et al.*, 2004). It was also found that plant plastid and animal mitochondrion showed varied levels of variability with animal mitochondrion having ~10- 30 times more nucleotide substitution (Wolfe *et al*., 1987) when compared to plants. In plants and fungus, COI was found to be too invariable with larger introns inhibiting its use as an ideal barcode for plants and fungi (Kress *et al*., 2005; Chase *et al*., 2007). Hence,

Kress and Erickson in 2007 came up with the use of plastid DNA and nuclear ribosomal intergenic regions to discriminate plant species. Fazekas *et al*., 2008 supported the idealness of seven plastid DNA regions for their ability to distinguish 92 species in 32 genera of land plants.

The major plant barcodes suggested include the coding regions, *rbcL, matK, rpoB, rpoC1*, the non coding intergenic spacers *psbA-trnH, atpF-atpH, psbK-psbI* and the nuclear *ITS* (CBOL, 2009; Kress *et al*., 2005; Kress and Erickson, 2007; Chase *et al*., 2007; Lahaya *et al*., 2008; Fazekas *et al*., 2008; Ford *et al*., 2009). The plant barcode regions are either from nuclear or plastidial / chloroplast genome. Theoretically the biparental nuclear genome is expected to give detailed information regarding the species, hybridisation etc. In barcoding the use of the mitochondrial region was dominant over the nuclear genome as it did not exhibit gene duplications common in nuclear genome (Ford *et al*., 2009). The only tested successful nuclear genes for plant barcoding are the nuclear internal transcribed spacer (*ITS*) and ribosomal DNA (rDNA) (Kress *et al*., 2005; Chase *et al*., 2007; Sass *et al*., 2007).

Among the two, *ITS* alone is being recommended and taken further as an efficient gene for barcoding studies by CBOL and others working groups in this area. The reason for the limited nuclear genes for barcoding may be the difficulty in obtaining genes having universal amplification and the conservation of functional genes across lineages. Presence of introns, low copy number and recombination events have restricted the use of nuclear genes for barcoding (Herbert *et al*., 2003). In the comparative study on the nuclear and plastidial genes, the nuclear genes exhibited greater variability than the plastid genes but were also accompanied by higher levels of heterozygosity, intra specific variation and retained ancient alleles (Pillon *et al*., 2013). These characteristics were not ideal for barcoding purposes and hence for the generation of diagnostic species specific markers. On the other hand, plastidial genes were able to delineate large number of species with greater number of diagnostic

species specific markers. Plastome sequencing increased the phylogenetic resolution at lower taxonomic levels (Parks *et al*., 2009).

2.5.1 Barcoding loci for land plants

2.5.1.1 Nuclear gene - *ITS*

Internal transcribed spacer (*ITS*) is a spacer DNA situated between the small subunit ribosomal RNA (rRNA) and large subunit rRNA genes. *ITS1* separates 16S and 5.8S ribosomal subunits and *ITS2* separates 5.8S and 28S ribosomal units (Ritland *et al*., 1993). *ITS* is a multi gene and encodes the nucleic acid of the ribosome. The region consists of two sections, *ITS1* and *ITS2,* and can be amplified separately using universal primers and this advantage is beneficial for DNA amplification even from poor quality/ degraded DNA samples. The locus is considered as the most useful one in phylogenetic studies of both plants and animals due to its high evolutionary significance and its high levels of discriminatory power at the species level accompanied by the amplification and sequencing ease (Baldvin *et al*., 1995; Alvarez *et al*., 2003).

Availability of universal primers, species discrimination, presence of multiple copies in cells, high universality and phylogenetic significance makes the locus ideal for barcoding studies (Kress *et al*., 2005; Sass *et al*., 2007; Vijayan and Tsou, 2010). However, difficulty in sequence recovery, secondary structure formation due to GC rich region, amplification of multiple sequences due to fungal contamination, presence of divergent paralogous copies of the spacer etc. are some of the disadvantageous in its use as an ideal barcoding gene (Ritland *et al*., 1993; Baldwin *et al*., 1995; Hollingsworth *et al* 2011).

2.5.1.2 Chloroplast/Plastid Genome

2.5.1.2 .1 *rbcL* (Ribulose 1, 5 bisphosphate carboxylase/oxgenase) gene

This gene is best characterised and the first gene to be sequenced from plants (Zurawski *et al*., 1981; Vijayan and Tsou, 2010). The function of *rbcL* gene is to code for the larger chain subunit of Ribulose 1, 5 bisphosphate carboxylase/oxgenase (RuBisCO). RuBisCo is an enzyme involved in the first major step of carbon fixation. *rbcL* is located in the chloroplast genome and is an ideal candidate for studying phylogenetic relationships among higher taxonomic levels (Les *et al*., 1991; Chase *et al*., 1993; Duvall *et al*., 1993). Encoded in the chloroplast genome, the *rbcL* gene is found to be 1430 bp in length. In comparison to the nuclear genes, this chloroplast gene is found to be ideal due to its functional constrain that reduces the revolutionary rate on non synonymous substitutions and thereby making it evolutionarily significant (Wolfe *et al*., 1987). The *rbcL* locus is reported to have difficulties in resolving closely related species due to its low variability (Gielly and Taberlet, 1994) but performs well in combination with other loci (CBOL Plant Working Group, 2009).

2.5.1.2 .2 *matK* (Maturase kinase) gene

The plastidial gene encodes the protein maturase that splices the group II introns. The gene is located within the intron of chloroplast gene *trnK* with a length of 1500bp. *matK* gene has high revolutionary significance with higher substitution rates at nucleotide and amino acid levels and its ubiquitous presence make it an ideal candidate (Hilu *et al*., 1997; Kelchner *et al.*, 2000). The evolution of *matK* is found to be three times greater to *rbcL* and six fold greater at the amino acid level (Johnson and Soltis, 1995). The features viz., its fast evolution, its function as a group II intron maturase involved in splicing mechanism make it a good candidate for phylogenetic studies (Barthet, 2006). However, the lower rate of amplification and poor species discrimination are problems associated with *matK* loci (Hollingsworth *et al.*, 2009).

2.5.1.2 .3 *rpoB* and *rpoC1* (RNA polymerase β subunit 1).

These genes encode the sub units of chloroplast RNA polymerase (Shinozaki *et al*., 1986; Serino *et al*., 1998). A higher level of non synonymous substitutions accompanied by higher substitution rates make these loci ideal for phylogenetic studies (Guisinger *et al*., 2008). The loci was among the seven loci tested by the Consortium for Barcode of Life (CBOL) plant working group due to its high amplification success, universality and quality of sequences, and mostly forms a part of barcoding studies in various plant groups individually or in combination (Chase *et al*., 2007; Hollingswoth *et al*., 2009). Lower species discrimination is one drawback associated with these loci (CBOL, 2009).

2.5.1.2 .4 *trnH- psbA* intergenic spacer

psbA gene encodes the D1 protein (also known as PsbA protein) in the core complex of photo system II. The gene occupies the position between the gene coding for the protein D, a constituent of the photosystem II and the gene of histidine transfer RNA (*trnH*) (Degtjareva *et al*., 2012). This spacer is one of the most variable among the angiosperms and is proposed along with *nrITS* for the barcoding of land plants (Kress *et al*., 2005). The problem of obtaining high quality bidirectional reads and alignment of the reads was easily overcome by the species variation and universal amplification shown by this locus. The length variation shown by this locus is due to the presence of the *rps19* gene or pseudogene within the *psbA* and *trnH* genes. The length varies as short as (~300bp) in some angiosperms resulting in lesser species discrimination and in some monocots (Schindel and Miller, 2005) while in conifers the length may exceed as long as (~1000bp) causing difficulty in the alignment (Chase *et al*., 2007, Hollingsworth *et al*., 2009).

2.5.1.2 .5 *atpF – atpH* intergenic spacer

The genes *atpF* and *atpH* encode the gene for ATP synthase subunits CFOI and CFOIII, respectively (Drager *et al*., 1993). The high universality makes it a supplementary locus

along with other major barcoding loci (Drager *et al*., 1993; Fazekas *et al*., 2008). *atpF-atpH* is preferred due to its higher frequency of bidirectional reads, few sequencing reads per amplification and few micro inversions (Fazekas et al., 2008). The locus is mostly used as a supplementary locus along with other main loci. The locus *atpH-atpF* is not routinely recommended by CBOL or any other plant groups due to its difficulty in aligning the sequences as well as its low discriminatory power to differentiate various species.

2.5.1.2 .6 *psbK-psbI* intergenic spacer

The genes *psbK* and *psbI* code for the low molecular mass polypeptides viz., K and I of photo system II (Meng *et al*., 1991). The spacer is considered as a supplementary locus along with other loci to increase the species discriminatory power. The locus is advantageous with its high PCR amplification success and sequencing ease but having the disadvantage of aligning the bidirectional unambiguous reads (CBOL, 2009).

2.5.2 Sequence repositories and consortia involved in DNA barcoding

DNA barcoding was an international move established mainly to promote the use of DNA barcoding technique to identify animal/ plant species on the basis of a single standard universal marker. The barcoded species from various working groups needs to be universally applicable and available to the public. This was made possible by the formation of different sequence repositories. The main sequence repositories and consortia involved for the promotion of barcoding studies are:

BOLD (The barcode of life data system) was created and maintained at University of Guelph in Ontario. It aids in the acquisition, storage, analysis and maintenance of DNA barcodes (Ratnasingham and Hebert, 2007). The major aim of this consortium is to build a reference barcode library. Currently it serves as the database of over 370,000 plant barcodes

representing 58,510 species of plants (http://www.barcodinglife.com). The BOLD system shares its services and data with the iBOL (International Barcode of Life), CBOL (Consortium for the Barcode of Life, GBIF (Global Biodiversity Information Facility) and NCBI GenBank (National Center for Biotechnology Information).

The Consortium for Barcode of Life (CBOL) is an international initiative developed with a goal to distinguish majority of the world species, using one or combinations of DNA regions and to produce large scale reference sequences library of life on earth (CBOL, 2009). The main aim was to develop specific barcode through DNA barcoding as a global standard to identify all life forms on earth. The consortium was established in May 2004, and promotes barcoding through working groups, networks, conferences, workshops, outreach and training. Currently the organisation accommodates 200 member organisations from 50 countries having its Secretariat office at Smithsonian Institution's National museum of Natural history in Washington DC (http://www.barcodinglife.com).

iBOL (International Barcode of Life project) (http://www.ibol.org/) is a consortium of scientists working from 25 nations for the creation of a reference barcode library which can act as a platform for the DNA based identification. The GenBank online genetic sequence database (http://www.ncbi.nlm.nih.gov/genbank/) is one of the most often and commonly used repositories of genetic information. GenBank contains over 108 million entries for over 260,000 named organisms and the sequences are identified using the BLAST program against the known and well characterized sequences.

The National Centre for Biotechnology Information (NCBI) web-based megablast algorithm with default settings are used to identify the query sequences. Identification could be made manually by considering the number of closely-related species, E-value, maximum identity with the sequences available in the database. The collaboration between the three major

consortia; The International Nucleotide Sequence Database (GenBank) USA, European Molecular Biology Laboratory (EMBL) Germany, and DNA Data Bank (DDBJ) Japan helps in the exchange of the DNA barcode sequence information with the DNA barcode CBOL database.

MMDBD (Medicinal Materials DNA Barcode Database) is a database which accommodates the DNA sequence information and key references of the medicinal materials available with the Pharmacopoeia of the People's Republic of China, American Herbal Pharmacopoeia and other related references (http://137.189.42.34/mherbsdb/index.php). MMDBD contains over 1658 species and 31,468 sequence information for distinguishing medicinal materials (plant, animal, and fungi) from their common substitutes and adulterants . The database accomodates sequences of multiple regions including four nuclear regions, four mitochondrial regions and seven chloroplast regions. It covers the information regarding adulterants, medical parts, photographs, primers used for obtaining sequences and key references.

Apart from these, there are many international barcoding initiatives for the development of public reference sequence libraries for different species. Marine barcode of life (MarBol), CBOL fungal working group, Bee barcode of life initiative (Bee-BOL), Coral reef barcode of life, European consortium for barcode of life (ECBOL), Fish barcode of life champaign (FISH-BOL), Health BOL, Lepidoptera barcode of life, Mammali a barcode of life campaign, Mosquito barcode initiative (MB), Polar barcode of life(Polar-BOL), Quarantine barcode of life (QBOL), Shark barcode of life (Shark BOL), Sponge barcoding project(Sponge BOL), Tephritid barcode initiative (TBI), Trichoptera barcode of life (Trichoptera BOL), CBOL protest working group (ProWG) are the main projects in collaboration with the barcoding initiatives.

2.5.3 Applications of DNA barcoding

DNA barcoding, the robust technique, had found its application in varied fields. Irrespective of the loci used, DNA barcoding is applicable in a number of fields including taxonomy (Lara *et al*., 2010), species discrimination (Fazekas *et al*., 2008), identification of cryptic and invasive species (Hajibabaei *et al*., 2006), phylogenetic studies, biodiversity and conservation studies (CBOL, 2009). Barcodes are also used for better understanding of the food chains ecosystems to unravel food webs and predator-prey relationships (Kaartinen *et al*., 2010) and in disease control. Barcoding also finds its application in quarantine issues (Bonants *et al*., 2010), illegal trade of endangered species (Eaton *et al*., 2010) and logged timber (Lowe and Cross, 2011) and authentication of wood samples of threatened and commercial timber trees (Nithaniyal *et al*., 2014).

DNA barcode can be used as a tool to compliment taxonomy where it can be used to create data on unknown taxa (Schindel and Miller, 2005) and also in discriminating the closely related species where taxonomic evaluation is difficult. Barcodes are able to document and confirm known species while uncovering lots of hidden variation, some of which may also lead to the description of new species and also in the estimation of species richness in poorly studied floras (Costion *et al*., 2011). In 2005, there were 33,000 records covering 12,700 species in the Barcode of Life Data Systems (BOLD) at the University of Guelph, Canada. Recently the data has increase to over 290,000 records, representing over 31,000 species, and the data is accumulating at an accelerating pace, showing the success of DNA barcoding initiative (http://www.boldsystems.org). The Environmental Protection Agency is testing barcoding to identify insects and other invertebrates in rivers and streams, critical indicators of environmental quality thereby helping the improvement of quality.

The US Department of Agriculture with DNA barcoding community in a joint venture is developing global database of DNA barcodes for fruit flies, an important agricultural pest. Trade of endangered timber species may also be halted by using barcodes to identify processed wood and lumber products. Trade of endangered timber species may also be halted by using barcodes to identify processed wood and lumber products. Among those most excited about barcoding technology are researchers concerned with bio security and agricultural quarantine issues. DNA barcoding could successfully identify illegally-obtained wildlife species and wildlife-derived products based on fragments and poorly preserved samples (https://www.sciencedaily.com/releases/2007/09/070914120856.htm). Apart from the above uses, barcoding is a tool for authentication and adulteration detection of food and forensic samples. The technique has greater applications in the field of authentication of medicinal plants, herbs and spices. It also acts as a tool to detect fraud means of admixing / adulteration in traded samples.

2.5.4 Establishing a universal barcoding loci for land plants

Kress et al., 2005, suggested two non coding spacer *trnH-psbA* and nuclear *ITS* after analysing *trnK-rps16, trnH-psbA, rp136-rps8, atpB-rbcL, ycf6-psbM, trnV-atpE, trnC-ycf6, psbM-trnD, trnL-F* and *rbcL* from a total of 99 species covering 80 genera and 53 families. Later studies by Kress and Erickson, 2007 confirmed the combination of *rbcL* and *psbA-trnH* to be ideal for land plants due to its high discriminatory power and ~93% amplification using single primer pair. The study was conducted using nine putative barcoding loci *viz., accD, matK, ndhJ, rpoB2, rpoC1, ycf5, ITS1, psbA-trnH* and *rbcL* across 48 genera of land plants. *rbcL and psbA-trnH i*ndependently exhibited high PCR amplification success with 95.8% and 92.7%, respectively. *matK* showed poor amplification success across the genera. Sequence quality especially to align bidirectional reads was found to be difficult with the *trnH-psbA* locus, but the limitation could be overcome by the high variability due to the presence of

diagnostic insertion/mutations. No single locus showed discrimination greater than 80% and so a two loci combination which gave maximum discrimination and amplification success was suggested.

trnL-trnF and 23S rDNA known as UPA (universal plastid amplicon) were suggested by Taberlet *et al*., 2007, but both the loci exhibited lower species resolution. *Chase et al., 2007 reviewed the suitability of using rpoC1, rpoB, matK, trnH-psbA, nrITS, trnL-F* and put forward two options viz., *rpoC1, rpoB* and *matK* or *rpoC1, matK* and *psbA-trnH* as barcodes for land plants by assessing the merits and demerits of each loci. In the studies using *nrITS, accD, ndhJ, matK, trnH-psbA, rpoB, rpoC1, ycf5* loci on identifying the members of Cycadales, *nrITS* showed more variability than the other loci used in the study (Sass *et al*., 2007). Other barcode working group including the Royal Botanical Gardens, Kew, UK (http://www.rbgkew.org.uk/barcoding) came up with two and three combinations of barcoding loci viz., *matK+rpoC1+ psbA-trnnH* and *matK+rpoC1+rpoB* (Chase *et al*., 2007).

Fazekas et al., 2008, included eight different loci, five coding *(rpoB, rpoC1, rbcL, matK* and 23S rDNA) and three non coding loci *(trnH-psbA, atpF–atpH*, and *psbK–psbI)* for analysing 92 species with 32 diverse genera of land plants. Species resolution using single locus ranged between 7% (23S *rDNA)* to 59 %(*trnH-psb). matK, psbA-trnH and psbK-psbI* loci were found to be difficult in aligning the bidirectional sequence reads. matK experienced greater difficulty in PCR amplification. *rbcL* was found to be the least problematic locus but the species resolution was less when compared to *matK. rpoB and rpoC1* were more difficult when compared to *rbcL* in amplification and sequence recovery and were least informative loci. The spacer regions *psbA-trnH and atpF-atpH* performed well when compared to the coding regions despite the issues in alignment of the bidirectional reads. Use of more than one locus increased species resolution. The combination *matK+atpF–*

atpH+psbK–psbI showed considerably high resolution with *rpoB+rpoC1+matK* the least. Fazekas *et al*., 2008, recommends the use of multi locus barcodes including one coding regions from *rpoB, rbcL, matK* and two from the non coding regions such as *trnH-psbA, atpF–atpH*. Loci *rpoC1* and *psbK-psbI* were not suggested due to low species variation and high PCR and sequencing failure, respectively.

Eight potential barcodes were compared using > 1600 samples from the two biodiversity hot spots at Mesoamerica and southern Africa and the barcode gap were determined between inter-intra specific variations using multiple accessions per species (Lahaya *et al*., 2008). The eight loci taken for the study are *rbcL, matK, psbA-trnH, ndhJ, accD, rpoB, rpoC1* and *ycf5* wherein *ndhJ* and *ycf5* were found to be not efficiently amplifying the orchids. *psbA-trnH* and *rbcL* had amplification problems in achlorophyllous *Hydnora johanis* but amplified in other parasitic plants. In the study the most variable locus with highest interspecific divergence was *psbA-trnH* locus but was associated with other amplification and alignment difficulties.

The second most variable locus was *matK.* in monophyletic recovering of species, *matK and psbA-trnH were* found to be equally best. Taking into account the overall performance matK locus using primers designed by Cuénoud *et al.,* 2002 was suggested best for DNA barcoding of plants (Lahaya *et al*., 2008). In a later study covering 101 taxa including 18 families of trees, shrubs, orchids and parasitic plants using the loci *atpF-atpH* and *psbK-psbI*, Lahaya *et al*., 2008, suggested the combination of *matK +psbA-trnH* locus or matK+ *psbK-psbI* would efficiently increase species discrimination than the variable *trnH-psbA* and *atpF-atpH* alone. In the multilocus barcoding system, slowly evolving loci will be delineating individuals into families, genera or groups and the more rapidly evolving loci differentiate species within those higher groups (Newmaster and Raghupathy 2009; Kress and Erickson, 2007).

Jaramillo *et al*., 2008 assessed the phylogeny of tropical genus *Piper* using *ITS* and the chloroplast intron *psbJ-pet A*. A sample size of 575 accessions corresponding to 332 species of *Piper* for *ITS* region and 181 accessions for *psbJ-pet A* region were taken. *ITS* locus proved to be ideal in discriminating the different clades of *Piper* genus. According to CBOL 2009, among 397 plant samples from angiosperm, gymnosperm and liverworts successfully amplified using the plastidial genes, the species discrimination ranged from 43% to 69% with *rpoC* the least and *psbA-trnH* the highest. The order of discrimination was *rpoC1<rpoB<atpF–atpH<rbcL<matK<psbK–psbI<trnH–psbA.* Universality among the coding regions was greatest with *rpoC1*, consistently amplifying with a single primer followed by *rbcL*. In non coding loci, *psbA-trnH* exhibited highest universality. With respect to species discrimination, in different groups, *matK, rbcL and psbA-trnH* performed equally well. No single loci exhibited good discrimination power and so a combination of barcoding genes was analysed.

Two loci combinations gave 59%-75% and three loci exhibited 70%-75% resolution of the species. Undertaking this study CBOL suggested the elimination of four barcode genes, *rpoC1, rpoB, atpF-atpH and psbK-psbI. rpoC1* and *rpoB* on grounds of low discriminatory power despite its universality and sequence quality. *atpF-atpH* was eliminated due to its low species resolution both singly and in combination whereas *psbk-psbI* showed good discriminatory power with least sequencing success. Remaining three loci, *rbcL, matK* and *psbA-trnH* showed desirable characters for a plant barcode. Ford *et al*., 2009, in his in silico analysis of 41 of 81 coding regions of *Nicotiana* plastid genome, shortlisted 12 barcoding regions and evaluated against a reference set of 98 land plant taxa. Studies by Starr *et al*., 2009 in the cosmopolitan group of *Carex* which represents one of the three largest groups of plant genera, using *matK, rbcL, rpoC1, rpoB and psbA-trnH*, revealed that no single or multi locus was able to resolve the species greater than 60%. According to the study *matK* was

found suitable considering its ability to resolve different species of the group. *rbcL* and *psbA-trnH* exhibited difficulties with PCR and sequence alignment while *rpoB* and *rpoC1* showed low species resolution among the *Carex* group.

Bruni *et al*., 2010 assessed the potential of different barcoding loci like *rpoB, matK, psbA-trnH* and nuclear regions *At103* and *sqd1* in distinguishing the poisonous plants from edible ones. *matK* and *At103* were proved to be ideal for their discrimination based on the amplification, sequencing success and resolution power of the tested loci. In plants, selection of a single ideal locus was difficult, all the loci suggested and tested were handicapped in one or the other way. The performance of each locus varied between taxa and family. In this context certain loci were given preference depending on its performance in a wide range of taxa. Combinations of loci were also suggested for the better resolution of species.

2.5.5 Authentication of plants using DNA barcoding

Newmaster and Ragupathy, 2009 tested the utility of barcoding on the sister species complex of pantropical *Acacia* (Mimosoideae, Fabaceae). All the three barcoding loci viz., *rbcL, matK* and *trnH-psbA* could distinguish the species of the *Vachellia* and *Acacia*. 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. *psbA-trnH* could distinguish ten species of Polygonaceae in Chinese Pharmacopoeia and also their adulterants. Li *et al*., 2010 utilized the technique to distinguish *Taxillus chinensis* from its adulterants. *rbcL, matK, psbA-trnH, ITS* and *ITS2* were the loci used for the study. Amplification and sequencing efficiency was 100% in case of *rbcL* and *psbA-trnH* when compared to the other three loci. *psbA-trnH* locus exhibited higher inter specific divergence and distinguished *T. chinensis* from other parasitic loranthus.

Guo *et al*., 2011 analysed DNA barcoding to discriminate the medicinal plant *Scutellaria baicalensis* belonging to the family Lamiaceae from its adulterants. The plant is a well known traditional Chinese medicine and due to over exploitation has resulted in the decline of its natural population and increased use of adulterant/substitute of related *Scutellaria* spp. viz. *S. amoena, S. rehderiana and S. viscidula.* The loci *matK, psbA-trnH* and *rbcL* loci were used in the study and the results suggested a tiered approach of *psbA-trnH* and *rbcL* differentiating *S. baicalensis* from its adulterants. Authentication of herbs of the family Aspargaceae and Asclepiadaceae mostly used in Ayurveda medicine was done by Rai *et al*., 2012. The authentication was based on *ITS2, mat K* and *rpoC1* region. *ITS2* proved to be efficient in identifying closely related species of Asclepiadaceae and could easily discriminate the substitutes and admixtures, but proved less informative in case of family Aspargaceae.

Identification of ancient *Olea europaea* L. and *Cornus mas* L. seeds were achieved using DNA barcoding. The results proved its viability in tracing the identity of ancient plant samples (Gismondi *et al*., 2012). *ITS2* locus has served as an identification tool in many of the plant species and its adulterants. The discrimination ability of *ITS2* locus among 6600 plant samples belonging to 4800 species and the results showed 92.7% discrimination success at species level. Cistanches herbs and its adulterants (Sun *et al*., 2012), identification of medicinal vines of Chinese pharmacopoeia and its adulterants which included 393 samples of 170 species from 22 genera and 13 families are some of the reports on plant identification/authentication using *ITS2* locus (Liu *et al*., 2012).

Processed medicinal plants used in the traditional medicine of South Africa were tested for authenticity by Mankga *et al*., 2013. Using *matK* and *rbcL* loci, 18 market samples were analysed and 16 samples gave good amplification and sequencing success. Among them 11 samples were proved to be authentic and 5 were mislabelled by the sellers. Authentication of

Salvia divinorum, source of a recreational drug salvinorin A was done using *rbcL* and *trnL-F*. *rbcL* locus differentiated it from other sources of drugs like *Nicotiana tobaccum* and *Cannabis sativa* and *trnL-F* distinguished it from other *Salvia* species like *S. greggi, S.chionophylla, S. microphylla, S. dolomitica, S. clevelandii, S. cedrosensis* (Murphy and Bola, 2013). *Cassia* species used in Indian traditional medicine were authenticated using *rbcL, matK. psbA-trnH* and *ITS2* loci. Twenty species of cassia were taken for the study and no single locus could differentiate all the species. A multi locus approach of *rbcL* + *psbA-trnH* was able to differentiate all the species (Purushothaman *et al*., 2014). Two different species of *Ocimum* were identified using *matK* primers. Two medicinally important *Ocimum basilicum* and *Ocimum tenuiflorum* could be distinguished by this method (Anbazhagan *et al*., 2014).

Raterta *et al*., 2014 conducted DNA barcoding to authenticate medicinal plants commercially available in Manila, Philippines and Quiapo. *matK, psbA-trnH, rbcL* and *ITS* loci were used for authenticating the plants. *psbA-trnH* was easily amplified and had a relatively high sequencing success, but could not give the best discriminatory power. On the other hand, *matK* gave good discriminatory power but poor amplification success. *ITS* was prone to fungal contaminations. The results proved the sequences of *matK* and *psbA-trnH* can be used as potential markers for identifying medicinal plants from the market that lack morphological features for species identification. Zhang *et al*., 2014 identified 111 medicinal materials collected from the herbal market of the three month fair of Dali region. The potential of *ITS2* and *psbA-trnH* were tested of which *ITS2* discriminated better at species level and *psbA-trnH* assigned species to the correct genus. The loci were able to identify 14 adulterants and 12 substitutions in the market samples tested. Identification and authentication of Chinese medicinal plant *Angelica sinensis* and its adulterants (Feng *et al*., 2010); *Ruta graveolens* and its adulterants (Al-Qurainy *et al*., 2011); oriental medicines, *Pericarpium Citri Reticulatae*

and *Citri Unshius Pericarpium* (Wang *et al*., 2012); Radix Astragali and its adulterants (Zheng *et al*., 2014) were done using *ITS* locus.

Wu *et al*., 2015, analysed the hidden assassins in traditional medicines containing aristolochic acids. In the study, 158 Aristolochiaceous samples covering 46 species and 4 genera as well as 131 non-Aristolochiaceous samples representing 33 species, 20 genera and 12 families were analyzed using DNA barcodes based on the *ITS2* and *psbA-trnH* sequences. BLAST1, nearest distance and N-J method based on *ITS2 and psbA-trnH* sequences differentiated aristolochiaceous and non- aristolochiaceous plant materials. A real time PCR assay was developed based on the *ITS2* sequences for rapid selection of aristolochiaceous plants. Moon *et al*., 2016, authenticated two traditional medicinal components Arisaematis Rhizoma and Pinelliae tuber using *rbcL* and *matK* loci. Both the loci were able to distinguish the genuine plant from its adulterants using polymorphic sites present in the sequences. *matK* sequence alignment revealed 45 and *rbcL* showed 28 polymorphic sites.

2.5.5.1 Authentication and *adulteration detection* of spices using DNA barcoding

In spices, DNA barcoding has proved to be efficient especially in case of powdered and processed samples. The technique helps in authentication of the product by certifying the commodity by tracking its origin and provenance of raw materials at different levels of their transformation (Galimberti *et al*., 2013). In case of spices, the medicinal values are attributed to the components present in the genuine sample. Any adulteration or admixing results in the decline of the perceived medicinal value and also may cause serious health hazards. For authentication and adulteration detection, genuine samples are collected, reference databases are created and compared with the data of traded/ commercial samples. Any change in the sequences or presence of extraneous sequences in the market samples is considered an

adulterant. Komatsu and Cao, 2003 utilised the variability in chloroplast *trn*K nucleotide sequences for the identification of five *Curcuma* species including turmeric (*C. longa*). SNP based species specific nucleotide sequence was developed to identify the drugs made from turmeric (*C.longa*) and other related species such as *C. zedoaria, C. aromatica* and *C. phaeocaulis* (Sasaki *et al*., 2004).

Jarret, 2008 has studied *Capsicum annuum* species using chloroplast DNA introns and waxy introns as candidates for barcoding. The loci used for the amplification consisted of *trnS-trnfM, trnL-trnT, trnF-trnL, trnD-trnT, trnC-rpoB, rps16, matK, trnH-psbA* and waxy regions. Out of the eight regions analysed, regions *trnL-trnT, trnF-trnL* and *trnH-psbA* could differentiate *C. annuum* from other taxa. The *waxy* region exhibited single base indels and substitutions which helped in differentiation of all taxa within the plant material. *trnH-psbA* along with *waxy* regions proved best for the barcoding of *Capsicum* spp. The potential of these two markers may be attributed to the combined effects of uniparental and biparental inheritance. Molecular analysis using nucleotide sequence of chloroplast DNA (cpDNA) for species identification of *Curcuma* rhizomes were found to be effective in distinguishing *C. zedoaria, C. aromatica and C. xanthorrhiza* from *C. longa* due to the polymorphism shown in the intergenic spacer between *trnS* and *trnfM* region (Minami *et al*., 2009). Analysis using 18S rRNA gene and *trnK* gene sequences were done for the identification of six *Curcuma* species and the results revealed SNPs (Single Nucleotide Polymorphism) that distinguished each *Curcuma* species separately (Cao *et al*., 2001).

de Mattia *et al*., 2011 have tested commercial kitchen spices belonging to the family Lamiaceae using DNA barcoding. A total of 64 samples from different spice groups including mentha, ocimum, origanum, salvia, thymus and rosmarinus were analysed using different DNA barcoding loci viz., *rpoB, rbcL, matK and trnH-psbA. trnH-psbA* proved to be the most variable among the loci followed by *matK*. The two loci *trnH-psbA* and *matK*

proved to be the best in differentiating the commercial samples of *Mentha aquatic* and *Ocimum basilicum* from its adulterants. *Salvia officinalis*, could also be distinguished from other related species using *psbA-trnH.* In case of origanum samples barcoding was not very effective.

Hayakawa *et al*., 2011 used chloroplast microsatellite regions to identify and characterise high curcumin content lines from *C.longa*. The chloroplast regions namely *matK*, *rpl16* intron2, *petB* intron1 and *petB* intron 2 were used in the study. These regions could differentiate *C.longa* from *C. zedoaria* and *C. aromatic*. The results showed that the higher curcumin content was unique to a certain haplotype lines of *C.longa.* Deng *et al.*, 2011 authenticated *C. longa* and its two related species *C. sichuanensis* and *C. chuanhuangjiang* using *psbA-trnH* locus. N-J tree and K2P methods were used in the phylogenetic and distance calculation between the species. *Illicium verum* (Illiciaceae), the Chinese star anise, were tested with the barcoding loci *matK, rbcL, psbA-trnH* and *ITS2* to differentiate it from the toxic adulterants in trade (Meizil *et al*., 2012). Twenty fresh samples and two market samples were analysed for the authentication of the sample from its adulterants. Among the four loci *psbA-trnH* with indels and variable sites exhibited highest inter-specific divergence and species discrimination clearly discriminating *I. verum* from its congeneric adulterants. *Crocus sativus* L., commonly known as saffron is a highly targeted commodity for adulteration due to its high economic value. Gismondi *et al*., 2013 conducted studies on authentication of saffron and to detect adulteration using *rbcL, matK psbA-trnH* and *ITS* loci single and in combination. The results suggested the success of barcoding as a tool to authenticate traded saffron especially to differentiate the Italian (IT) and Spanish (ES) *sativus* species. The study also gave valuable insight to the existence of barcode gene intra specific divergences. Black pepper powder, one of the most important spice of trade and commerce was successfully authenticated and adulterants were detected using DNA barcoding (Parvathy

et al., 2014). In the study using three barcoding loci (*psbA-trnH, rbcL, rpoC1*), *psbA-trnH* proved to be the ideal loci in detecting black pepper adulteration with chilli. Two out of nine market samples were found to be adulterated with chilli. The study demonstrated the efficiency of the technique to detect the presence of chilli as low as 1g in 200 g of black pepper powder.

Swetha *et al*., 2014 demonstrated DNA barcoding as an efficient tool for screening of adulterants in traded samples of Cinnamon. *Cinnamomum verum* (true cinnamon) was found to be adulterated with other species such as *C. cassia* and *C. malabatrum*. In a study involving ten market samples, seven proved to be adulterated with *C.cassia*. The loci *rbcL, matK* and *psbA-trnH* were used for amplification of the samples, out of which the amplification and sequencing success was 100% for *rbcL* and *psbA-trnH* while *matK* failed to amplify the market samples. *rbcL* showed higher interspecific divergence and *psbA-trnH* exhibited lower interspecific divergence. Out of the three loci, *rbcL* locus proved to be efficient in tracing out adulterants in traded cinnamon.

Traded turmeric powder (*C. longa*) when tested with the barcoding loci *rbcL, matK* and *ITS* for authenticity showed the presence of adulterants/fillers. *ITS* proved to be the ideal locus with a higher discriminatory power. Out of 10 market samples, four samples were found to be adulterated. The adulterants were wild *Curcuma* species (*C. zedoaria*), cassava starch and other fillers like rye, wheat and barley (Parvathy *et al*., 2015). A study on *Myristica fragrans* (Sangihe Nutmeg) using *matK* locus proved the in efficiency of a single locus *matK* to differentiate between different species of *Myristica (Myristica fatua, M. maingayi*, and *M. globosa*), and could only be used for the genus level differentiation within the family. Only few nucleotide differences was observed between different species of *Myristica* and this could be the possible reason for the failure of *matk* locus (Tallei and Kolondam, 2015).

However, Swetha *et al*., 2016, proved the barcoding technique to be very efficient in the authentication and adulteration detection in market samples of Nutmeg (*Myristica fragrans).* *rbcL* and *psbA-trnH* loci were used to differentiate between *M. fragrans* and its adulterant *M. malabarica. rbcL* locus proved less informative in differentiating the two species, whereas *psbA-trnH* locus was very efficient in discriminating the two species with sixty four polymorphic sites and nine indels. Out of the five market samples analyzed, three were found to be adulterated with *M.malabarica*. Soffritti *et al.,* 2016, tried the authentication and adulteration detection of the expensive spice 'saffron'. *matK* and *rbcL* primers enabled the adulteration detection in traded saffron with *Buddleja officinalis, Gardenia jasminoides, Curcuma longa, Carthamus tinctorius* and *Calendula officinalis.* Based on the polymorphic sites, species specific markers were developed which could detect adulteration at very low concentrations of extraneous matter.

2.5.5.2 Authentication of other important commodities of trade and health using barcoding

Commodities of trade and health are of utmost importance. The quality and purity of traded drugs and health products need special authentication and certification. Commodities used for health purposes such as drugs and other value added health compliments are to be authenticated, elsewhile may lead to serious health issues and allergic reactions in susceptible individuals. Barcoding has played a significant role in the authentication and adulteration detection of such commodities. Some of the related reports are detailed below. Srirama *et al*., 2010 studied on the species admixtures in raw drug trade of *Phyllanthus* using *psbA-trnH* locus. 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 related species like *Phyllanthus debilis, Phyllanthus urinaria, Phyllanthus maderaspatensis,* and

Phyllanthus kozhikodianus. The locus could easily differentiate and authenticate 16 species of the *Phyllanthus* used in trade. The locus could easily differentiate and authenticate 16 species of the *Phyllanthus* used in trade.

Kumar *et al*., 2011 screened adulterants in olive oil using *psbA-trnH* and *matK* barcodes. The primers designed were able to detect adulteration of olive oil using canola and sunflower oil and an adulteration up to 5% could be easily detected. Study on authentication of herbal teas using barcoding loci *rbcL* and *matK* revealed adulteration. Thirty five percent of the samples generated barcodes for unlisted ingredients (Stoeckle *et al*., 2011). These unlisted ingredients may be allergic and not suitable for certain individuals who consume herbal teas and may lead to other severe health issues. Kool *et al*., 2012, demonstrated the functionality, efficacy and accuracy of using DNA barcoding for the identification of commercialised medicinal plants in Southern Morocco. One hundred and eleven root samples were tested using *rpoC1, psbA-trnH, matK* and *ITS* loci and could identify adulterations in eight traded samples belonging to six herbal products.

Wallace *et al*., 2012, tested the adulteration and authenticity of varied natural health products. Among the ginseng products tested, the entire American ginseng (*Panax quinquefolius)* was authentic. Fifty percent of the samples labelled as Korean ginseng (*Panax 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. In case of other products tested, *Acetea asiatica* was detected in product labelled “Black Cohosh” (*Actaea racemosa*). Barcodes generated from a sample commercially labelled as Echinacea (*Echinacea purpurea*), matched to sequence from walnut family (Juglandaceae). Green tea (*Camellia sinensis*) extracts and two capsules of Korean ginseng failed to match the commercial labels.

The herbal dietary supplement Black cohosh (*Actaea racemosa*) used for treatment of menopausal symptoms were authenticated from its related adulterant species. Using the barcoding locus *mat K*, 27 out of 36 dietary supplements tested were found to be authentic black cohosh. The remaining nine samples (25%) were adulterated and had sequences identical to that of three Asian *Actaea* species (*A. cimicifuga, A. dahurica,* and *A. simplex*) (Baker *et al*., 2012). Barcoding of herbal constituents were tested from herbal juices using *matK* locus. The technique proved to be an efficient one for the routine screening of different juices and health drinks (Mahadani and Ghosh, 2013). The role of DNA barcoding in forensic sample analysis of moassel products were proved efficient by the Alcohol and Tobacco section at the Science and Engineering Directorate of the Canada Border Services Agency (CBSA) using *rbcL* and *matK* locus. The presence of tobacco was identified in more than 60 commercial moassel products seized from ‘‘hookah bars’’(Carrier *et al*., 2013). Saw palmetto *(Serenoa repens),* herbal dietary supplement, was authenticated using *rbcL* and *matK* based barcoding (Little and Jeanson, 2013). Out of the total tested samples, 85% supplements were found to contain *S. repens*, 6% had adulterant species, and 9% of the supplements could not be determined conclusively.

The reports on adulteration and contamination of North American herbal products had made a stir among the public and media of Northern America. The study encompassed 44 herbal products from 30 different species of herbs representing 12 companies and a multi tired approach using *rbcL +ITS2* loci was performed. The results suggested a gross adulteration/ substitution and contamination of the herbal samples. Fifty nine percent of the samples generated barcodes for plants species that were not listed in the contents of the pack pointing to the substitution or contamination. Product substitution occurred in 33out of the 44 products and only 2 out of the 12 companies sold authentic products. Filler species like rice, wheat etc were also detected in the traded products (Newmaster *et al*., 2013).

Authentication of the herbal dietary supplements made from *Ginkgo biloba* L. were analysed using *matK* locus. Thirty one out of 37 supplements tested contained identifiable *G. biloba* sequences and six contained fillers without any detectable *G. biloba* sequences (Little, 2014). Long *et al*., 2014, successfully authenticated 33 kinds of non-Camellia teas and 4 kinds of teas (Camellia) using *rbcL, matK, psbA-trnH* and *ITS2* loci. DNA barcoding could successfully dissolve an international trade dispute case of roasted barley tea. Consignment containing roasted barley tea imported from China was rejected stating adulteration. Barcoding using *rbcL, matK, psbA-trnH* and *ITS2* loci of the rejected commodity revealed substitution and adulteration. Of the 13 batches of samples tested, 1 batch was substituted with *Morus* species and 2 batches had only *Hordeum vulgare* while 10 had *Hordeum vulgare* admixed with *Morus* spp., *Triticuma* spp. *Avena sterilis* and *A. fatua* (Jian *et al*., 2014).

DNA barcoding for the quality assessments of traditional Chinese medicine (TCM) occupying the herbal markets was done by Hermann and Wink, 2014. Authentication of 37 herbal drug samples representing 28 species were done using *rbcL* locus and the locus proved efficient in aligning 75% of the drugs to their species level and 25% to their genus level by BLAST analysis. One drug sample, *Fraxinus rhynchophylla* was found to be substituted with *Arctium lappa.* Bruni *et al*., 2015 suggested the applicability of barcoding in evaluating food safety. Processed honey was subjected to adulteration detection using *rbcL* and *psbA-trnH* loci. The sequences recovered from the four honey samples tested contained 39 plant species of genus *Castanea*, *Quercus*, *Fagus* and other herbal taxa. One out of the four samples showed sequences from *Atropa belladonna*, a toxic plant. Kumar *et al*., 2015 assessed the authenticity of the traditional herbal product “Bala” (*Sida cordofolia* and *S. rhombifolia*) available in south Indian markets using *psbA-trnH* and *ITS2* barcodes. Out of the 10 traded samples, only two belonged to the Bala group while the remaining eight were admixtures. Palhares *et al*., 2015 analysed 257 herbal samples from 8 species from the Brazilian market for the quality control

using DNA barcoding. High percentage (71%) of substitution and admixing of the plant samples were reported. *Hamemelis virginiana* was found to be substituted with samples of genus *Lantana* and *Solanum*. *V. officinalis* was substituted with plants of genus *Ageratum* and *Cissampelos* due to their easy availability in Brazil. The study also revealed the use of *Vernonia condensata* as an adulterant of *Peumus boldus.* Admixture of *H.virginiana* with *Tilia samples* and *Passiflora incarnate* with *Senna alexandrina* were also identified. Similar morphology and easy availability triggered adulteration in this case.

Authentication of herbal drug *S. cordifolia* was done by construction of a reference library using *rbcL, matK, psbA-trnH* and *ITS2* markers, out of which *psbA-trnH* and *ITS2* were found to be the best two marker combination for species identification of the *S. cordifolia* samples based on the intra-species and inter-species divergence. The study showed that none of the market samples belonged to the authentic species, *S. cordifolia*. Seventy-six per cent of the market samples belonged to other species of *Sida*. The predominant one was *Sida acuta* (36%) followed by *S. spinosa* (20%), *S. alnifolia* (12%), *S. scabrida* (4%) and *S. ravii* (4%). The remaining 24% of the samples were from other genera such as *Abutilon* sp. The observation is in contrast to the belief that medicinal plants are generally substituted or adulterated with closely related species (Vassou *et al*., 2015).

Seethapathy *et al*., 2015 assessed product adulteration in natural health products for laxative yielding plants like *Cassia, Senna* and *Chamaecrista* in South Indian markets. Barcoding of the products was done using *ITS2, matK, rbcL* and *psbA-trnH* of which *ITS2* showed higher interspecific divergence and successful discrimination of the plant species. *Cassia fistula* species sold in market was authentic. *S. auriculata* was sold as *S. alexandriana* and *S. tora* in different regions while *S. occidentalis* was mislabeled as *S. tora*. Study on the commercial Rhodiola products revealed not only species diversity but potential safety issues in the trade

and health. The barcoding potential of *matK, rbcL, psbA-trnH* and *ITS2* in species identification was assessed for 82 voucher samples from 10 species of *Rhodiola*. *psbA-trnH* and *ITS2* effectively discriminated all the species. Due to higher inter specific divergence shown by *ITS2,* the locus was selected to be the best.

Analysis using *ITS2* from 100 Rhodiolae Crenulatae Radix et Rhizoma decoction piece samples purchased from drug stores and hospitals revealed only thirty six samples (40%) to be authentic *R. crenulata*. Thirty five samples were found to be to *R. serrata,* nine samples were *R. rosea,* seven samples were *R. gelida,* two were *R. quadrifida* and one was *R. Fastigiata* (Xin *et al*., 2015). A strategy incorporating DNA barcoding and protein structure analysis was developed to authenticate herbal powder collected from an herbalist. The aim was to identify and authenticate the unknown/ unlabelled herbal sample (herbal mixtures) and the results proved it to be genuine *Cassia javanica* L. (Sheth and Thaker, 2015). The study proved DNA barcoding to be efficient in authenticating unknown samples/mixtures with the help of a common reference database. *ITS* barcoding was used for the identification of consumer relevant mushrooms used in dietary supplements. By using this technique, the dietary supplement manufacturing companies could demonstrate the accuracy of their labelled ingredients for products that contain fungi (Raja *et al*., 2017).

Barcoding as a tool for adulteration and authenticity testing of food and its derived products have proved efficient not only in plants based but also in other areas also. Barcode based Fish authentication, Fish Barcode of Life Initiative (FISH-BOl) (Galimberti *et al*., 2013) have more than 70,000 reference sequences for authentication. Authentication of canned meat and meat products is also routinely used in inspections and quality controls (Kane *et al*., 2016; Quinto *et al*., 2016).

MATERIALS AND METHODS

The study was conducted at the Crop Improvement and Biotechnology Division of the Indian Institute of Spices Research (IISR), Kozhikode, Kerala during the period 2012 to 2016. The spice samples viz., Group-I Black Pepper, Group-II Turmeric and Group-III Chilli (reference/ standard samples and their probable adulterants) used in the experiments were made in the laboratory by powdering the whole spice in a Cyclotech 1093 sample mill followed by grinding with liquid nitrogen to fine powder. All the samples were preserved in sealed covers and stored at -20˚C.The market samples were procured from local market and represented different firms. The brief outline of the study for three groups of spices is mentioned in the below steps:

- ❖ Collection of genuine and powdered market samples.
- ❖ Isolation of genomic DNA from genuine spice powders/whole commodities.
- ❖ Primer designing/ selection and optimisation of PCR parameters for the amplification of selected barcoding regions (four coding genes *matK*/ *rbcL*/*rpoB*/*rpoC1*, three noncoding spacers *atpF–atpH*/ *psbA–trnH*/ *psbK–psbI* and internal transcribed spacers of nuclear ribosomal DNA (*ITS*)).
- ❖ Sequencing, data analysis, selection of ideal barcode loci
- ❖ Detection of plant based adulterants in spice market samples.
- ❖ Validation of the results by cloning and simulation studies.
- ❖ Generation of barcode databases of the spices and their closely related adulterants and deposition of the sequences in the common databases.

3.1 Group I. BLACK PEPPER

3.1.1Collection of samples

3.1.1.1 Standards/reference samples of black pepper

Leaf/ berries from five accessions of *Piper nigrum* L. (Collection Nos: 6834, 6857, 6833, 6835, 6849) were obtained from the Germplasm Repository maintained at the ICAR-Indian Institute of Spices Research (IISR) Experimental Farm , Peruvannamuzhi, Kozhikode, Kerala and used as reference samples for the study.

3.1.1.2 Standard /reference samples-probable adulterants of black pepper

Five accessions of wild *Piper* species., viz., *Piper attenuatum* Buch-Ham (Collection Nos: 5469, 4661, 4634, 7077, 6951) and *Piper galeatum* (Miq.) C. DC. (Collection Nos: 347, 6980, 6904, 6815, 6984) were collected from the Germplasm Repository. Papaya seeds (*Carica papaya* L.) were collected and dried from mature papaya fruits procured from the local market in Kozhikode. Chilli (*Capsicum annuum* L.) samples were procured from local market, Kozhikode as well as from the Kerala Agricultural University, Thrissur.

3.1.1.3 Test samples/market samples of black pepper powder

Twelve popular branded samples of black pepper powder were procured from the local market. Three different batches of the same brand were also procured to validate the adulteration of the specific brand (Table1).

3.1.1.4 Simulated samples

Model blends/ simulations of black pepper berries (*P. nigrum*) with *P. attenuatum*, *P. galeatum*, papaya seeds and chillies were made on the basis of weight (Paradkar *et al*., 2001).

Table 1. Details of the market samples of black pepper powder used in the study

Sl.No.	Code	Brand name of black pepper powder	Manufacturer/ Marketed company	Batch/ lot No.	Price per 100gm (Rs.)
1.	P1	EASTERN	Eastern Condiments Pvt. Ltd, Adimali, Kerala,	Lot no. - DB6C007.	92.00/-
2.	P2	NIRAPARA	KKR Food Products, Kaladi, Kerala	,Lot no.- 140/11	61.10/-
3.	P3	MELAM	M.V. J. Foods Pvt. Ltd., Cochin, Kerala),	Lot no. - 08602.	78.00/-
4.	P4	PANDA	Panda Foods Pvt. Ltd., Wyanad, Kerala,	Lot no.-CMC113	71.50/-
5.	P5	RANI	Rani Food Products, Vatakara, Kerala,	Lot no. - 3001.	70.00/-
6.	P6	PRIME	Manufacturer not given on the packet	Lot no.- 005	76.00/-
7.	P7	MTR	MTR foods Pvt Ltd, bangalore	Lot no: 13K14B1955	82.00/-
8.	P8	SARAS	Saras spices, Kizhakkambalam, Aluva, Ernakulum	Lot no.-08H13	76.00/-
9.	P9	QUALITY	Quality foods Pvt .Ltd., Panavally, Alapuzha, Kerala	Lot No. 11:09	70.00/-
10.	P10	NADAN	Anu traders, Edathala north, Aluva , Ernakulam.	Lot No.A4	66.00/-
11.	P11	SWAMI'S	Green Valley condiments, Ayroor, Ernakulam, Kerala	Lot no.-F2	56.50/-
12	P12	NRS	New Rava stores, Court road, Calicut, Kerala	Lot no.- S8PG	58.00/-

Blends of dried black pepper berries and chillies were simulated in the ratio of 1:10, 1:25, 1:50, 1:100, and 1:200 to check the sensitivity of the barcoding technique. Representative pictures of various simulated and genuine materials are given in (Figure 1).

3.1.2 Isolation and quantification of genomic DNA from samples

3.12.1 DNA isolation using manual method

Total genomic DNA from genuine reference samples (*P. nigrum*), probable adulterant species *P. attenuatum*, *P. galeatum* and other powdered market samples of black pepper was isolated using the protocol described by Dhanya *et al*., (2007). The regents used are prepared as per Annexure I. Simulated samples of black pepper with probable adulterant species *P. attenuatum*, *P. galeatum* and papaya seeds were also done using the same protocol described below:

a. One gram of powdered samples was ground to a finer form using mortar and pestle.
b. The powder was thoroughly suspended in 6 ml of CTAB extraction buffer (3%CTAB, 100 mM Tris-HCl (pH-8.0), 20 mM EDTA (pH-8.0), 1.5 M NaCl) into 50 ml Oakridge tube.
c. The tubes were incubated at 65 ^{o}C in water bath for 30 min with intermediate shaking.
d. Allowed the tube to cool to room temperature, followed by addition of one third volume of potassium acetate solution. The contents were gently mixed and incubated on ice for one hour.
e. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly by inversion and centrifuged at 10,000 g for 15 min at 4 ^{o}C using Dynamica, UK centrifuge.
f. The aqueous phase was transferred to a fresh Oakridge tube and equal volume of 30% PEG, was added, mixed gently and incubated on ice for 30 min to one hour.
g. The samples were centrifuged at 12,000 g for 20 min at 4 ^{o}C and the supernatant were discarded.

h. The DNA pellets were washed with 70% ethanol, dried and dissolved in nuclease free water. RNase (10 µgml^{-1}) was added and incubated in water bath for 30 min at 37 ^{o}C.

i. Equal volume of chloroform: isoamylalcohol was added and mixed for 10 min and centrifuged at 8000 g for 15 min at 4 ^{o}C.

j. The aqueous phase was transferred to a fresh tube and to it equal volume of ice cold ethanol was added followed by incubation at -20 ^{o}C for one hour to precipitate DNA.

k. Centrifuged at 8000 g for 10 min at 4 ^{o}C and the supernatant were discarded. Dried and dissolved the DNA pellets in nuclease free water and DNA samples were stored at -20 ^{o}C.

3.1.2.2 DNA isolation from simulated samples of black pepper with chilli (Kit method)

DNA isolation from simulated samples of black pepper with chilli and black pepper leaf (Collection Nos: 7077, 6951, 6815, 6984) samples was done using the DNeasy plant mini kit (Qiagen, Germany). All the reagents used are supplied along with the kit. The procedure is as follows:

a. The sample materials were disrupted (≤ 100 mg wet weight or ≤ 20 mg lyophilised tissue) using the mortar and pestle.

b. Added 400 µl buffer AP1 and 4 µl RNase A to the micro centrifuge tubes. Vortex and incubated for 10 min at 65 ^{o}C. The tubes were inverted 3-4 times during incubation for even mixing of the contents.

c. Added 130 µl buffer AP2, mixed well and was followed by incubation on ice for 5 min.

d. The lysate was pipetted into a QIA shredder minispin column in a 2ml collection tube and centrifuged for 2 min at 20,000 g. Flow-through fraction after centrifugation was transferred into a new tube without disturbing the pellet.

e. The new tube containing the flow-through was mixed well by pipetting with 1.5 volumes of buffer AP3/E.

f. About 650 µl of the mixture was transferred into a DNeasy minispin column containing a 2 ml collection tube. The content were centrifuged for 1min at ≥ 6000g and discarded the flow thorough. This step was repeated with the remaining samples.

g. The spin column was placed into a new 2 ml collection tube, and 500 µl buffer AW was added. Centrifuged for 2 min at 20,000g.

h. The spin column was transferred to a new micro centrifuge tube and added 100 µl buffer AE for elution. Incubated for 5 min at room temperature and then centrifuged for 1 min at ≥ 6000 g. DNA was eluted to the micro centrifuge tube and stored at -20 ^{o}C.

3.1.2.3 DNA quantification using Spectrophotometric analysis

The isolated DNA was quantitatively and qualitatively checked using spectrophotometric analysis (Sambrook and Russell, 2001). DNA quantification based on spectrophotometer was done by measuring absorbance of DNA at 260 nm using a Biophotometer plus (Eppendorf, Germany). The concentration of DNA in the samples was calculated in terms of microgram per microlitre (µ gml^{-1}).

$$\text{Concentration of DNA (µg/ml)} = \frac{\text{Absorbance at 260 nm X 50 x dilution factor}}{1000}$$

DNA quality was checked by estimating the absorbance ratio of DNA at 260 nm and 280 nm (A260/A280)

3.1.2.4 DNA quantification using Agarose gel electrophoresis

Quantity of the isolated DNA was estimated by comparing the intensity of the DNA band with that of a standard λ marker (Genei, Bangalore, India). Agarose gel electrophoresis was carried out using SUB series Horizontal electrophoresis unit (Hoefer, USA) using a programmable power supply PS300-B (Hoefer, USA). The regents used in the study are prepared as per Annexure II.

a. The gel trays were cleaned thoroughly using tissue paper dipped with ethanol.

b. The tapes were fixed to the ends of gel trays and the combs were placed horizontally.

c. 0.8 % Agarose in 1 X TBE was prepared and the solution was boiled in a microwave oven until all the Agarose particles were completely dissolved. The agarose was allowed to cool (60°C).

d. 2 µl of ethidium bromide (10 mg ml^{-1}) was added to the gel and was poured to the gel tray.

e. The tape and comb were removed after 20 minutes and the gel was placed in to the electrophoresis tank. 1 X TBE was poured until the gel was fully immersed.

f. 2µl of DNA samples mixed with gel loading dye for loading of the samples to the wells. A standard marker DNA of known concentration was also loaded in a separate lane. The gel was run at 50 - 60 volts.

g. The DNA bands were visualized on a UV transilluminator and documented the gel using a Syngene Gel Documentation system.

3.1.3 Primer selection and PCR standardisation of the barcoding loci

The barcoding loci and the primer selection were done on the basis of universal acceptance, primers suggested by CBOL and literature studies. The primer sequences were checked for self complimentarity, melting temperature and GC content using Primer 3plus software. Primers were procured from IDT (Integrated DNA Technologies, USA) in the lyophilised form. The lyophilised form of primers was further reconstituted into nuclease free water.

3.1.3.1 Stock preparation of primers

a. The lyophilised powder was centrifuged at high speed (~13000g) for 10 min.

b. Adequate quantity of water was added to the vial (n moles x 10µl) and kept on shaker for 1 hour for dissolving the primer to a final concentration of 100µM.

c. 10 µl of the above primer stock was added to 90 µl water to give a 10 nM/ µl solution.

The details of the primers used in this study are provided in Table 2.

3.1.3.2 Optimisation of the PCR components

By keeping the buffer concentration constant (1X), concentrations of all other PCR components (Template DNA, dNTP's, *Taq* DNA polymerase, primer and $MgCl_2$) were tested in varying proportions.

a. Template DNA concentration- A concentration of 10-50 ng DNA was used.

b. *Taq* DNA polymerase- The enzyme concentration taken was 0.25U-1 U (Genei, Bangalore, India)

c. dNTP's- Final concentrations of 0.1 mM to 0.3 mM were tried (Genei, Bangalore, India).

d. $MgCl_2$- A final concentration between 1.0- 2.5 mM per reaction was checked (Genei, Bangalore, India).

e. Primers- concentrations of 5-20 picomoles per reaction.

The PCR reaction protocol was standardised by varying the above components in different proportions. PCR conditions for the barcoding loci were standardized by varying the temperature profiles. Gradient PCR was done to standardise the annealing temperature for each barcoding loci. The reaction was performed in an Eppendorf vapo protect thermal cycler. The amplified products were resolved in 1% agarose gel along with a 100bp ladder as a size marker (Fermentas, UK). The barcoding loci which gave consistent amplification pattern with genuine spice samples, their adulterants and market samples were selected for further studies. The resolved bands were gel eluted by agarose gel electrophoresis using

Table 2. Primers used in the study

Sl.No.	Primer name	Sequence	Reference
1	*rbcL a-f* *rbcL a-r*	5’ ATGTCACCACAA ACA GAGACTAAAGC3’ 5’ GTAAAATCA AGTCCACCGCG 3’	Kress and Erickson, 2007
2	*nrpoC1-2* *rpoC1-4*	5’ GGCAAAGAG GGAAGATTTCG3’ 5’ CCATAAGCATATCTTGAGTTG G 3’	Ford *et al*., 2009
3	*trnH-2* *psbA-F*	5’ CGCGCATGGTGGATT CACAATCC 3’ 5’ GTTATGCATGAACGTAATGCT C 3’	Tate and Simpson, 2003
4	*matK* 390F matK 1326R	5’CGATCTATTCATTCAATATTTC 3’ 5’ TCTAGCACACGAAAGTCGAAGT 3’	Cuenoud *et al*., 2002
5	*matK*3F_KIM *matK* 1R_KIM	5’ CGTACAGTACTTTTGTGTTTACGAG 3’ 5’ ACCCAGTCCATCTGGAAATCTTGGTTC 3’	Vijayan andTsou, 2010
6	*ITS-2* *ITS-3*	5’-GCTGCGTTCTTCATCGATGC-3’ 5’-GCATCGATGAAGAACGCAGC-3’	Urbatsch *et al.*, 2000
7	*rpoB* *rpoBr*	5’-ATGCAACGTCAAGCAGTTCC-3’ 5’-CCGTATGTGAAAAGAAGTATA-3’	CBOL, 2009
8	*atpF* *atpH*	5’-ACTCGCACACACTCCCTTTCC-3’ 5’-GCTTTTATGGAAGCTTTAACAAT-3’	Lahaye *et al.*, 2008
9	*psbK* *psbI*	5’-TTAGCCTTTGTTTGGCAAG-3’ 5’-AGAGTTTGAGAGTAAGCAT-3’	Lahaye *et al.*, 2008

QIAquick gel extraction kit (Qiagen, Germany). All the reagents used were supplied along with the kit.

3.1.3.3 Gel elution protocol

a. The specific DNA fragments from the agarose gels were excised with a sterile, clean, sharp scapel.
b. The gel slice was weighed in a colourless tube and mixed with buffer QG in the ratio, 3 volumes of buffer QG: 1 volume gel (100 mg gel~100 µl). The maximum amount of gel per spin column was 400mg.
c. Incubation was performed at 50^0C for 10min and intermediate vortexing of the tube was done every 2-3 min to dissolve the gel.
d. One volume of isopropanol was added to the sample and mixed well.
e. The QIAquick spin column was placed in a 2 ml collection tube and centrifuged for 1min.The flow-through was discarded and the QIAquick spin column was placed back into the same tube.
f. Followed by the addition of 500 µl buffer QG to the QIAquick spin column and centrifugation for 1min. The flow-through was discarded and the QIAquick spin column was placed back to the same tube.
g. 750 µl of buffer PE was added to the column for wash, centrifuged for 1 min and the flow-through was discarded.
h. Centrifugation of the column was done for 1min to remove residual wash buffer.
i. The column was placed into a clean 1.5 ml microcentrifuge tube. 50 µl of elution buffer EB was added to the centre of the QIAquick membrane and incubated at room temperature for 5 min.
j. The purified DNA was collected in the microcentrifuge tube by centrifugation.

3.1.4 Detection of adulteration in the market samples of black pepper

The sequences were BLAST analysed against the NCBI (National Centre for Biotechnology Information) database and the authenticities were checked. The market samples which showed sequence similarity towards probable adulterants / other than genuine samples were considered adulterated.

3.1.4.1 Sequencing and data analysis

All the purified PCR products were custom sequenced at Scigenome labs (Kochi, Kerala). 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. The sequences were sequenced bidirectionally and the contigs were assembled from the forward and reverse sequence reads using DNA Baser (version 3.4) software. BLAST (Basic Local Alignment Search Tool) analysis was done against the nucleotide database of GenBank to confirm the sequence originality (Atschul *et al*., 1997).

Sequences obtained for the coding loci were translated using the translate tool of ExPASY, a SIB Bioinformatics Resource Portal (Gasteiger *et al*., 2003) and their identity was checked by Protein BLAST tool. The generated sequences were aligned using the online tool ClustalW (Larkin *et al*., 2007) or MUSCLE algorithm (Edgar, 2004) and edited manually by Bioedit (Hall, 1999). Further data analysis was done using Mega 5.2 (Tamura *et al*., 2011). All positions containing gaps and missing data were eliminated from analysis. The pair wise genetic distances, the inter specific and intra specific distances for the barcoding loci were calculated using the Kimura 2 parameter of MEGA 5.2. Two loci DNA barcode approach (*rbcL + psbA-trnH, rpoC1+psb-trnH*) was done to increase the species resolution in case of

the black pepper (Group I). The multilocus barcodes were constructed by concatenating the sequences using Genious 4.7.4 software (Drummond *et al*., 2006).

3.1.5 Validation studies on adulteration of black pepper

Cloning was done to validate the adulteration in the market samples that showed maximum similarity to the probable adulterant sequences or sequences other than the *P. nigrum*. Simulated samples were used as positive control for validating the results. The bands specific to chilli, black pepper (from genuine reference samples -control), putative market samples and simulated samples were amplified using the ideal locus and cloned to validate the adulteration in the market samples.

3.1.5.1 Simulation

Simulations were done using *P. nigrum* with *P.attenuatum, P.galeatum*, papaya seeds and chilli. The simulated samples of black pepper with their probable adulterants were PCR amplified, cloned and sequenced. Different proportions (1:10, 1:5 1:50, 1:100, and 1:200) of simulated samples were used to check the sensitivity of the barcoding loci to detect adulteration at band level itself.

3.1.5.2 Cloning

The gel purified PCR product was cloned to pGEMT cloning vector system (Fermentas, USA) (Figure 2).

3.1.5.2.1Ligation to the vector

Ligation reaction was done using the Promega ligation protocol as per the manufacturer's instructions. The quantity of reagents used for a single ligation reaction is given in Table 3. The quantity of DNA fragment (insert) to the vector was taken in the ratio of 1:3 in the ligation reaction. The calculation of insert was done using the below formula.

$$\frac{\text{Size of the insert (bp) x Amount of vector (ng) x Insert :vector molar ratio (3)}}{\text{Size of vector (bp)}} = \text{ng of insert}$$

The ligation mixture was kept at 16^0C for 1 hour and then at 4^0C overnight.

Table 3. Ligation mixture components

Sl.no	Component	Volume
1	2X buffer	5 µl
2	PGEMT vector	1 µl
3	PCR product	3 µl(variable)
4	T4 ligase	1 µl
5	Water	0 µl
6	Total	10µl

3.1.5.2.2 Transformation

Bacterial transformation was done using manual method by giving heat shock treatment. All the regents used for the experiment were prepared as per Annexure 3.

Revival of the culture:-The bacterial culture JM109 was streaked on LB agar plate by quadrant streaking to obtain singe colonies. The plates were incubated at 37 ^{o}C overnight.

Competent cell preparation: - The competent cells of JM109 were prepared using the Genei competent cell preparation kit as per the manufacturer's protocol as given below. All the reagents were supplied along with the kit.

a. 4-5 colonies of *E.coli* JM109 were inoculated in a 25 ml LB broth (supplemented with 100 µl ampicillin for 100 ml broth).
b. The inoculated broth was incubated at 37 ^{o}C for 3 hours in a shaking incubator at 220 rpm.
c. 10 ml of the culture was centrifuged at 35000 rpm for 15 min at 4 ^{o}C.

d. The supernatant was discarded and 3.2 ml of solution A was added to the pellets and mixed well.

e. The contents were chilled on ice for 20 min and further centrifuged at 35000 rpm for 15 min at 4 ^{o}C.

f. 500 µl of solution A was added to the pellets and resuspended carefully.

g. 150 µl was dispensed to each pre- chilled microcentrifuge tubes and stored at -80^{o}C.

Heat shock treatment

a. Tubes containing the competent cells were kept on ice.

b. 10 µl of ligated product was added to the competent cells and incubated on ice for 40min.

c. Tubes were removed from ice and immediately placed in water bath at 42 ^{o}C for 90 sec and quickly placed back on ice for 5 min.

d. 250 µl of fresh LB broth was added to the tubes and kept at 37 ^{o}C for one hour incubation in a shaking incubator.

e. LB agar plates were prepared by adding X-gal, IPTG and ampicillin as per the prescribed final concentration. The contents mixed well and poured in to a sterile petri plates and allowed to solidify for 5-10 min. The plates were further kept at 37^{o}C for incubation.

f. Plated the suspension in different volumes to the LB agar plates using a sterile L-rod and incubated at 37 ^{o}C overnight.

3.1.5.2.3 Screening and selection of colonies

Blue / white selection was adopted for screening of the recombinants. The white and light blue tinged bacterial colonies were picked using sterile toothpick and inoculated on to LB agar-ampicillin plates. The plates were numbered grid wise, assigning each clone a particular number in the plate. The sub cultured plates of recombinant clones were incubated at 37°C overnight and stored at 4°C for about one week.

Transformed clones were screened by colony PCR (modified Gussow and Clackson, 1989) using vector specific M13 forward and reverse primers. The colonies were picked using a sterile tooth picks and resuspended in 10 µl sterile water and kept for boiling in a water bath for 3 min. About 1 µl of this was used as template for PCR. The components and their volume used for colony PCR given in Table 4. Thermal cycling conditions for colony PCR were 94^{o} C for 3 min followed by 30 cycles of denaturation at 94^{o} C for 1 min, primer annealing at 48^{o} C for 1 min, primer extension at 72^{o} C for 1 min and final extension at 72^{o} C for 5 min. Amplified products were resolved on a 1.5% agarose gel and visualized using Syngene gel documentation system. Positive transformants were identified based on the insert size.

Table 4.Colony PCR reaction components

Sl.no	PCR component	Reaction volume (Total volume 25 µl)
1.	Nuclease free water	18.67 µl
2.	10X *Taq* assay buffer with15mM $MgCl_2$	2.50 µl
3.	10mM dNTP mixture	0.50 µl
4.	M13 Forward primer (2.5 pico moles μl^{-1})	1.00 µl
5.	M13 reverse primer (2.5 pico moles μl^{-1})	1.00 µl
6.	*Taq* DNA polymerase (3 U μl^{-1})	0.33 µl
7.	Template	1. 00 µl

3.1.5.2.4 Isolation of plasmid DNA

a. Plasmid DNA was isolated using QIA prep spin miniprep kit (Qiagen, Germany). All the regents were supplied along with the kit. The colonies were inoculated in 5 ml of LB broth containing ampicillin and kept for incubation at 37^{o} C in a shaking incubator overnight.

b. 1-5ml of overnight bacterial culture was pelleted by centrifugation at ≥ 8000rpm for 3min at room temperature.

c. The pellets were resuspended in 250 µl of buffer P1 and transferred to a microcentrifuge tube. 250 µl of buffer P2 was added to it and mixed thoroughly by inverting the tubes until the solution became clear.

d. 350 µl of buffer N3 was added and mixed thoroughly by immediately inverting the tubes and was centrifuged for 10 min at 13000 rpm at room temperature.

e. The supernatant was applied to QIA prep spin column by decanting. Centrifuged for 30-60 sec and discarded the flow through.

f. The column was washed by adding 750 µl buffer PE and the centrifugation was done for 30-60 sec. Later the flow through was discarded and the column was transferred to a new collection tube. Centrifugation was performed for 1min to remove residual wash buffer.

g. The spin column was placed in a clean 1.5 ml microcentrifuge tube and 50 ml elution buffer was added to the centre of the Qia prep spin column. Incubation for 3-5min was done and centrifuged at 13000 rpm at room temperature for 1min. Eluted products were collected in the microcentrifuge tube.

3.1.5.2.5 Sequencing and data analysis

The plasmids were custom sequenced at Scigenome labs (Kochi, Kerala) using ABI 3730 XL sequencing machine. The sequence analysis was done as per the earlier prescribed methods by removing the vector sequences.Vector sequences were identified using NCBI tool Vecscreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and trimmed using Bioedit software (Hall, 1999). Sequence identity searches and multiple sequence alignment were done to detect the adulteration in the powdered market (test) samples.

3.1.5.3 HPLC analysis for adulteration detection

HPLC (High Performance Liquid Chromatography) were performed to reconfirm chilli adulteration in black pepper market sample. Piperine and capsaicin (Sigma Aldrich) were used as authentic standards. Three market samples (two putative adulterated market samples MS3 and MS9, and one negative control sample MS4) along with a simulated sample (1:100) were taken in duplicates for analysis. The HPLC analysis was carried out at Phytochemistry Division, CMPR (Centre for Medicinal Plants Research) Kottakkal Arya Vaidya Sala, Kottakkal, Kerala.

The samples, 25 gm each were weighed to 500 ml boiling flasks. 200 ml rectified spirit was added and refluxed gently for 5 hours. After refluxing the flasks were cooled and ~3 to 4 ml of the contents were filtered using 0.45 µm syringe filter into stoppered test tubes. 10 µl of the sample was injected in to HPLC apparatus. HPLC analysis was carried out using Shimadzu LC10A, connected with the photo diode array detector and RP-C-18 coloumn. Acetonitrile: 1% acetic acid (60:40) of HPLC grade was used as mobile phase with detector wavelength at 280 nm. 10 µl of the sample was injected at a flow rate of 0.8 ml min^{-1}.

3.1.6 Barcode generation

DNA baser software package was used to generate consensus sequences and contigs. Initial screening/homology searches of the sequences were carried out using BLAST algorithm (Atschul *et al*., 1997) to identify the closest matching sequences in the nucleotide database of GenBank to confirm the authenticity of sequences taken as reference samples. The sequences were aligned using Clustal W (Larkin *et al*., 2007) and trimmed and edited using BioEdit (ver.7.2.5).

3.1.6.1 Submission of sequences in NCBI

Bankit tool was used to submit the sequences to the NCBI following the below given criteria:

The sequences should have more than 200 nucleotide base pair length (except for introns, non coding RNA, microsatellites, ancient DNA). Sequences should not be a non-contiguous sequence and not artificially aligned. The sequences should be single sequences, not a mix of molecule types, such as mix of genomic and mRNA sequence.

Steps

a. Bankit account was created through My NCBI login. Basic information regarding authors, institution, working title of the paper and correspondence was provided.
b. Bankit submissions retain the information and are displayed once the sequence is accepted in the database. Details regarding the origin, name of the organism, authority, isolation source was entered in the space provided.
c. Sequences were checked for open reading frames (ORFs). Only sequences with uninterrupted ORFs were selected. Sequences was made in FASTA format and pasted in the space given or uploaded.
d. The amino acid sequences for the coding regions were taken using EXpasy translate tool.
e. Sequence features like CDS, gene, protein product information, and amino acids sequences was entered and submitted.
f. Once submitted, a submission ID is given and the verification process takes 2-4 weeks depending on the sequence and the details provided.
g. A bankit NCBI accession number was given after all clarifications if any and after the final confirmation regarding the uniqueness of the sequence.

Group II. TURMERIC

3.2.1 Collection of samples

3.2.1.1 Standards/reference samples of turmeric

Whole fresh rhizomes of five accessions of *C. longa* L. (IC Nos. 143, 119, 126, 360, and 361) collected from the ICAR-Indian Institute of Spices Research (IISR) Experimental Farm at Peruvannamuzhi, Kozhikode, Kerala, served as the reference samples for the experiment.

3.2.1.2 Standard /reference samples-probable adulterants of turmeric

The probable adulterants of turmeric were selected based on earlier reports. Four accessions of C. *zedoaria* Rosc. / *Curcuma zanthorrhiza* Roxb. (IC Nos: 465, 760, 765, and 1517) were obtained from the Germplasm Repository maintained at the ICAR- Indian Institute of Spices Research, Kozhikode, Kerala, India. Five samples of cassava (*Manihot esculenta* Crantz*)* were obtained from the local market, Kozhikode, at the time of the study. The samples were sun dried, powdered and used for further analysis.

3.2.1.3 Test samples/ market samples

Popular brands of turmeric powder from ten different firms were procured. Three different batches of the same brand were also procured to validate the adulteration of the specific brand (Table 5).

3.2.1.4 Simulated Samples

Model blends of *C. longa* with *C. zedoaria* and with cassava were made on the basis of weight to check the sensitivity of the technique. Representative pictures of sample materials and model blends are given (Figure 3).

Table 5. Market samples of turmeric powder used in the study

Sl.No	Code	BRAND NAMEOF TURMERIC POWDER	Manufacturer/ Marketed company	Batch/ lot No.	Price per 100gm
1.	T1	EASTERN	Eastern Condiments Pvt. Ltd., Adimali, Kerala,	Lot no. – MC22A102	₹ 12.35/-
2.	T2	NIRAPARA	KKR Food Products, Kaladi, Kerala.	Lot no.- 136/11	₹ 13.65/-
3.	T3	SWAMI'S	Green Valley condiments, Ayroor, Ernakulam, Kerala	Lot no.- F2	₹ 15.00/-
4.	T4	MELAM	M.V. J. Foods Pvt. Ltd., Cochin, Kerala	Lot no. - 10406	₹ 11.68/-
5.	T5	DOUBLE HORSE	Manjilas Foods Pvt. ltd., Sashtri road, Nellikkunnu, Thrissur, Kerala	Lot no. – AA100020	₹ 11.80/-
6.	T6	SAICO	Saico Foods , Pantheerankavu, Kozhikode, Kerala.	Lot no. –SS04	₹ 13.25/-
7.	T7	SEVANA	Sevana group of companiesChampanoor, Angamali, Kerala	Lot no. –U102	₹ 11.80/-
8.	T8	KAULA	Kaula Agro Foods Pvt. Ltd., kodanad, kerala	Lot no. – 14KS3OD001	₹ 16.50/-
9.	T9	DEVON	Devon Foods Ltd., Malloossery, Chungo, Kottayam, Kerala	Lot no. –T12/13	₹ 17.00/-
10.	T10	SAKTHI	Sakthi masala Pvt. Ltd., Mamarathupalayam, Erode, TamilNadu.	Lot no. – TURICDEX111	₹ 13.54/-

3.2.2 Isolation and quantification of genomic DNA from samples

3.2.2.1 DNA isolation from turmeric powder and their probable adulterants using manual method

Total genomic DNA from genuine turmeric samples, market samples, cassava and simulated samples was isolated using the method described by Remya *et al*., (2004). Reagent preparation for the protocol for DNA isolation was done as per Annexture1.

a. 2 g of powdered sample was mixed with 16 ml of extraction buffer (3%CTAB, 100 mM Tris-HCl (pH-8), 20 mM EDTA (pH-8), 1.5 M NaCl) in a 50 ml Oakridge tube. 0.3 % β-mercaptoethanol was freshly added to the buffer.
b. The tubes were kept for overnight incubation in shaking water bath at 37 ^{o}C.
c. Equal volume of chloroform: isoamylalcohol (24:1) was added to the tubes and mixed thoroughly by inversion for 15 min and centrifuged at 10,000g for 15 min.
d. The aqueous phase was transferred to a fresh Oakridge tube and mixed with equal volume of 100% ice cold ethanol.
e. The DNA was precipitated by incubating the tubes at -20 ^{o}C for 2 hours. The precipitated DNA was further centrifuged at 10,000g for 5min and the pellets were washed using 70% ethanol.
f. The DNA pellets were dried and dissolved in minimum volume of nuclease free water. RNase (10 $\mu g ml^{-1}$) was added and incubated at 37 ^{o}C for 30min in water bath.
g. Equal volume of phenol: chloroform: isoamylalcohol (25: 24: 1) was added and mixed for 15 minutes. Centrifuged at 10,000g for 15 minutes at 4°C. The DNA supernatant was taken out and the extraction was repeated again.

h. Aqueous phase was separated and mixed with chloroform: isoamylalcohol (24:1) and centrifuged at 10,000g for 15 minutes at 4°C.

i. The aqueous phase after centrifugation was separated and mixed with equal volume of chilled 100 % ethanol. Incubated at 4°C for 1 hour and centrifuged at 8,000 rpm for 5 minutes.

j. The supernatant was discarded and the pellets are carefully dried under vacuum. The DNA pellets was further dissolved in minimum volume of water and stored at -20^{o}C.

3.2.2.2 DNA quantification using spectrophotometric analysis

The isolated DNA was quantitatively and qualitatively checked using spectrophotometric analysis (Sambrook and Russell, 2001) and the detailed procedures are explained in section 3.1.2.3 of Chapter 3.

3.2.2.3 DNA quantification using agarose gel electrophoresis

Quantity of the isolated DNA was estimated by comparing the intensity of the DNA band with that of a standard λ marker (Genei, Bangalore, India). Agarose gel electrophoresis was carried out using SUB series Horizontal electrophoresis unit (Hoefer, USA) using a programmable power supply PS300-B (Hoefer, USA). The detailed procedures are explained in section 3.1.2.4 of Chapter 3.

3.2.3 Primer selection and PCR standardisation of the barcoding loci

The barcoding loci and the primer selection were done on the basis of universal acceptance, primers suggested by CBOL and literature studies.

The primer sequences were checked for self complimentarity, melting temperature and GC content using primer 3plus software. Primers were procured from IDT (Integrated DNA Technologies, USA) in the lyophilised form. The lyophilised form of primers was further reconstituted into nuclease free water.

3.2.3.1 Stock preparation of primers

Primers were prepared as per 3.1.3.1section**.** The details of the primers used in this study are provided in Table 2.

3.2.3.2 Optimisation of the PCR components

PCR reactions and the cycling conditions were standardised by varying the concentrations of PCR mixture components (Template DNA, dNTP's, *Taq* DNA polymerase, primer and $MgCl_2$). The detailed procedures are explained in section 3.1.3.2 of Chapter 3.

3.2.3.3 Gel elution protocol

The gel elution protocols are done as per the methodology explained in section 3.1.3.3 of Chapter 3.

3.2.4 Detection of adulteration in the market samples of turmeric

3.2.4.1 Sequencing and data analysis

All the purified products were custom sequenced at Scigenome labs (Kochi, Kerala) using ABI 3730 XL sequencing machine. Bidirectional sequencing was done and the contigs were assembled from the sequences using DNA baser (version 3.4) software. Sequence analyses were performed as per section 3.1.4.1.

3.2.5 Validation studies on adulteration of turmeric

The sequences obtained were BLAST analysed against the NCBI (National Centre for Biotechnology Information) database and the authenticities were checked. The market samples which showed sequence similarity towards probable adulterants / other than genuine samples were considered adulterated.

The band specific to the ideal locus from the putative market samples, reference sample and the simulated samples were cloned to validate the adulteration in the market samples.

3.2.5.1 Simulation

Model blends of reference samples with the probable adulterants (*C. zedoaria* and cassava starch) were prepared. The simulated samples for turmeric powder with their probable adulterants were PCR amplified, cloned and sequenced.

3.2.5.2 Cloning

The gel purified PCR product was cloned to pGEMT cloning vector system (Fermentas, USA) (Figure 2).

3.2.5.2.1 Ligation to the vector

Ligation reaction was done using the Promega ligation protocol as per the manufacturer's instructions. The quantity of reagents used for a single ligation reaction is given in Table 3. The quantity of DNA fragment (insert) to the vector was taken in the ratio of 1:3 in the ligation reaction. The calculation of insert was done using the formula detailed in section 3.1.5.2.1 of Chapter 3.

3.2.5.2.2 Transformation

Bacterial transformation was done using manual method by giving heat shock treatment. All the regents used for the experiment were prepared as per Annexure 3. The detailed procedures are explained in section 3.1.5.2.2 of Chapter 3.0.

3.2.5.2.3 Screening and selection of colonies

The screening and selection of colonies were done as per the protocols explained in section 3.1.5.2.3 of Chapter 3.

3.2.5.2.4 Isolation of plasmid DNA

The isolation of plasmid DNA was done as per the protocols explained in section 3.1.5.2.4 of Chapter 3.

3.2.5.2.5 Sequencing and data analysis

The plasmids were custom sequenced at Scigenome labs (Kochi, Kerala) using ABI 3730 XL sequencing machine. The sequence analysis was done as per the earlier prescribed methods by removing the vector sequences.Vector sequences were identified using NCBI tool Vecscreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and trimmed using Bioedit software (Hall, 1999). Sequence identity searches and multiple sequence alignment were done to detect the adulteration in the powdered market (test) samples.

3.2.6 Barcode generation

DNA baser software package was used to generate consensus sequences and contigs. Initial screening/homology searches of the sequences were carried out using BLAST algorithm (Atschul *et al*., 1997) to identify the closest matching sequences in the nucleotide database of GenBank to confirm the authenticity of sequences taken as reference samples. The sequences were aligned using Clustal W (Larkin *et al*., 2007) and trimmed and edited using BioEdit (ver.7.2.5).

3.2.6.1 Submission of sequences in NCBI

Bankit tool was used to submit the sequences to the NCBI following the below given criteria:

The sequences should have more than 200 nucleotide base pair length (except for introns, non coding RNA, microsatellites, ancient DNA). Sequences should not be a non-contiguous sequence that is not artificially aligned. It should be single sequences, not a mix of molecule types such as mix of genomic and mRNA sequence.

The detailed method is explained in section 3.2.6.1 of Chapter 3.

3.3 Group III Chilli

3.2.1 Collection of samples

3.2.1.1 Standards/reference samples of chilli

Four varieties of *Capsicum annuum* L. (chilli) were collected from Kerala Agricultural University, Thrissur. The varieties procured were PB7, Vellakanthari, PKM1 and Anugraha.

3.2.1.2 Standard /reference samples-probable adulterants of chilli

Probable plant based adulterants of chilli include *Ziziphus nummularia* (Burm.f) Wight & Arn. ('choti ber') and *Beta vulgaris* L. (beetroot). Choti ber is rarely found in Kerala market and hence was procured from the ICAR-National Research Centre on Seed Spices, Ajmer, Rajasthan. Five different varieties of beet root were procured from the local market, flaked, sun dried, powdered and used.

3.2.1.3 Test samples/ market samples

Popular brands of chilli powder from seventeen different firms were procured. Three different batches of the same brand were also procured to validate the adulteration of the specific brand (Table 6).

3.2.1.4 Simulated Samples

Model blends of *C. annuum* with beet root and 'choti ber' were made in the general ratio on weight basis. The samples were sun dried, powdered and used for further analysis. Representative pictures of the samples and model blends are depicted in Figure.4.

3.2.2 Isolation and quantification of genomic DNA from samples

3.2.2.1 DNA isolation from chilli powder and their probable adulterants using manual method

Total genomic DNA from genuine chilli samples, probable adulterants samples, market samples and simulated samples were isolated using the protocol described by Dhanya *et al* ., (2008).

a. 2g of powdered sample was weighed and mixed with 10 ml of 95% alcohol in an Oakridge tube for 5 min at room temperature.
b. The tubes were centrifuged at 5000 g for 5 min and discarded the supernatant. The pellets were again washed with 10ml of 95% alcohol and centrifuged.
c. The sample pellets were dried in airflow chamber and further homogenised with 14 ml of extraction buffer (3%CTAB, 100 mM Tris-HCl (pH-8), 20 mM EDTA (pH-8), 2 M NaCl). 0.3% β- mercaptoethanol was freshly added to the extraction buffer.
d. The incubation was done at 37°C in water bath for 1 hour with intermittent shaking.
e. Equal volume of phenol: chloroform: isoamyl alcohol was added and mixed thoroughly for 10min followed by centrifugation at 8000 g for 15 min at 4°C.
f. The aqueous phase was transferred to a fresh tube and the extraction was repeated.
g. Equal volume of chloroform: isoamyl alcohol was added and mixed by inversion for 10min andcentrifuged at 8000 g for 15min at 4°C.
h. Aqueous phase was transferred to fresh tube and equal volume of ice cold ethanol was added. The tubes were incubated at - 20°C for 30 min for DNA precipitation.
i. DNA precipitate formed as white layers were spooled out and washed with 70% ethanol.
j. The pellets were dried and dissolved in minimum volume of nuclease free water.
k. RNase (10 µ gml^{-1}) was added and incubated at 37 °C for onehour.
l. Equal volumes of phenol: chloroform: isoamyl alcohol (25: 24: 1) were added and mixed for 15 min andcentrifuged at 10,000 g for 15 min at 4°C.
m. Aqueous phase was transferred and mixed with equal volume of chloroform: isoamyl alcohol (24:1), centrifuged at 8000 g for 5 min.

Table 6. Details of the market samples of chilli powder used in the study

Sl.No	Code	Brand nameof black pepper powder	Manufacturer/ Marketed company	Batch/ lot No.	Price per 100gm
1.	C1	EASTERN	Eastern Condiments Pvt. Ltd, Adimali, Kerala,	Lot no. – EAL2A953	₹ 18.25/-
2.	C2	SEVANA	Sevana group of companies Champanoor, Angamali, Kerala	Lot no. –U115	₹ 12.00/-
3.	C3	SAICO	Saico Foods , Pantheerankavu, Kozhikode, Kerala	Lot no. –SS09	₹ 12.00/-
4.	C4	NIRAPARA	KKR Food Products, Kaladi, Kerala.	Lot no. –105/12	₹ 14.95/-
5.	C5	MELAM	M.V. J. Foods Pvt. Ltd., Cochin, Kerala	Lot no. –01102	₹ 16.50/-
6.	C6	DOUBLE HORSE	Manjilas Foods Pvt. ltd., Sashtri road, Nellikkunnu, Thrissur, Kerala	Lot no. – 096L26	₹ 16.50/-
7.	C7	SWAMI	Green Valley condiments, Ayroor, Ernakulam, Kerala	Lot no. –E12300205158	₹ 17.60/-
8.	C8	KAULA	Kaula Agro foods Pvt. Ltd., kodanad, kerala	Lot no. –14KS29D004	₹ 17.00/-
9.	C9	DEVON	Devon Foods Ltd., Malloossery, Chungo, Kottayam	Lot no. –CLP-10	₹ 16.00/-

10.	C10	SAKTHI	Sakthi masala Pvt. Ltd., Mamarathupalayam, Erode, TamilNadu	Lot no. – CH12CNOX111	₹ 26.00/-
11.	C11	EASTERN	Eastern Condiments Pvt. Ltd, Adimali, Kerala	. Lot no. –MAK3F007	₹ 22.50/-
12	C12	MELAM	M.V. J. Foods Pvt. Ltd., Cochin, Kerala	Lot no. – 01402	₹ 28.00/-
13	C13	AACHI	Aachi Spices and Foods (P) Ltd., Ambattur, Chennai, Tamil Nadu.	Lot no. –B072T	₹ 24.00/-
14	C14	PRO NATURE	Pro Nature Organic Foods Pvt.Ltd., Jayanagar, Bangalore. Karnataka.	Lot no. –131441	₹ 40.00/-
15	C15	NRS		Lot no. –0AE23	₹ 17.00/-
16	C16	BROWN TREE	Brown Tree retail Pvt. Ltd., Ashok Nagar, Chennai, Tamil Nadu.	Lot no. –360	₹ 36.00/-
17	C17	MTR	MTR Foods Pvt Ltd, bangalore.	Lot no. –13l21C194	₹ 25.00/-

n. The aqueous phase was transferred to a new tube and mixed with equal volume of ice cold 100% ethanol and incubated for 2 hours at -20°C.
o. DNA pellets were obtained by centrifugation at 10000g for 5 min. The pellets were dried and dissolved in 500µl of nuclease free water.
p. 200 µl of 30% PEG 6000 solution was added to the DNA sample and kept for incubation at room temperature for 30 min.
q. The tubes were centrifuged at 14000g for 15 min and the pellets were washed using 80 % ethanol.
r. Pellets were dried, dissolved in nuclease free water and stored at -20°C.

3.2.2.2 DNA quantification using spectrophotometric analysis

The isolated DNA was quantitatively and qualitatively checked using spectrophotometric analysis (Sambrook and Russell, 2001) and the detailed procedures are explained in section 3.1.2.3 of Chapter 3.

3.2.2.3 DNA quantification using agarose gel electrophoresis

Quantity of the isolated DNA was estimated by comparing the intensity of the DNA band with that of a standard λ marker (Genei, Bangalore, India). Agarose gel electrophoresis was carried out using SUB series Horizontal electrophoresis unit (Hoefer, USA) using a programmable power supply PS300-B (Hoefer, USA). The detailed procedures are explained in section 3.1.2.4 of Chapter 3.

3.2.3 Primer selection and PCR standardisation of the barcoding loci

The barcoding loci and the primer selection were done on the basis of universal acceptance, primers suggested by CBOL and literature studies.

The primer sequences were checked for self complimentarity, melting temperature and GC content using primer 3plus software. Primers were procured from IDT (Integrated DNA Technologies, USA) in the lyophilised form. The lyophilised form of primers was further reconstituted into nuclease free water.

3.2.3.1 Stock preparation of primers

d. The lyophilised powder was centrifuged at high speed (~13000g) for 10min.

e. Adequate quantity of water was added to the vial (n moles x 10µl) and kept on shaker for 1 hour for dissolving the primer to a final concentration of 100µM.

f. 10 µl of the above primer stock was added to 90 µl water to give a 10nM/ µlsolution.

The details of the primers used in this study are provided in Table 2.

3.2.3.2 Optimisation of the PCR components

PCR reactions and the cycling conditions were standardised by varying the concentrations of PCR mixture components (Template DNA, dNTP's, *Taq* DNA polymerase, primer and $MgCl_2$). The detailed procedures are explained in section 3.1.3.2 of Chapter 3.

3.2.3.3 Gel elution protocol

The gel elution protocols are done as per the methodology explained in section 3.1.3.3 of Chapter 3.

3.2.4 Detection of adulteration in the market samples of chilli

3.2.4.1 Sequencing and data analysis

All the purified products were custom sequenced at Scigenome labs (Kochi, Kerala) using ABI 3730 XL sequencing machine. Bidirectional sequencing was done and the contigs were assembled from the sequences using DNA baser (version 3.4) software. Sequence analyses were performed as per section 3.1.4.1. of Chapter 3.

3.2.5 Validation studies on adulteration of chilli

The model blends of chilli with probable adulterants *viz*., choti ber and beet root were done using the powdered samples. The band specific to the ideal locus from the market samples, simulated samples and reference sample were cloned to validate the adulteration in the market samples.

3.2.5.1Simulation

Model blends of reference samples of chilli with the probable adulterants viz., choti ber and beetroot were prepared on the basis of weight. Different proportions of simulated samples (1:10, 1:25, 1:50, 1:100, and 1:200) were used to check the sensitivity of the barcoding loci to detect adulteration at band level.

3.2.5.2 Cloning

The gel purified PCR product was cloned to pGEMT cloning vector system (Fermentas, USA) (Figure 2).

3.2.5.2.1 Ligation to the vector

Ligation reaction was done using the Promega ligation protocol as per the manufacturer's instructions. The quantity of reagents used for a single ligation reaction is given in Table3. The quantity of DNA fragment (insert) to the vector was taken in the ratio of 1:3 in the ligation reaction. The calculation of insert was done using the formula detailed in section3.1.5.2.1 of Chapter 3.

3.2.5.2.2 Transformation

Bacterial transformation was done using manual method by giving heat shock treatment. All the regents used for the experiment were prepared as per Annexure 3. The detail procedures are explained in section 3.1.5.2.2 of Chapter 3.

3.2.5.2.3 Screening and selection of colonies

The screening and selection of colonies were done as per the protocols explained in section 3.1.5.2.3 of Chapter 3.

3.2.5.2.4 Isolation of plasmid DNA

The isolation of plasmid DNA was done as per the protocols explained in section 3.1.5.2.4 of Chapter 3.

3.2.5.2.5 Sequencing and data analysis

The plasmids were custom sequenced at Scigenome labs (Kochi, Kerala) using ABI 3730 XL sequencing machine. The sequence analysis was done as per the earlier prescribed methods by removing the vector sequences.Vector sequences were identified using NCBI tool Vecscreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and trimmed using Bioedit software (Hall, 1999). Sequence identity searches and multiple sequence alignment were done to detect the adulteration in the powdered market (test) samples.

3.2.6 Barcode generation

DNA baser software package was used to generate consensus sequences and contigs. Initial screening/homology searches of the sequences were carried out using BLAST algorithm (Atschul *et al*., 1997) to identify the closest matching sequences in the nucleotide database of GenBank to confirm the authenticity of sequences taken as reference samples. The sequences were aligned using Clustal W (Larkin *et al*., 2007) and trimmed and edited using BioEdit (ver.7.2.5).

3.2.6.1 Submission of sequences in NCBI

Bankit tool was used to submit the sequences to the NCBI following the below given criteria:

The sequences should have more than 200 nucleotide base pair length (except for introns, non coding RNA, microsatellites, ancient DNA). Sequences should not be a non-contiguous sequence that is not artificially aligned. The sequences should be single sequences, not a mix of molecule types, such as mix of genomic and mRNA sequence. The detailed method is explained in section 3.2.6.1 of Chapter 3.

Fig 1.Representative pictures of sample materials and model blends of Group I
a) *P.nigrum*, b). *P.attenuatum*, c). *P.galeatum*, d). Papaya seeds, e). *C.annuum*, f). simulated sample (Papaya: Black pepper 1:9), g).simulated sample (Chilli: Black pepper 1:9), h). Market sample

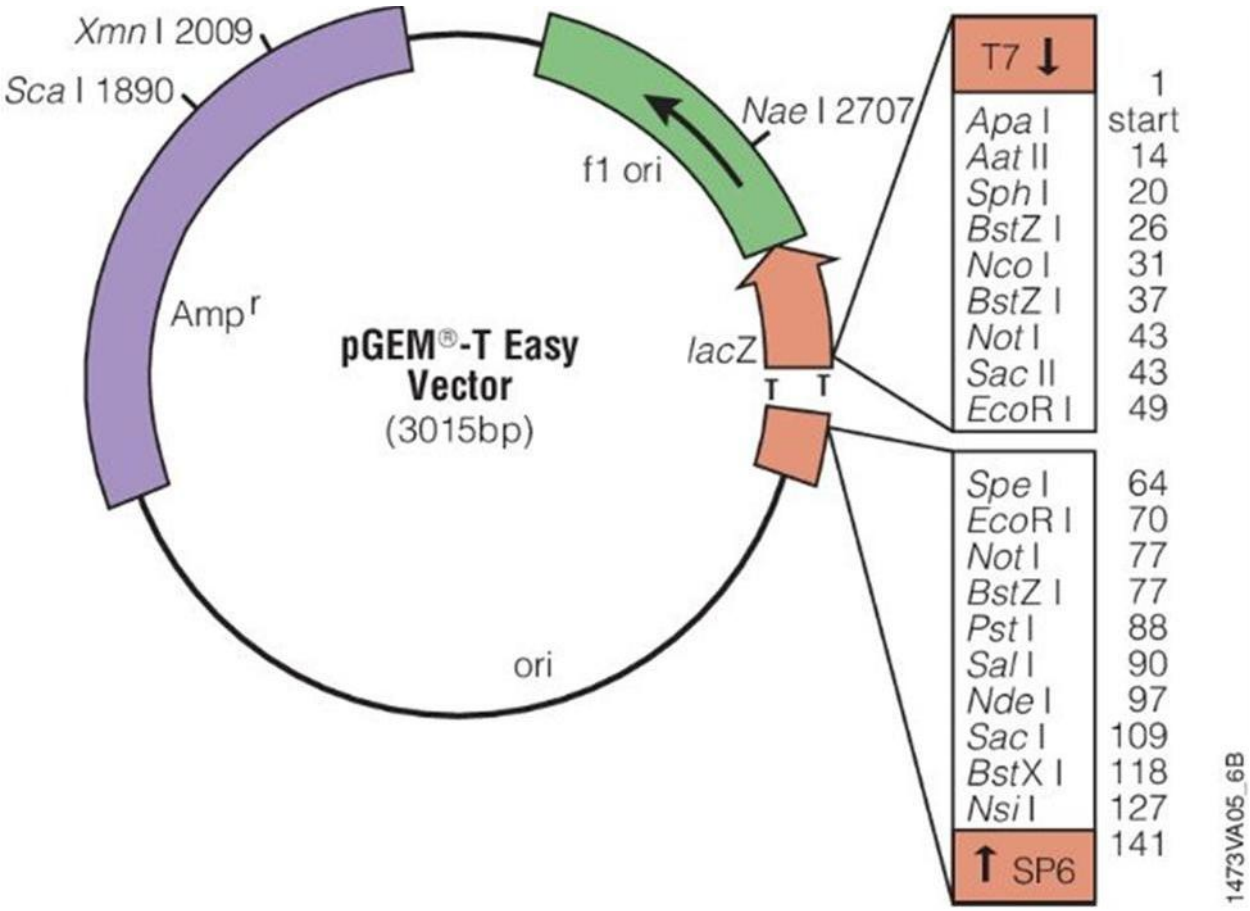


Figure2. pGEMT cloning vector system (Fermentas, USA)

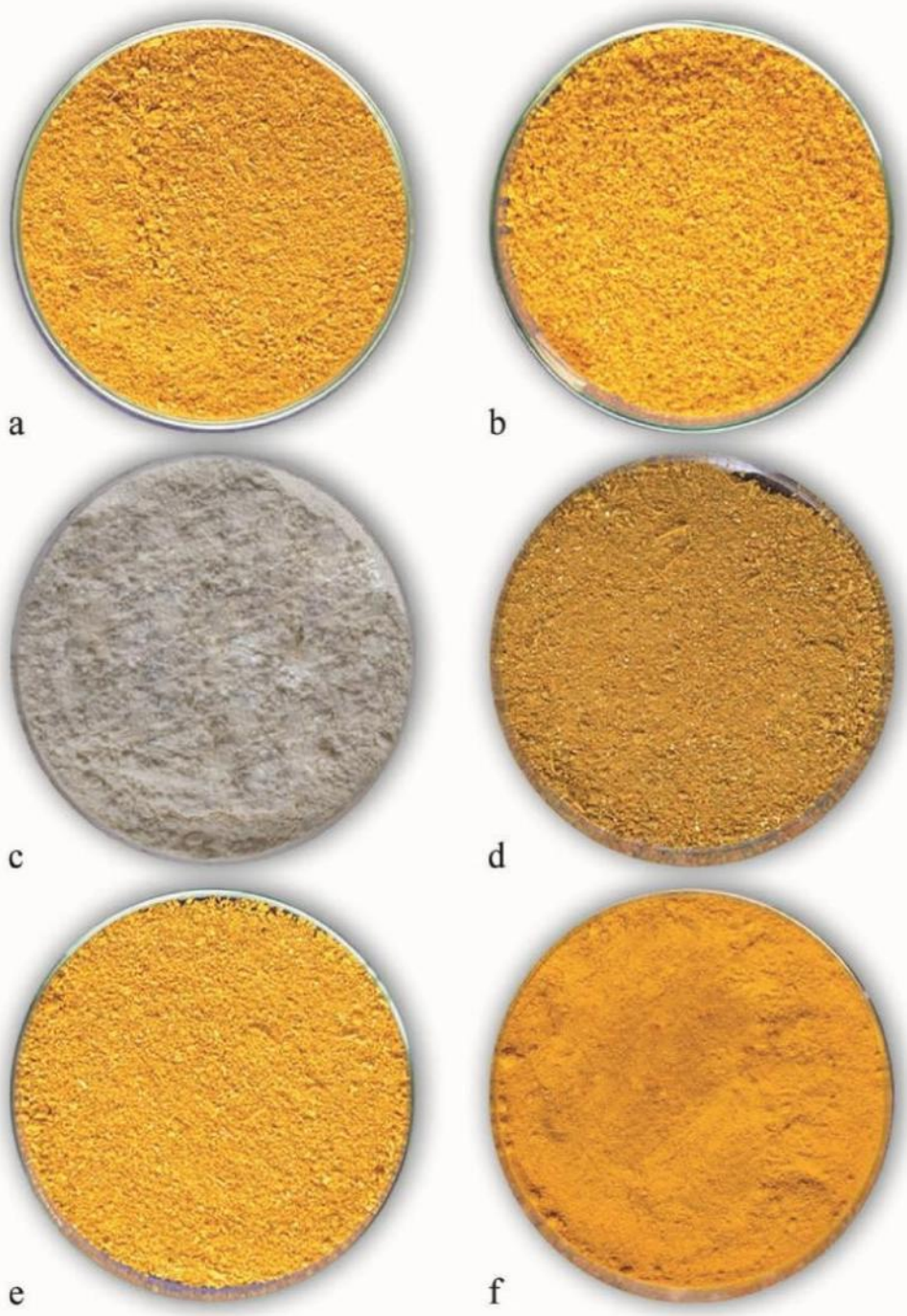


Fig 3.Representative pictures of sample materials and model blends of Group II

a) *C. longa*, b). *C.zedoaria*, c). Cassava starch, d). Simulated sample (Cassava starch: Turmeric1:9), e). simulated sample (C. zedoaria: C. longa1:9), f). Market sample

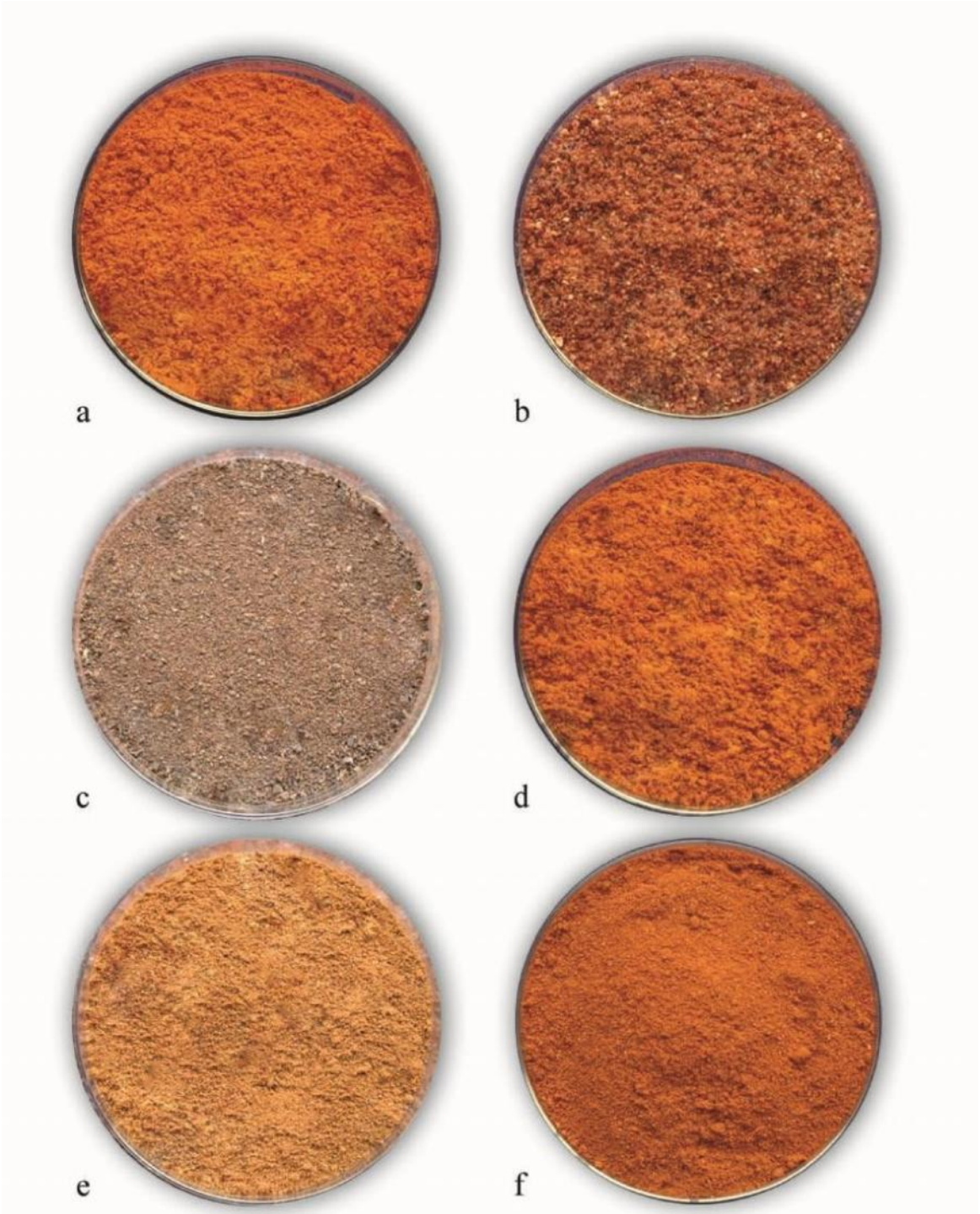


Fig 4. Representative pictures of sample materials and model blends of Group III

a) C. annuum, b). Choti ber, c). Beetroot, d). simulated sample (Choti ber: Chilli1:9), e). simulated sample (Beet root: Chilli1:9), f). Market sample

RESULTS

4.1 Group I. Black pepper

4.1.1 Collection of samples

The reference species of *P. nigrum, P.attenuatum* and *P.galeatum* was collected from the Experimental Farm, ICAR-IISR which serves as the reservoir of Germplasm Repository for *Piper* species. The germplasm of *Piper* species consisted of plants collected from different geographical locations and were thus rich in diversity and variability. These different accessions served to obtain maximum intra specific sequences of *P. nigrum* for the SNP studies.

4.1.2 Isolation and quantification of genomic DNA from their probable adulterants and market samples.

Genomic DNA was isolated from genuine black pepper samples (3.1.1.1), probable adulterants of black pepper viz., *P. attenuatum, P. galeatum,* papaya seeds (3.1.1.2) and market samples (Table 1) using manual method showed an absorbance ratio (A260/A280) between 1.8 to 1.9 indicating good quality DNA, free of proteins, polysaccharides and RNA contamination. The DNA yield was ranged between 18.6 to 34.2 µg g^{-1} and quality of isolated DNA showed conspicuous bands of high molecular weight with little shearing in the gel analysis for all the sample tested (Figure 5).

Similarly, DNA isolation using Qiagen kit method for simulated chilli-black pepper samples yielded 20.0 to 30.0 µ g g^{-1} of DNA from dried tissue and showed conspicuous bands of high molecular weight with little shearing in the gel analysis.

4.1.3 Standardization of PCR parameters for the commodity under study and their adulterants using barcoding primers.

The standardised PCR reaction protocol for the different barcoding loci contained 10-50 ng of DNA, 1X assay buffer with 1.5 mM $MgCl_2$, 1 mM dNTP, 1 picomol μl^{-1} each of forward and reverse primers and 1 U *Taq* DNA polymerase.

The PCR reaction conditions were different for all the barcoding loci tested (Table 7). The initial denaturation temperature was ranged between 92˚C to 95˚C at 1 to 5 min for all the barcoding loci tested. The longest denaturation time of 5 min was observed in *ITS*, *atp*F-*atp*H and *psb*K-*psb*I at constant temperature 94˚C. The annealing temperature was ranged between 48 ˚C to 59˚C at 20 to 90 sec for all the loci tested. The lowest annealing time of 20 sec was observed with matK-3F/1R. The final extension time also varied between all the loci and a longest of 10 min was observed in *rbcL* and *ITS* barcoding loci.

4.1.4 Selection of an ideal barcode locus

4.1.4.1 Barcode success in black pepper

PCR amplification success, sequencing efficiency, occurrence of SNP and sequence analysis were considered to assess the efficiency of the barcoding loci. Of the different barcoding loci tested, *psbA-trnH*, *rbcL*, *rpoC1* and *matK* locus were shortlisted for further studies in the Group I, based on the recommendations of CBOL, review of literature studies, the amplification consistency and sequencing success.

The PCR success rate of *psbA-trnH*, *rbcL*, and *rpoC1* were found to be 100% for *Piper* species and adulterants. The *matK* primers (3F /1R and 390F/1326R) failed to give consistent amplification and so were ruled out from further analysis. Sequencing success of *rbcL* and *rpoC1* was found to be 100% where as in case of *psbA-trnH* it was 60% due to the difficulty

Table 7. PCR reaction conditions standardised for the barcoding loci

Loci	PCR reaction condition					
	Initial denaturation (min.)	**Denaturation (sec.)**	**Annealing (sec.)**	**Extension (sec.)**	**Final Extension (min.)**	**Total number of cycles**
matK 3F/1R	94^0C -1	94^0C -30	52^0C -20	72^0C -50	72^0C -5	35
matK390F/1326R	94^0C -1	94^0C -30	48^0C -30	72^0C -30	72^0C -7	26
rbcL	95^0C-4	94^0C -30	55^0C -90	72^0C -90	72^0C -10	35
ITS	94^0C -5	94^0C -30	56^0C -45	72^0C -45	72^0C -10	40
rpoB	94^0C -1	94^0C -30	48^0C -45	72^0C -45	72^0C -5	35
atpF- atpH	94^0C -5	94^0C -30	59^0C -45	72^0C -40	72^0C -5	35
psbK-psbI	94^0C -5	94^0C -30	54^0C -30	72^0C -40	72^0C -5	35
rpoC1	94^0C -1	94^0C -30	50^0C -40	72^0C -40	72^0C -5	40
psbA-trnH	92^0C -1	94^0C -90	52^0C -90	64^0C -90	64^0C -8	35

in aligning the bidirectional reads. The chromatogram showed distinct peaks with low background noise and the peaks were non-overlapping and broad.

4.1.3.2. Identification of the ideal barcoding loci

An important criterion of an ideal barcode is its discriminatory power and the conserved nature within species with sufficient variation among species. Discriminatory power depends mainly on the greater number of variable sites and Single Nucleotide Polymorphisms (SNPs). In case of *rbcL* locus, *P. attenuatum* was discriminated from other *Piper* species by the SNP at position 234. *rpoC1* locus were able to discriminate *P. nigrum* from the adulterants at position 11. The locus *psbA-trnH* discriminated *P. galeatum* from the other *Piper* species *(P. nigrum* and *P. attenuatum)* at position 48, after aligning and trimming the sequences with Bioedit software. MUSCLE (Multiple Sequence Comparison by Log- Expectation) alignment of reference sequences and probable adulterant species was done to detect mismatches and SNPs. The SNPs distinguishing the *Piper* species are given in the (Figure 6a, 6b & 6c.).

The intraspecific and interspecific distances were calculated based on the K2P parameter. The non coding locus *psbA- trnH* exhibited higher inter and intra specific distance than the coding *rbcL* and *rpoC1* loci. But no single locus was able to resolve all the three *Piper* species. So a two locus approach using *rbcL + psbA-trnH* and r*poC1 + psbA-trnH* was adopted. This multi locus approach showed better resolution of species by increasing the species variation. Intra and inter specific divergence for the candidate barcodes were analysed and the distance was calculated for the candidate barcodes singly and in combination (Table 8).

The average intra specific distance of both loci combinations was 0.001 and the average inter specific distances were 0.009 and 0.008, respectively for *rbcL + psbA-trnH* and r*poC1 +*

Table 8. Analysis of intra and inter specific divergence for the candidate barcodes for group I samples.

Distance	**Locus**				
	Black pepper	***rbcL***	***rpoC1***	***psbA-trnH***	***rbcL+psbA-trnH***	***rpoC1+psbA-trnH***
Intra-specific distance	*P. nigrum*	0	0	0	0.001± 0.001	0.001± 0.001
	P. attenuatum	0	0	0.003± 0.003	0.001± 0.001	0.001± 0.001
	P. galeatum	0	0	0	0	0
Inter-specific distance	*P. nigrum* & *P.attenuatum*	0.002± 0.002	0.002± 0.002	0.002± 0.002	0.003± 0.002	0.002± 0.002
	P. nigrum & *P. galeatum*	0	0	0.008± 0.009	0.002± 0.001	0.002± 0.001
	P.attenuatum& *P.galeatum*	0.002± 0.002	0.002± 0.002	0.010± 0.009	0.004± 0.002	0.004± 0.002

psbA-trnH thereby indicating *rbcL + psbA-trnH* combination to be a better two locus barcode than *rpoC1 + psbA-trnH* in resolving the probable adulterants of *Piper* species.

4.1.4 Tracing out the plant based adulterants using DNA barcoding technique.

4.1.4.1 Authentication of reference samples and their adulterants.

The PCR amplification of *rbcL* and *rpoC1* loci yielded 600 bp and 500 bp sized fragments (Figure 7 & 8), respectively in all genuine samples of *P. nigrum, P. attenuatum, P. galeatum* papaya seeds, *C. annuum* and market samples. The locus *psbA-trnH* yielded a 350 bp size fragment in all the *Piper* species and 600 bp sized fragments for *C. annuum* and papaya seed. Amplification of *Piper* species, *C. annuum* and market samples are given Figure 9. The authenticity of all the sequences was cross-checked with the available sequences in the National Centre for Biotechnology Information (NCBI) and the Barcode of Life Data (BOLD) system using BLAST analysis. BLAST searches of the *Piper* species revealed maximum (100%) similarity to the respective sequences available in NCBI for all the three loci tested. This confirmed the authenticity of the reference sequences taken for the study.

4.1.4.2 Adulteration detection in commercial samples.

The sequences generated for the reference samples were used for identifying the adulterants in the commercial samples. All the twelve market samples except two gave 100% sequence similarity towards *P. nigrum* by BLAST analysis. The sequences of market samples did not correspond to the probable adulterants viz., *P. attenuatum, P. galeatum* and papaya seeds. The two distinct market samples viz., P3 and P9 gave 600 bp fragment and 350 bp (Figure 10) for *psbA-trnH* locus and the sequence analysis showed maximum identity to *Capsicum annuum* sequences of NCBI database, implying the probable adulteration of the sample with chilli. BLAST analysis of all the sequences from all the three barcoding loci proved P3 and P9 market samples are adulterated with chilli.

MUSCLE alignment of *psbA-trnH* locus with the sequence alignment between *Piper* species and all their adulterants (Figure11) showed variation among *Piper* and other plant based adulterants. The phylogenetic N-J tree constructed using the *psbA-trnH* locus with K2P parameter containing 1000 boot strap values clustered the P3 and P9 samples with *C. annuum* (Figure 12). All other market samples clustered with *Piper nigrum*.

4.1.5 Validation Studies

4.1.5.1 Cloning and Simulation

To validate the adulteration of the market sample, *P. nigrum* sample (control), simulated sample of black pepper with chilli and the two putative market samples were PCR amplified using *psbA-trnH* primers. The reference sample produced a single band of 600 bp size. The putative adulterant market samples and the simulated samples gave two distinct bands of 600 bp and 350 bp sizes. The specific bands of 600 bp and 350 bp were excised and gel purified. The purified products were cloned to pGEMT cloning vector and transformed to JM109 competent cells by heat shock method.

Approximately 70-100 single blue white colonies were observed in each plate (Figure 13). About 15-20 colonies were sub cultured to new LB agar grid plate and the presence of desired DNA fragment in each white colony was confirmed using colony PCR. Amplification of the white colonies with M13 forward and M13 reverse primers yielded products of ~550bp and ~800bp in all the positive clones of P3, P6 market samples and the simulated sample. Positive recombinant clones of reference sample (*P. nigrum*) yielded products of ~550 bp in size and non-recombinant negative clones yielded a product of ~ 200bp in size. Plasmid DNA was isolated from these selective plates. The plasmid sequencing of the reference samples (control samples) corresponded to *P. nigrum* species. Sequences that

showed maximum sequence identity with other species (adulterants) rather than with *P. nigrum* were analyzed along with the reference sequences by BioEdit and MEGA to detect the SNPs. Multiple sequence alignment (MSA) tool was used for comparing the sequences against the maximum identity sequence. The sequences from simulated and adulterant market samples (P3 & P9) revealed the presence of both *P. nigrum* and *C. annuum* sequences. BLAST searches of the plasmid sequences from the two market samples revealed 100% identity towards *C. annuum* sequences. Out of the twelve market samples taken, two market samples were found to be adulterated with chilli. The sequences of the adulterated market sample are given in (Table 9).

psbA-trnH proved to be best locus in adulteration detection of black pepper market samples. Band level detection of adulteration of the market samples was possible with this locus as pepper yielded a band of 350 bp and chilli yielded one of 600 bp size. This difference in the fragment size was further verified using model blends and clear bands distinguishing black pepper from chilli were observed at 350bp and 600bp, respectively. Simulated samples of varying proportion were amplified to detect the sensitivity of the technique. Adulteration even at very low level (0.5% i.e.1:200 ratio) could be detected with this method (Figure 14). The presence of chilli as low as 1g in 200 g of black pepper powder was detected using this technique.

4.1.5.2 High Performance Liquid Chromatography (HPLC) analysis.

HPLC data supported the adulteration of the two market samples with chilli (Table10). HPLC profile of these market samples and standards are given in Figure15a to 15f. Adulterated market samples P3 & P9 (Figure 15d, 15e) gave two characteristic peaks corresponding to piperine and capsaicin. No adulteration was detected in the negative control (P1) as evident from Figure15c. Chromatographic profile of the simulated sample

(Figure15f) was similar to those reported for adulterated samples. Similar peaks were observed for adulterated market samples and the simulated samples and the peak corresponded to the capsaicin and piperine standards. The chromatographic profiles thus reconfirmed the adulteration of black pepper market samples with chilli.

4.1.6. Construction of reference library and barcode generation

Consensus sequences and contig generation were accomplished by a DNA baser software package. The sequences were aligned using Clustal W, trimmed and edited using BioEdit (ver.7.2.5).

Barcode sequences were generated for all the genuine *Piper* samples and probable adulterants for all the three barcode loci. The sequences generated were deposited in the National Centre for Biotechnology Information (NCBI) and are also available with the Barcode of Life Data (BOLD) system. Sequences were assigned Gen bank accession ID (Table 11).

Table 9. Nucleotide sequences of ***psbA-trnH*** amplicon cloned from the adulterated market samples of black pepper

Market Sample	Nucleotide sequences
P3	CGCGCATGGTGGATTCACAATCCACTGCCTTGATCCACTTGGCTACAT CCGCCCCCTCGCCTACTTACATTCCATTTTTACATTATTTAAATTAGAA AACAAAAGATTCAAGTTCGAATATTTCTCTTCTTTCTTATTTGAATGAT ATTATTATTTCAAAGATGAGAATATGAATCAAAGATCAGAATCTGAAG TAAAAATTAATTTTTTTTTTGAAATGAAATAAAAAAGATATAGTAACA TTAGCAAGAAGAGGAACAAGTTATATTTCTATAATTTTCAATAAATAC ATACAAAATGAAAATAGAATACTCAATCCTGAATAAATGATAAATGC ATGCAAATATCCTCTCTTTCTTTTTCTATAATGTAAACAAAAAAGTATA TGTAAGTAAAATACTAGTAAATTACTAAATAAAATAAAAAGAAAAAA AGAAAGGAGCAATAGCACCCTCTTGATAAAACAAGAAAATGATTATT GCTCCTTTCTTTTCAAAACCTCCTATAGACTAGACTGGGATCTTATCCA TTTGTAGATGGAGCTTCGATAGCAGCTAAGTCTAGAGGGAAGTTATGA GCATTACGTTCATGCATAAC
P9	CGCGCATGGTGGATTCACAATCCACTGCCTTGATCCACTTGGCTACAT CCGCCCCCTCGCCTACTTACATTCCATTTTTACATTATTTAAATTAGAA AACAAAAGATTCAAGTTCGAATATTTCTCTTCTTTCTTATTTGAATGAT ATTATTATTTCAAAGATAAGAATATGAATCAAAGATCAGAATCTGAAG TAAAAATTAATTTTTTTTTTGAAATGAAATAAAAAAGATATAGTAACA TTAGCAAGAAGAGGAACAAGTTATATTTCTATAATTTTCAATAAATAC ATACAAAATGAAAATAGAATACTCAATCCTGAATAAATGATAAATGC ATGCAAATATCCTCTCTTTCTTTTTCTATAATGTAAACAAAAAAGTATA TGTAAGTAAAATACTAGTAAATTACTAAATAAAATAAAAAGAAAAAA AGAAAGGAGCAATAGCACCCTCTTGATAAAACAAGAAAATGATTATT GCTCCTTTCTTTTCAAAACCTCCTATAGACTAGACTGGGATCTTATCCA TTTGTAGATGGAGCTTCGATAGCAGCTAAGTCTAGAGGGAAGTTATGA GCATTACGTTCATGCATAAC

Table 10. Retention time and area of piperine and capsaicin in black pepper powders vis-à-vis standards.

Sample	Retention time	Area %
Piperine (standard)	11.680	100
Capsaicin (standard)	11.136	100
Sample 1-Market(negative control)	11.563	68.77
Sample 2-(Market sample P3)	11.296 11.712	33.92 33.16
Sample 3-(Market sample P9)	11.179 11.499	1.60 39.10
Sample 4-Simulated sample(1:100)	11.179 11.648	1.02 46.17

Table 11 *Piper* sequences submitted to NCBI /GenBank

Species	Locus		
	rbcL	***rpoC1***	***psbA-trnH***
	Piper nigrum		
IC.No. 316834	KF278651	KF278650	KF278656
IC.No. 316857	KF278652	KF365253	KF278657
IC.No. 316833	KF278653	KF365254	KF416315
IC.No. 316835	KF278654	KF365255	KF278658
IC.No. 316849	KF278655	KF365256	KF416316
	Piper attenuatum		
IC.No. 315469	KF365245	KF365249	KF676708
IC.No. 314661	KF365246	KF365250	KF676709
IC.No. 314634	KF365247	KF365251	KF676710
IC.No. 317077	KF365248	KF365252	KF676711
IC.No. 316951	KF676713	KF676714	KF676712
	Piper galeatum		
IC.No. 310347	KF676715	KF676720	KF676703
IC.No. 316980	KF676716	KF676721	KF676704
IC.No. 316904	KF676717	KF676722	KF676705
IC.No. 316815	KF676718	KF676723	KF676706
IC.No. 316984	KF676719	KF676724	KF676707

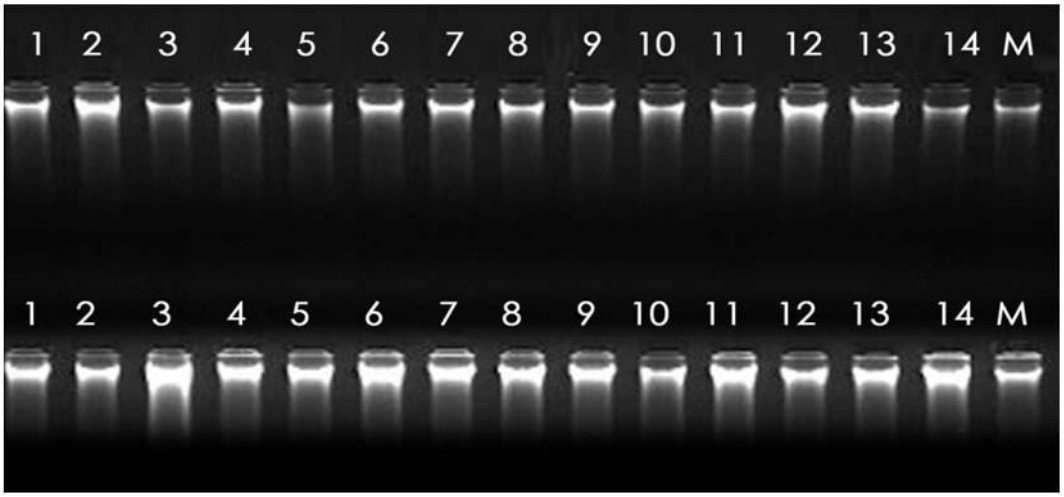


Figure 5. Total genomic DNA isolated from black pepper, probable adulterants and market samples. (**A**- Lanes1 to 5- *P. nigrum* accessions, lanes 6 to 10- *P.attenuatum* accessions, lanes11 to 14- *P. galeatum* accessions, lane M- marker (Human genomic DNA, Genei). **B**- Lane-1 *C.annuum*, lane-2 pappaya seeds, lanes 3 to 14 - market samples (P1-P12), lane M- marker (Human genomic DNA, Genei)

Figure 6a: MUSCLE alignment of ***rbcL*** **locus** showing sequence variation among *Piper sp.*

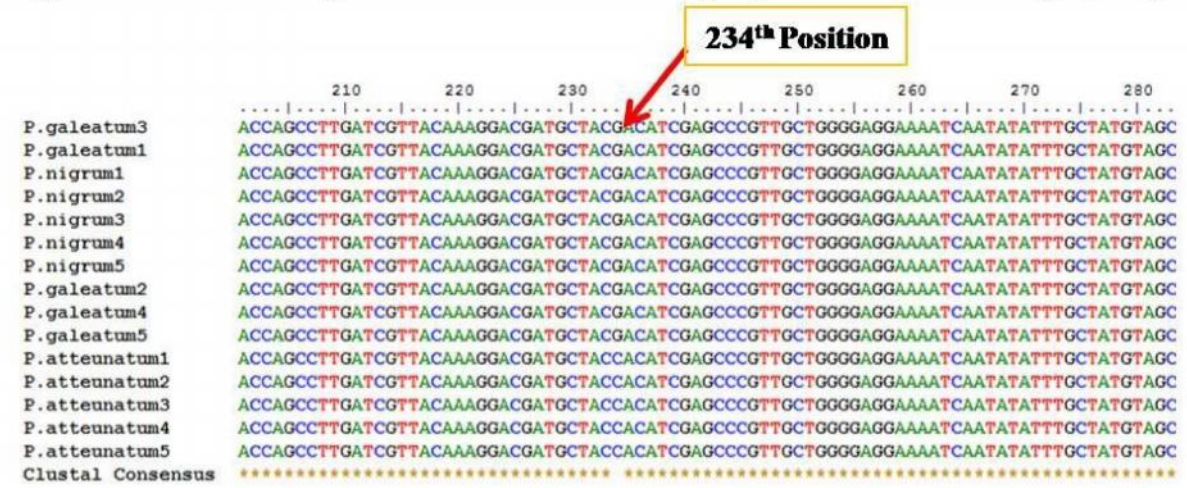


Figure 6b: MUSCLE alignment of ***rpoC1*** **locus** showing sequence variation among *Piper sp.*

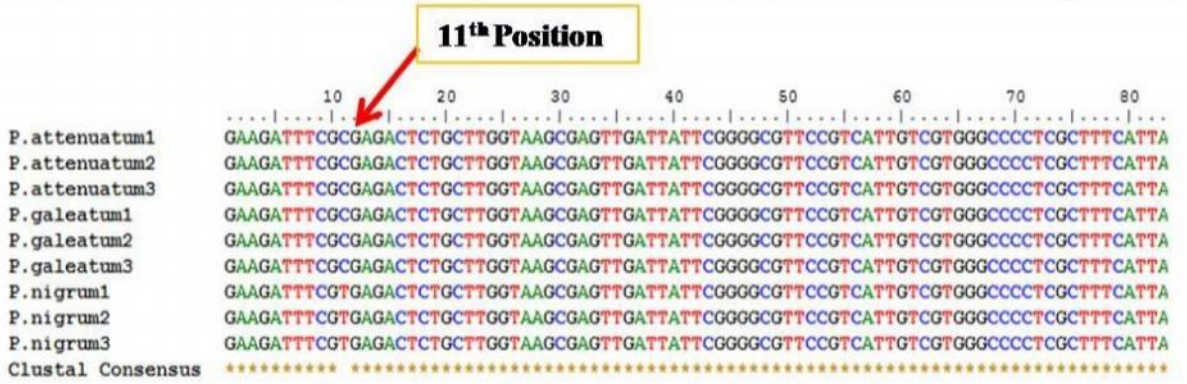
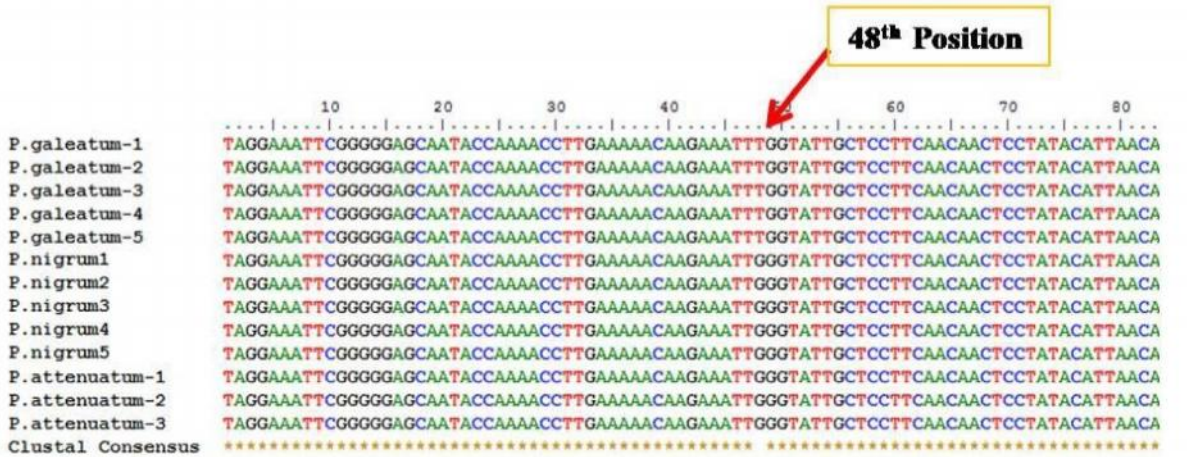


Figure 6c: MUSCLE alignment of ***psbA-trnH*** **locus** showing sequence variation in *Piper sp.*



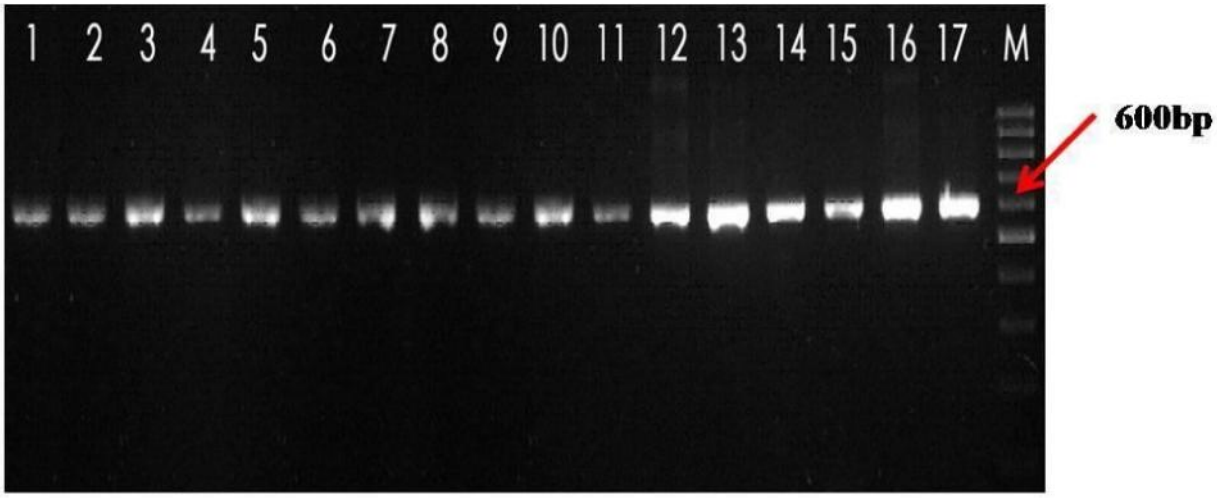


Figure 7. Amplification of *rbcL* locus in Group I samples (Lane1- *P. nigrum*, lane2-*P.attenuatum*, lane-3 *P. galeatum*, lane-4 pappaya seed, lane-5 *C.annuum*, lanes 6 to 17- Market samples (P1 to P12), lane M- Marker (100 bp ladder).

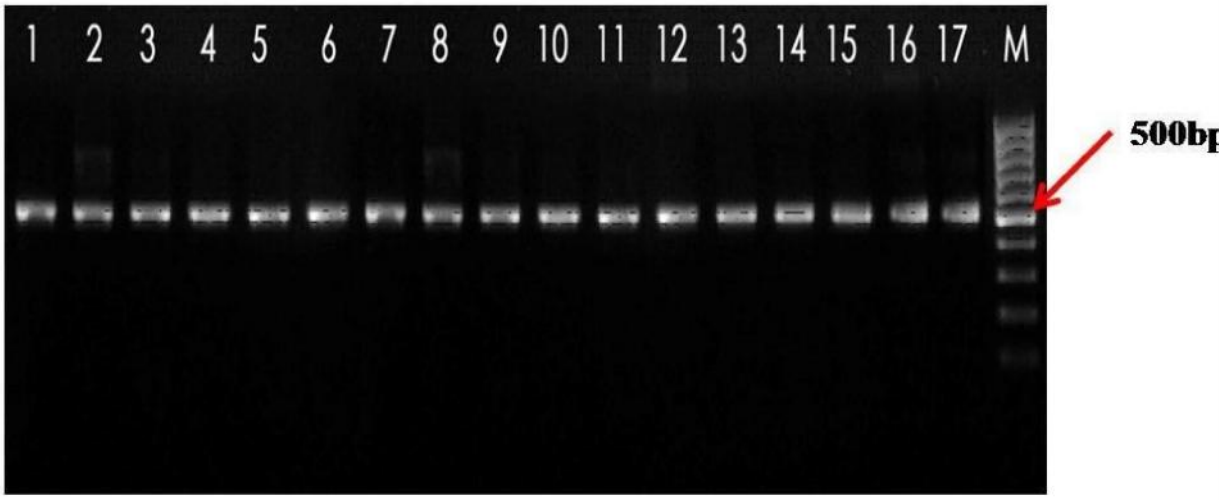


Figure 8. Amplification of *rpoC1* locus in Group I samples (Lane-1 *P. nigrum*, lane-2 *P.attenuatum*, lane-3 *P. galeatum*, lane -4 pappaya seeds, lane-5*C.annuum*, lanes 6 to17 market samples (P1-P12), lane M- marker (100 bp ladder).

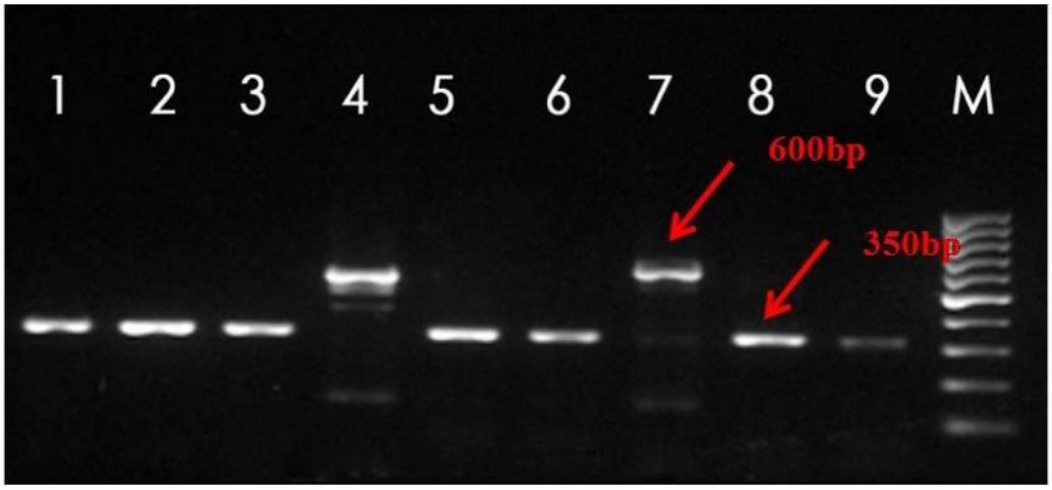


Figure 9. Amplification of ***psbA-trnH*** **locus** in Group I samples .(Lane1- *P. nigrum*, lane 2-*P.attenuatum*, lane 3- *P. galeatum*, lane 4 -*Capsicum annuum*, lane 5-P1, lane 6 -P2, lane 7-P3, lane 8- P4, lane 9-P5, lane M- marker 100bp ladder)

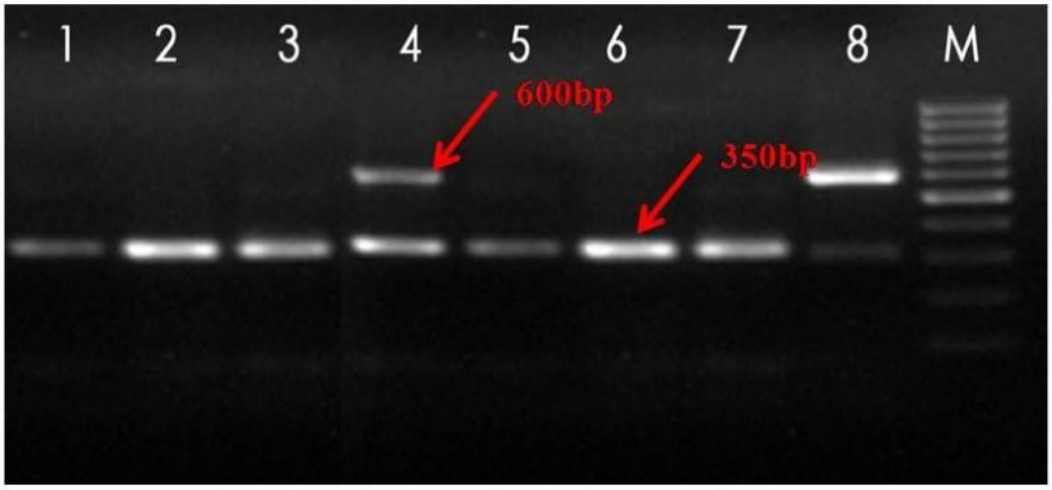


Figure 10. Amplification of *psbA-trnH* locus in market samples of Group I (Lane 1-P6, lane 2- P7, lane 3- P8, lane 4- P9, lane 5 -P10, lane 6- P11, lane-7-P12, lane 8-P3, lane M-marker (100bp ladder).

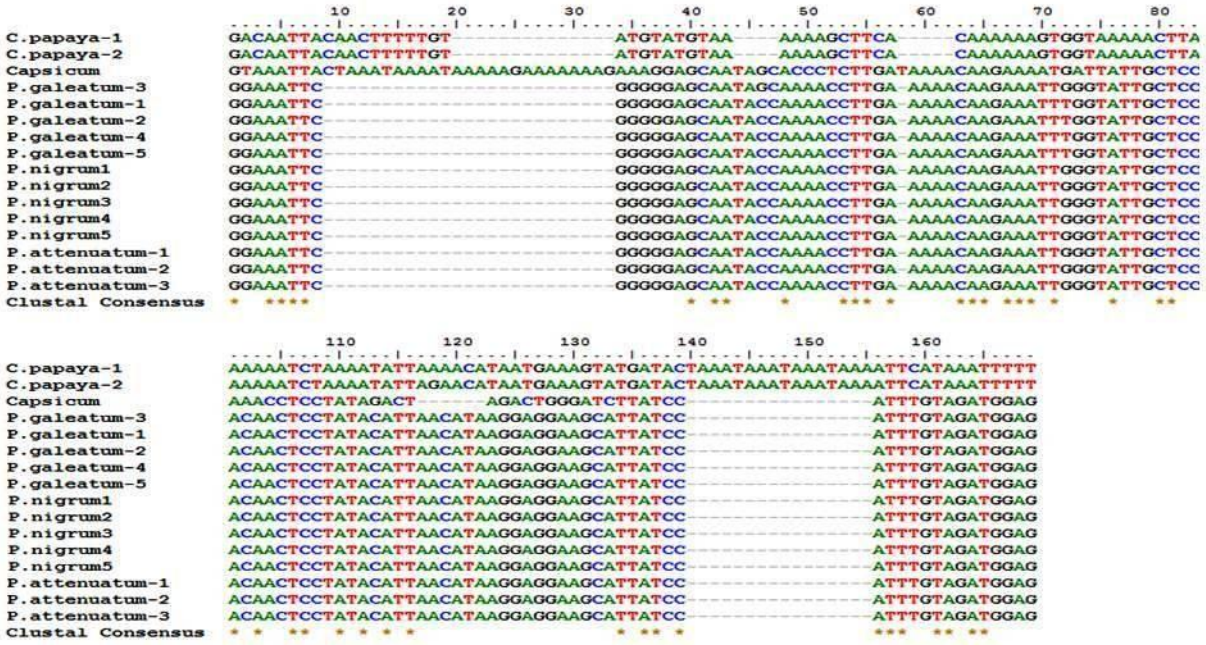


Figure 11: MUSCLE alignment of ***psbA-trnH*** sequences depicting the indels and polymorphic sites in *Piper* species and their adulterants.

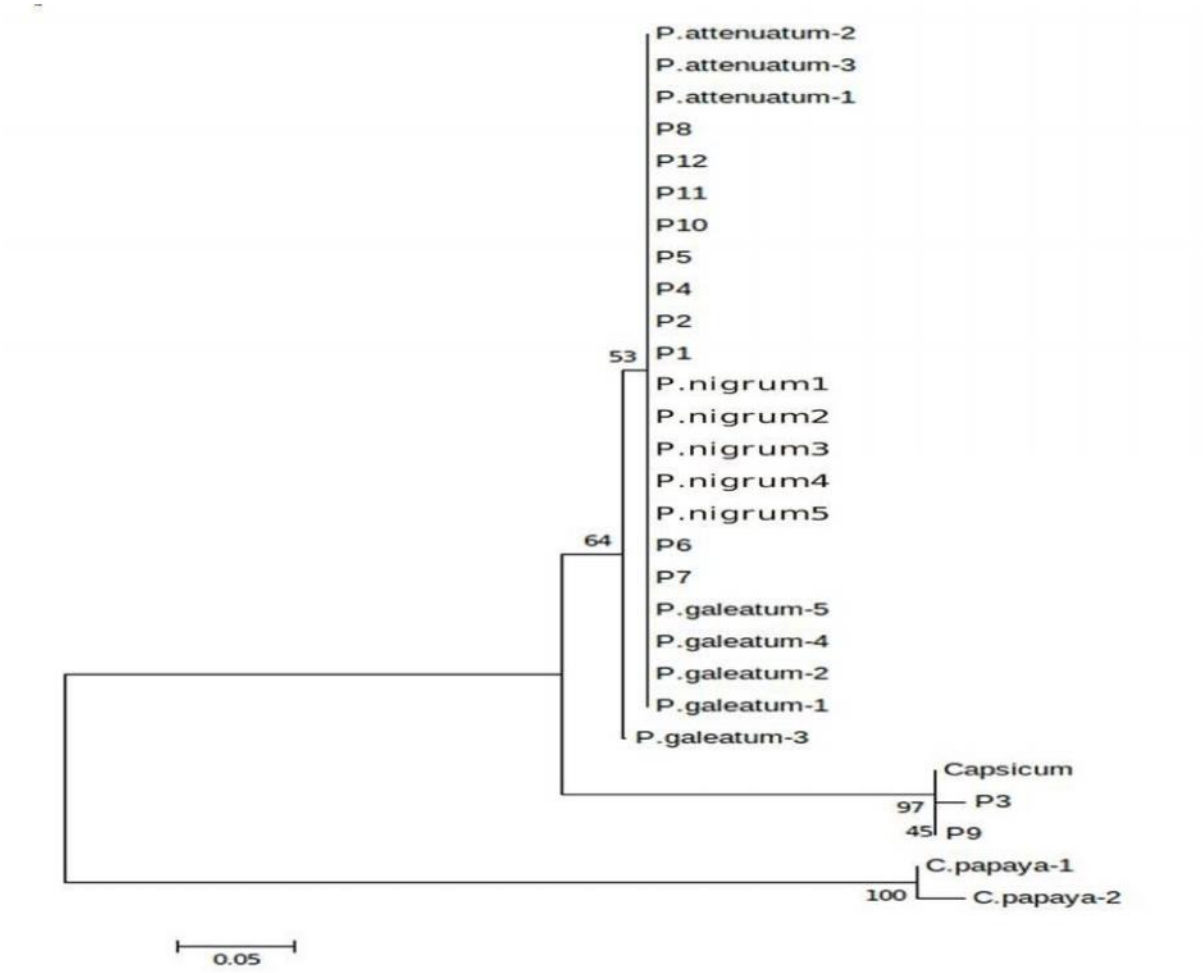


Figure 12. The N-J tree constructed from *psbA-trnH* sequences of Group I samples based on the K-2-P distance

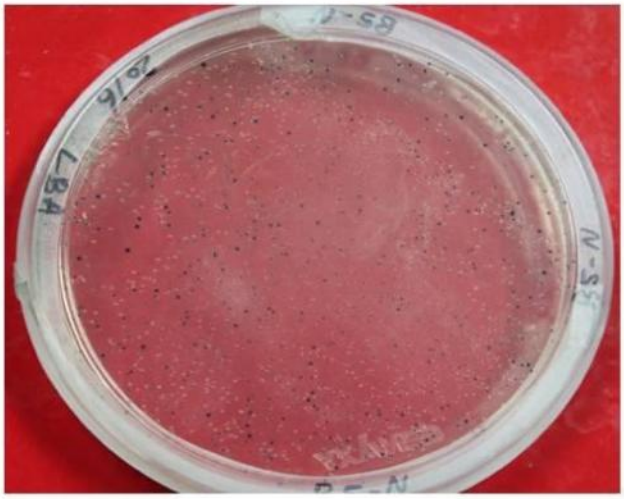


Figure 13. LB plates showing blue/white colonies after transformation of Group I samples

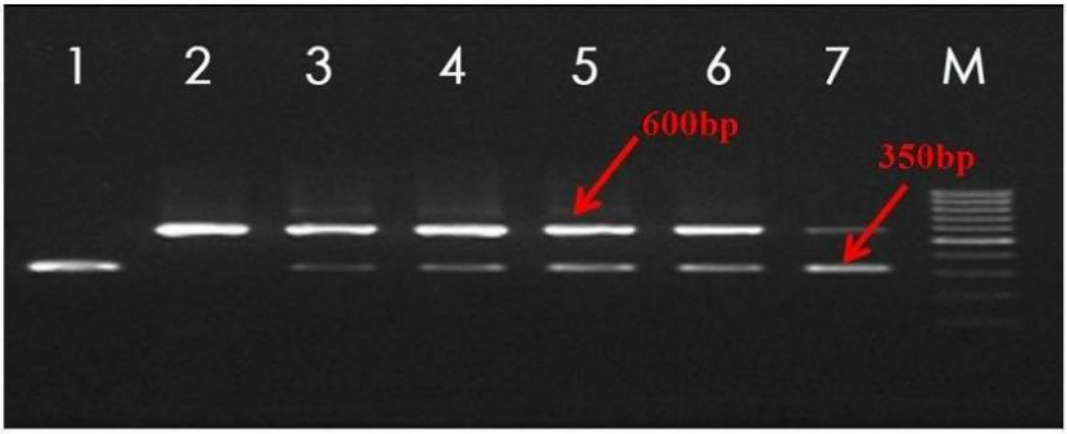


Figure 14. Amplification of adulterant specific bands in simulated samples and controls with *psbA-trnH* locus.(Lane 1- *Piper nigrum* (negative control), lane 2-*Capsicum annum* (positive control) lane- 3simulated sample (10 % adulteration), lane -4 simulated sample (4% adulteration), lane -5 simulated sample (2% adulteration), lane -6 simulated sample (1% adulteration), lane -7 simulated sample (0.5% adulteration), lane M-marker (100bp ladder).

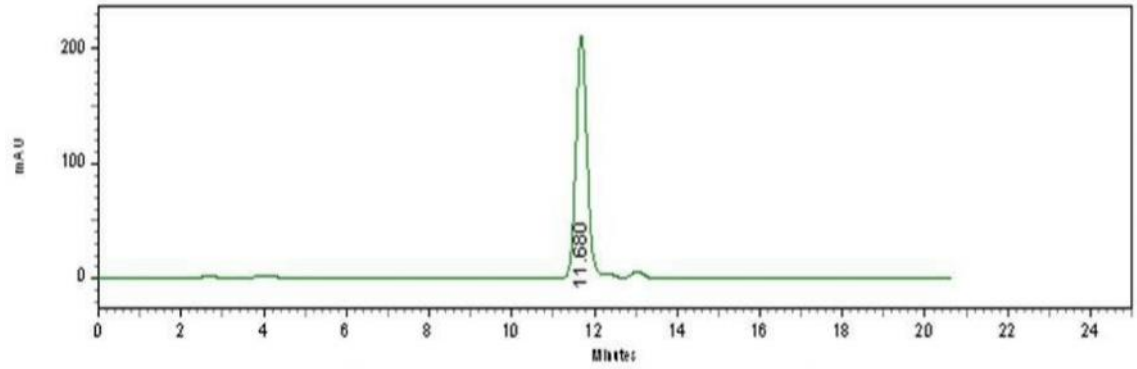


Figure 15a. Chromatogram of piperine standard (60ppm)

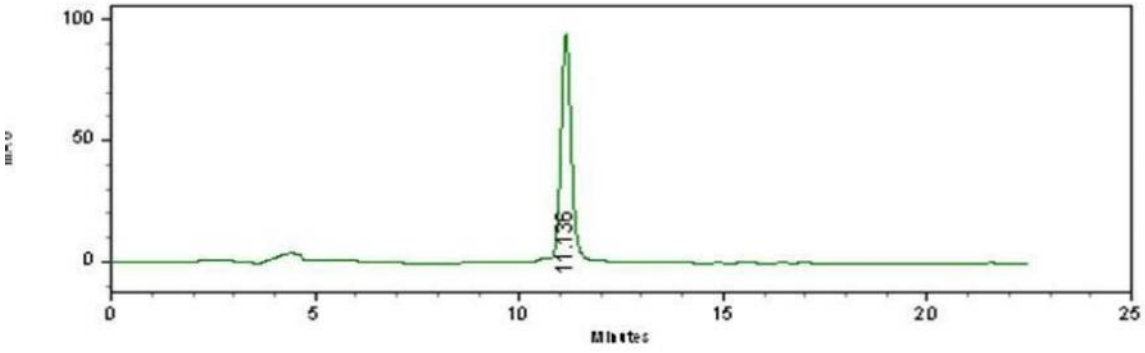


Figure 15b . Chromatogram of capsaicin standard (100 ppm)

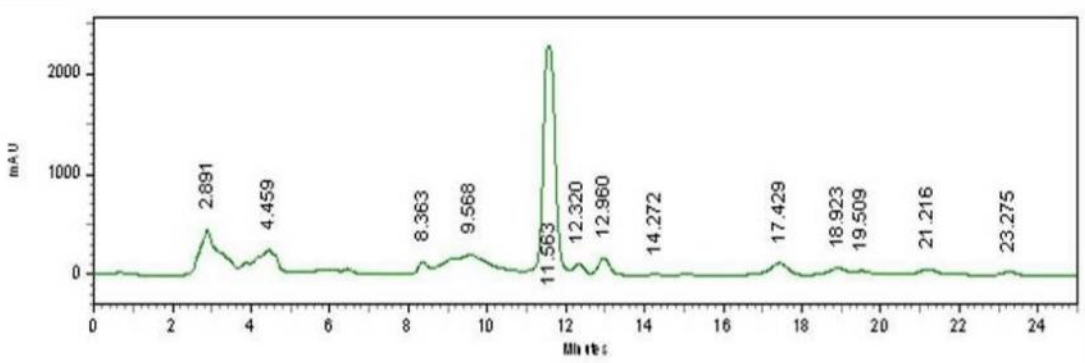


Figure 15c. Chromatogram of negative control

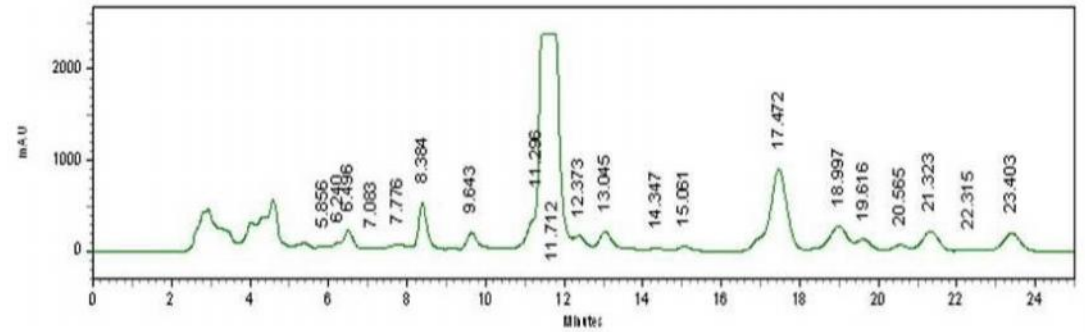


Figure15d. Chromatogram of adulterated sample (MS-3)

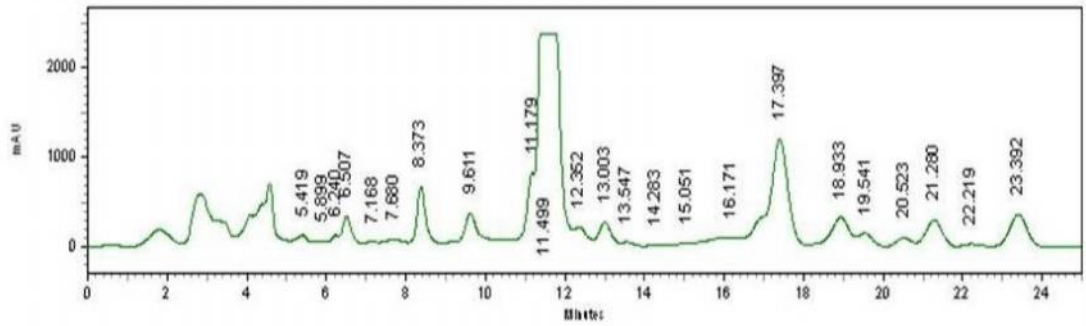


Figure 15e.Chromatogram of adulterated sample (MS-9)

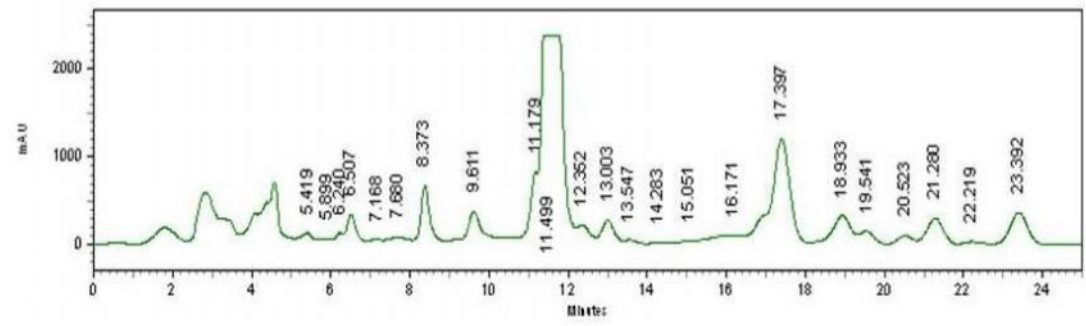


Figure 15f. Chromatogram of the simulated sample (1:100).

4.2 Group II. Turmeric

4.2.1 Sample collection

Collection of reference species of *C. longa* and *C. zedoaria* was from the Germplasm Repository of ICAR-IISR which served as the reservoir of various *Curcuma* species collected from varied geographical regions and was rich in diversity and variability. This was an added advantage for SNP analysis.

4.2.2 DNA isolation from turmeric, their probable adulterants and market samples

Total genomic DNA from *C. longa* and their probable adulterants viz., *C. zedoaria* and cassava starch was isolated using the protocol of Remya *et al*., (2004). The same protocol was used in isolating DNA from the simulated samples also. The absorbance ratio at A260/A280 was found to be between 1.70 - 1.80. This ratio indicated insignificant amounts of contaminating proteins and polysaccharides. The DNA yield was found to be between 10- 12 µg per gram of dried tissue. Market samples showed reduced yield of DNA, 6-10 µg per gram of dried tissue. DNA profiling using agarose gels showed conspicuous bands of DNA, with minimum shearing for the DNA isolated from turmeric, their probable adulterants and market samples (Figure 16).

4.2.3 Standardization of PCR parameters

The standardised PCR reaction components were same as of group I (Section 4.1.2). PCR conditions standardised for the barcoding loci were given in Table 7.

4.2.4 Selection of an ideal barcode locus for turmeric and their adulterants.

4.2.4.1 Barcode success in turmeric

The barcoding loci *viz*., *ITS*, *rbcL*, *psbA-trnH* and *matK* were selected for the Group II. Amplification and sequencing efficiency of *rbcL* and *ITS* were found to be 100% and yielded

a sequence length of 600 bp and 500 bp, respectively for all the samples analyzed (Figure 17a & 17b; 18a & 18b). The sequences generated for these two loci were of good quality with non-overlapping peaks and less back ground noise. The sequences were aligned bi directionally and the contigs were generated using DNA baser software. PCR amplification success with two different primer sets of *matK* was 0% despite using different concentrations of the DNA and less stringent conditions, proving the locus not to be suitable candidate for DNA barcoding for *Curcuma* species. Amplification and sequencing of *psbA-trnH* in turmeric was also found to be difficult. Sequence assemblies of the bi directional reads were difficult with premature termination of the sequence reads. The locus *psbA-trnH* did not show consistent amplification for the standardized conditions.

4.2.4.2 Identification of the ideal barcode loci in turmeric group

Considering the discriminatory power of the two loci to differentiate between the genuine *C. longa* and *C. zedoaria*, *ITS* proved to be the best locus. *ITS* locus showed greater variability in the sequences between *C. longa* and their adulterant. MUSCLE alignment of the *ITS* sequences showed greater number of variable sites and SNPs (Figure.19) by discriminating between *C. longa* and its common adulterant, *C. zedoaria* whereas *rbcL* locus did not show variability in discriminating the two species of *Curcuma.* Inter and intra specific distance was calculated using the K2P parameter using MEGA5 (Table 12). The locus *ITS* exhibited higher inter and intra specific distance than the coding *rbcL*. *rbcL* locus was unable to resolve *C. longa* from its adulterant species. The intra specific distance calculated for *rbcL* locus was zero for the two *Curcuma* species whereas for *ITS* locus it was 0.016± 0.005 and 0.026±0.005 for *C. longa* and *C. zedoaria*, respectively. The inerspecific distance for the locus *rbcL* was zero and for *ITS* was 0.677± 0.066, clearly stating the *rbcL* not an ideal locus to discriminate the *Curcuma* species. The interspecific distance calculated for *ITS* was greater than its intra

specific distance, which is an important criteria for barcode selection. Thus *ITS* proved to be a better locus in differentiating *C. longa* from its adulterant species.

Table 12. Analysis of intra and inter specific divergence for the candidate barcodes of Group II Turmeric

Distance	**Loci**		
	Turmeric	***rbcL***	***ITS***
Intraspecific distance	*C. longa*	0	0.016± 0.005
	C. zedoaria	0	0.026±0.005
Interspecific distance	*C. longa* & *C. zedoaria*	0	0.677± 0.066

4.2.5 Tracing out the plant based adulterants from market samples of turmeric

4.2.5.1 Authentication of reference samples and their adulterants

PCR amplification of reference samples yielded bands of 600bp for *rbcL* and 500bp for *ITS* locus, respectively. The species specific barcodes generated using these two loci were authenticated by sequence comparison and BLAST analysis with the NCBI database. The BLAST analysis of the sequences from the accessions of *C. longa* and *C. zedoaria* yielded 100% identity with the respective *C. longa* and *C. zedoaria* sequences of NCBI database, confirming the authenticity of the reference species.

4.2.5.2 Adulteration detection in commercial samples of turmeric powder

The sequences of the reference sample and the NCBI database sequences were used for authenticating the commercial market samples. Band level discrimination was not possible in

case of turmeric group since both the loci could not discriminate the genuine *C. longa* with the probable adulterant species at the band level.

Out of ten market samples taken, four was found to be adulterated. Sequence analysis of T5 and T8 revealed the presence of cassava starch, a probable adulterant. Maximum identity of market sample T4 was with *C. zedoaria* sequences. Presence of fillers like *Triticum aestivum* L. (wheat) and *Hordeum vulgare* L. (barley) was detected in sequence of the market sample, T7. The sequence analysis of all the other market samples except these four gave 100% identity to *C. longa* sequences for both the loci. The presence of these adulterants in the four market samples viz., T4, T5, T7 and T8 were reconfirmed by cloning and simulation studies.

The N-J tree (Figure 20) constructed on the basis of K2P with 1000 boot strap values clustered the T5 and T8 samples with the cassava sample while the market sample T4 had maximum affinity with the *C. zedoaria* samples. Market sample T7 which contained fillers grouped distinctly. All the other market samples clustered along with *C. longa* reference sequences. The adulteration of market samples could be clearly visualised in the phylogenetic tree constructed using MEGA software.

4.2.6 Validation of the results

4.2.6.1 Cloning and simulation studies

Detection of adulteration in market samples of turmeric was not possible by the band length comparison.The putative adulterated market samples, standard reference sample and simulated sample were PCR amplified using *ITS* forward and reverse primers and the products were gel eluted. Eluted products were purified and transformed to pGEMT cloning vector using JM109 competent cells by heat shock method.

Approximately 60-100 single blue white colonies were observed in each plate (Figure. 21). Recombinant white colonies were picked and sub cultured. Colony PCR yielded products of ~700 bp in all the positive clones of T4, T5, T7 and T8 market samples and the simulated samples (*C. longa : C. zedoaria* and *C. longa*: cassava), respectively. Positive recombinant clones of reference sample yielded products of the same size as that of the putative adulterants and simulated sample. Non-recombinant negative clones yielded a product of ~200 bp in size.

Plasmid DNA was isolated and sequenced bi directionally. BLAST analysis of the reference sample gave maximum identity (100%) to the *C. longa* sequences of NCBI. Analysis of the sequences from simulated sample (*C. longa*: *C. zedoaria*) corresponded to *C. longa* and *C. zedoaria* sequences, respectively. Out of the 10 different plasmids taken from simulated sample for sequencing, 6 sequences showed 100% identity towards *C. longa* and the 4 sequences corresponded to *C. zedoaria.* Analysis of the sequences from simulated sample of *C. longa* and cassava corresponded to *C. longa* and cassava sequences, respectively. Out of 10 different plasmids taken from simulated sample for sequencing, 7 sequences showed 100% identity towards *C. longa* and the 3 sequences corresponded to cassava.

The market samples T5 and T8 showed sequences that corresponded to *C. longa* and cassava. Market sample T4 showed sequence identity towards *C. longa* and *C. zedoaria* while the T7 sequences corresponded to wheat, rye and barley along with *C. longa.*Out of the ten market samples studied, four market samples were found to be adulterated. The sequences of the adulterated market sample are given in Table 13. This confirms the adulteration of market samples of turmeric with *C. zedoaria*, cassava starch and other fillers.

Table 13. Nucleotide sequences of *ITS* amplicon cloned from the adulterated market samples of turmeric powder

Market Sample	Nucleotide sequences
T4	CCGCAGAACGTTCTGAGTCTTTGACGCAGTTGTGCCCGAGGCCTTGTG GTCGAGGGCACGCCTGCTTGGGTGTCATGACATCGTCGCTTTTGCTCC ATGCTTCGTCGGCATTGAGCGCGGAAGTTGGCCCCGTGTGCCCTCGGG CACAGTCGGTCGAAGAAGTGGGTAGTCGGTAATCGTCGAGCACGATG GACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGTTT TGGGATGAGTCCTCCAGAGACCCTGTGTGATGATTGCGGAGTCACGTG AAAGCGCCCCGTCAATCATTTGCGGCCCCAAGTCAGGCGAGACCACCC GCCGAGTTTAAGCATATAAATAAGCGGAGGAGGAGAAACTTACGAGG ATTCCCCTAGTAACGGCGAGTGAACCGGGATCAGCCCAGCTTGAGAAT TTGACGGTTGAATCCTTTTCGAATTGTAGTTGAAAAAAAACCTATACA TCATCAA
T5	TATGCGATACTGGTGTGAATTGCAGAATCCCGCGAACCATCGAGTTTT TGAACGCAAGTTGCGCCCGAAGCCATCCGGCCGAGGGCACGTCTGCCT GGGTGTCACGCAACCGTCGCCTCCAACCCCTTCGCCCCGTGGCGGGGG GCGCGGGGGCGGACGTTGGCCTCCCGTGTGCAGCGCGCACGCGGCTG GCCCAAAAGCAGAGTCCTCGGCGGCGATCGCCACGGCTATCGGTGGTT GGAAGACCCTCGGACACGGCCGTGGGCGAACGTCTGCCGAACGGGAC CCCGAGACCCCCGAGCGTTCCCAACGGAATGCTCCGACCGCGACCCCA GGTCAGGCGGGAACACCCGCTGAGTTTAAGCATATCAATAAGCGGAG GAAAAGAAACTTACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGG AAGAGCCCAGCTTGAGAATCGGGCGCCCTCGGCGTCCGAATTGTAGTC TGGAAAAGCGTCA
T7	GTCTTTGACGCAAGTTGCGCCCGAGGCCACTCGGCCGAGGGCACGCC TGCCTGGGCGTCACGCCAAAACACGCTCCCAACCACCCTCATCGGGAT TCGGGATGCGGCATCTGGTCCCTCGTCTCTCAAGGGACGGTGGACCGA AGATTGGGCTGCCGGCGTACCGCGCCGGACACAGCGCATGGTGGGCG TCTTCGCTTTATCAATGCAGTGCATCCGGCGCGCAGCTGGCATTATGG

	CCTTTGAACGACCCAACAAACGAAGCGCACGTCGCTTCGACCGCGAC CCCAGGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATAAATAAGC GGAGGAGAAGAAACTTACAAGGATTCCCCTAGTAACGGCGAGCGAAC CGGGAGCAGCCCAGCTGA
T8	GCAGTTGTGCCCGAGGCCCTTGGGGTGAAGGCACGTCTGCCTGGGTGT CACTGAACCGTCCCCTTTTGCTCCTTGCTTCCGCGGCGTTGGGCGCGGA GGTTGGCCTTGCCCTCCCGGGTGCAGCGCGCGTGCGTCTGGCACAAAA CCAGAGTCCTCGGCGGCGATCGGCGAGGGTATCGGTGGTTGGAAATCC TTCGGACACGGCCGTGGGCGAACGTCTGCCGAACGGGAACCCGAGAC CCCCGAGCGTTCCCAACGGAATGCTCCGACCGCGACCCCAGGTCAGGC GGGAACACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAA ACTTACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAGAGCCC AGCTTGAGAATCGGGCGCCCTCGGCGTCCGAATTGTAGTC

4.2.7. Construction of reference library and barcode generation

The sequences generated by amplifying the barcoding loci viz., *ITS* and *rbcL* were submitted with the NCBI. Barcodes were generated for all the reference sequence of *C. longa* and *C. zedoaria* and the sequences were assigned Gen bank accession ID (Table 14).

Table 14 *Curcuma* sequences submitted to NCBI

Species	Locus	
	rbcL	***ITS***
C. longa		
IC.No. 143	KM226968	KM226973
IC.No. 119	KM226969	KM226974
IC.No. 126	KM226970	KM226975
IC.No. 360	KM226971	KM226976
IC.No. 361	KM226972	KM226977
C. zedoaria		
IC.No. 760	KM977977	KU936065
IC.No. 465	KM977978	KU936066
IC.No. 1517	KM977979	KU936067
IC.No. 765	KM977980	KU936068

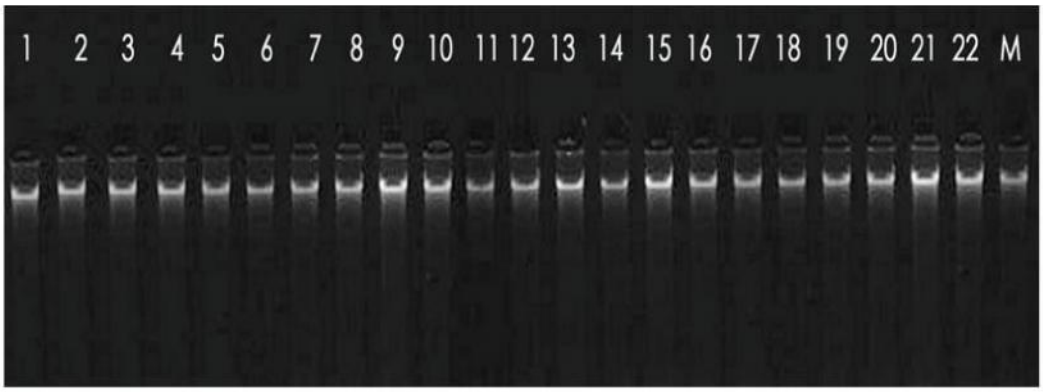


Figure 16. Total genomic DNA isolated from turmeric, probable adulterants and market samples (Lanes 1 to 5 *C. longa* accessions, lanes 6 to 9 *C. zedoaria* accessions, lane10 to 12 cassava starch, lane 13 to 22 market samples (T1 to T10), lane M-marker (Human genomic DNA, Genei).

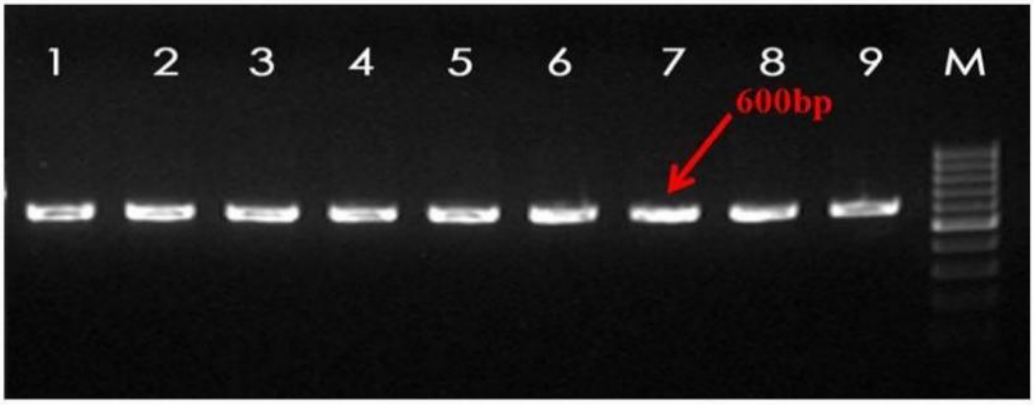


Figure 17a. Amplification of *rbcL* locus in *C. longa* and *C. zedoaria*. (Lanes 1 to 5-*C. longa* accessions, lanes 6 to 9- C. *zedoaria* accessions, lane M- 100bp ladder.

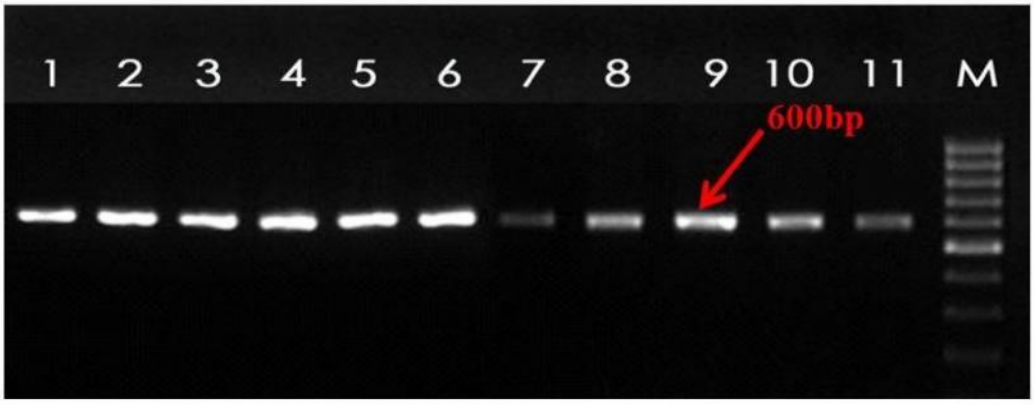


Figure 17b. Amplification of *rbcL* locus in cassava starch and market samples of turmeric. (Lane 1- cassava starch, lanes 2 to 11 market sample (P1 to P10), Lane M-marker (100bp ladder).

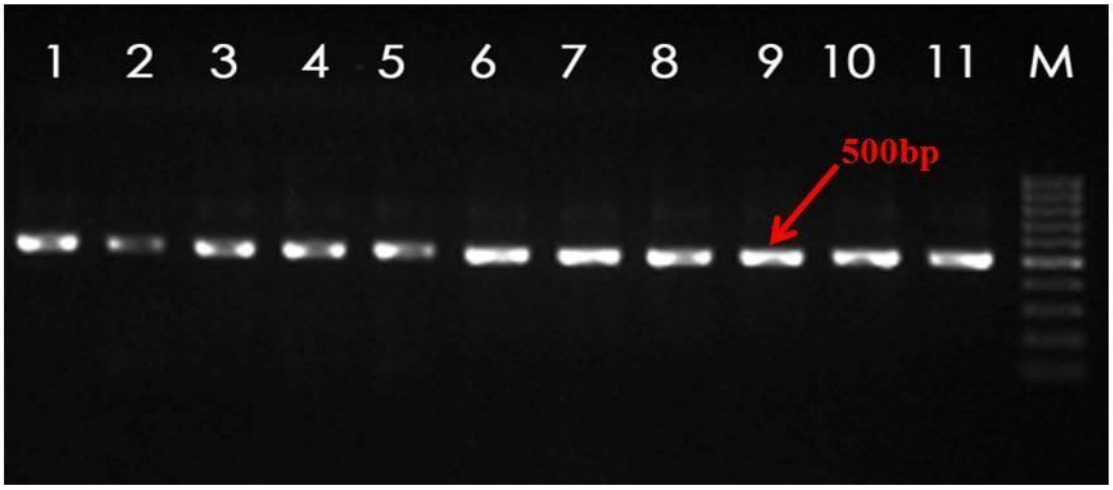


Figure 18a. Amplification of *ITS* locus in *C. longa*, *C. zedoaria* and cassava starch. Lanes 1- 5 *C. longa* accessions, lanes 6- 9 C. *zedoaria* accessions, lanes 10-11 Cassava starch, lane M- marker (100bp ladder).

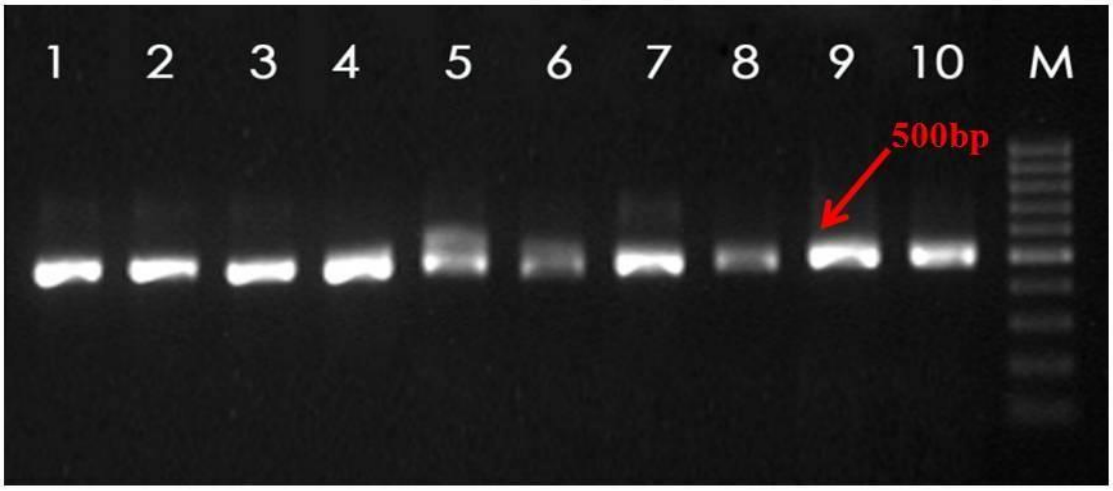


Figure 18b. Amplification of *ITS* locus in market samples of turmeric.(Lanes 1-10 market samples (P1-P10), lane M- marker (100bp ladder).

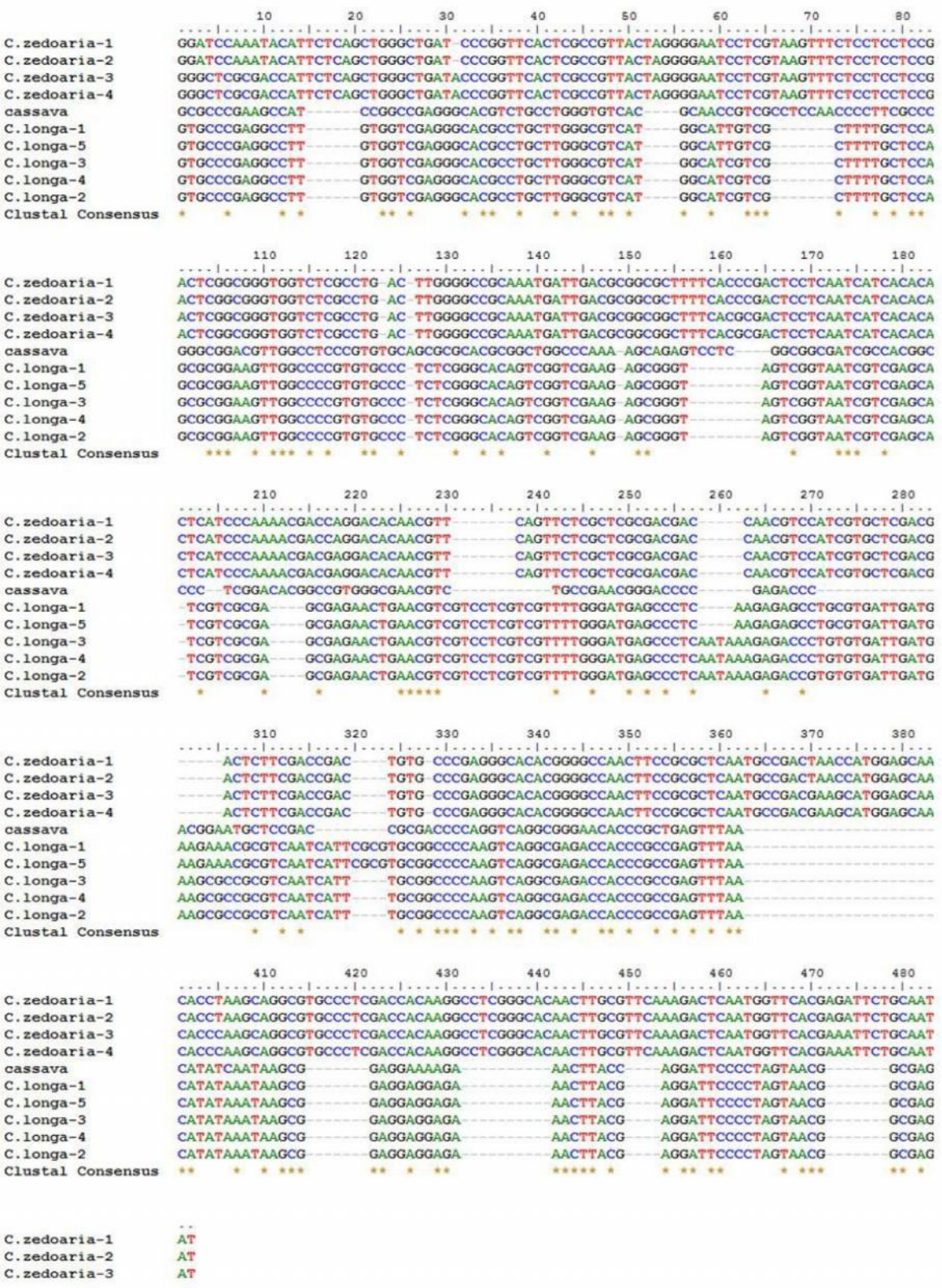


Figure 19. MUSCLE alignment of *ITS* sequences depicting the indels and polymorphic sites in *C. longa* and their adulterants.

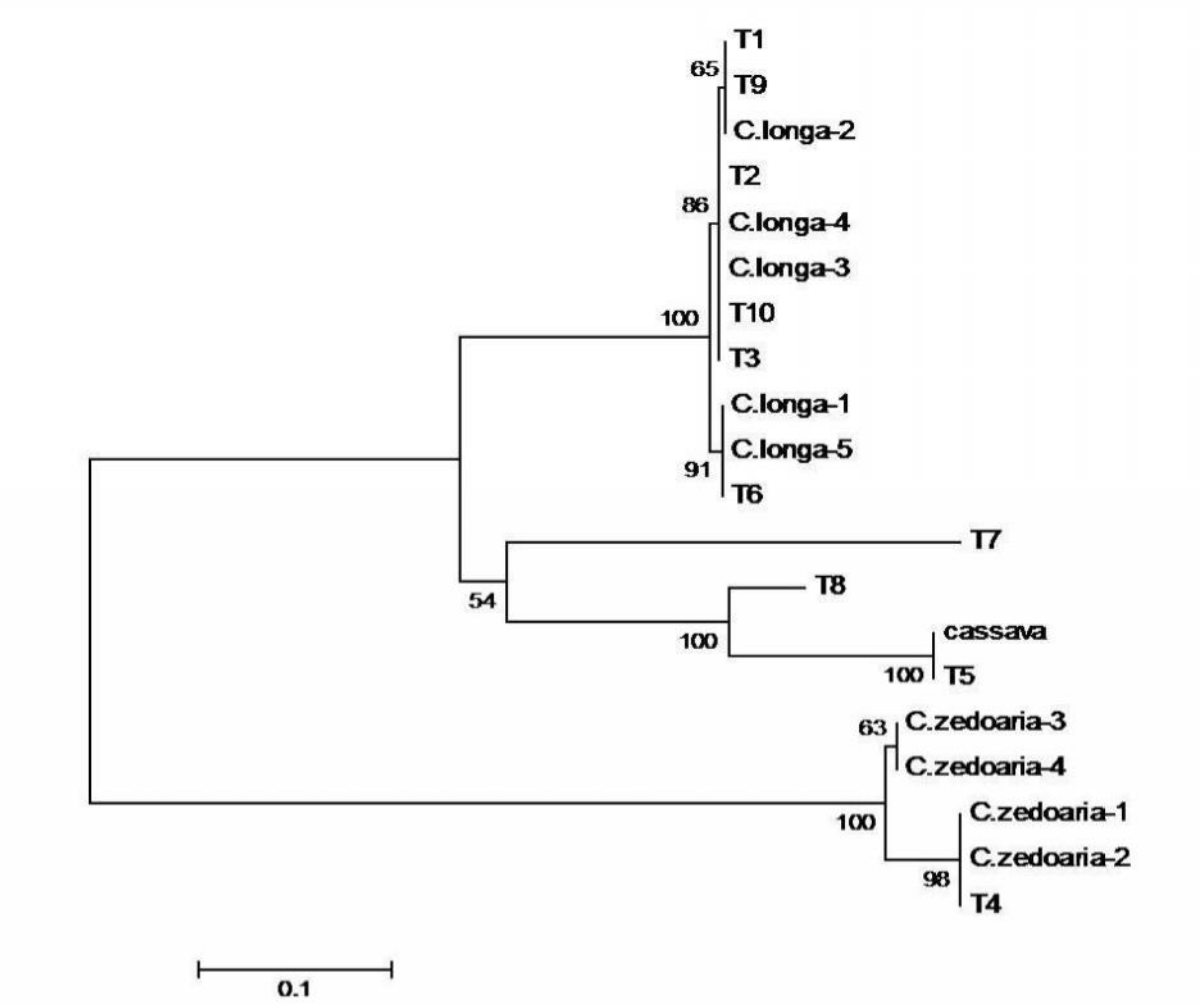


Figure 20. The N-J tree constructed from *ITS* sequences of group II samples based on the K-2-P distance

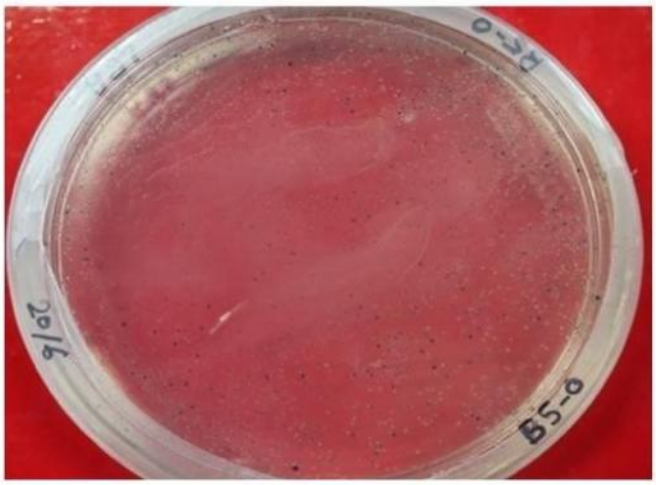


Figure 21. LB plates showing blue/white colonies after transformation of Group II samples

4.3 Group III. Chilli

4.3.1 Sample collection

Collection of reference species of chilli was from Kerala Agricultural University. Four different varieties of chilli were used for the study.

4.3.2 Genomic DNA isolation from chilli, their probable adulterants and market samples

Total genomic DNA from chilli, the probable adulterant samples and simulated samples was isolated using the protocol of Dhanya *et al*., 2008. The ratio of absorbance at A260/A280 was found to be between 1.73 - 1.90. The ratio indicated good quality DNA. The total yield of DNA was found to be between 5-15µ g per gram of dried tissue. The genomic DNA isolated was of high quality and showed good amplification success (Figure 22).

4.3.3 Standardization of PCR parameters

The standardised PCR reactions components were same as of group I (Section 4.1.2). PCR conditions standardised for the barcoding loci were given in Table 7.

4.3.4 Selection of an ideal barcode locus for chilli and their plant based adulterants.

4.3.4.1 Barcode success in chilli

From the eight barcoding loci, depending on the amplification success and literature studies, *matK, rbcL* and *psbA-trnH* loci were shortlisted for further studies. Samples showed 100% PCR and sequencing successes for all the three loci. The PCR amplification yielded product of ~600bp for *rbcL* (Figure 23a & 23b) and *psbA-trnH* (Figure 24a & 24b) and ~900bp amplicon *f*or *mat K* (Figure. 25a & 25b).

Bidirectional sequencing of the amplified PCR product was done. The sequences generated for these loci were of good quality with non-overlapping peaks and less back ground noise. The sequences were aligned bi directionally and the contigs were generated using DNA baser software.

4.3.4.2 Identification of the ideal barcode loci in chilli

psbA-trnH proved to be the ideal loci in discriminating *C. annuum* varieties from their adulterants. The band level identification of the adulterants was possible using this particular locus (Figure. 24a) .The locus yielded 600bp for chilli varieties, 500pb for 'choti ber' and 350bp for beetroot. This enabled easy identification of the adulterants at the band level itself. The MUSCLE analysis of *psbA-trnH* locus (Figure. 26) to discriminate chilli varieties from their related adulterants showed enough variation to discriminate *C. annuum* from 'choti ber' and beetroot. The locus exhibited greater variation with more number of SNP's and indels.

4.3.5 Adulteration detection in commercial samples of chilli powder

4.3.5.1 Authentication of reference samples

PCR amplification of capsicum varieties (reference samples) yielded 600bp for *rbcL* and *psbA-trnH* and 900bp for *matK* loci, respectively. The species specific barcodes generated using these three loci were authenticated by sequence comparison and BLAST analysis with the NCBI database. The results of the analysis yielded 100% identity with the respective species, confirming the authenticity of the reference species taken for the study.

4.3.5.2 Adulteration detection in commercial samples of chilli powder

The sequences of the reference sample and the NCBI database sequences were used for authenticating the commercial market samples. Out of seventeen market samples taken, none

was found to be adulterated with plant based adulterants. Sequence analysis of all the market samples gave 100% identity to *C. annuum* sequences for all the three loci. The absence of plant based adulterants in the market samples were confirmed by cloning of the market samples along with reference sample and simulated samples. The phylogenetic tree (N-J tree) constructed on the basis of K2P parameter with 1000 boot strap values using *psbA-trnH* locus clustered all the market samples to *C. annuum* (Figure 27).

4.3.6 Validation of the results

4.3.6.1 Cloning and simulation studies

PCR amplification and gel elution of the bands corresponding to *psbA-trnH* locus of market samples, one standard reference sample and simulated samples (chilli: choti ber and chilli: beetroot) were transformed to pGEMT cloning vector using JM109 competent cells by heat shock method.

Cloning yielded approximately 50-70 single blue white colonies in each plate (Figure 28). White recombinant colonies were picked and sub cultured. Colony PCR yielded products of ~800 bp in all the positive clones of market samples and reference sample. Chilli: choti ber simulated sample yielded products of 800bp and 700bp. The chilli: beetroot yielded 800bp and 550 bp fragments. Plasmid isolation and bi directional sequencing showed maximum sequence identity with *C. annuum* for reference samples. BLAST analysis of the sequences from simulated samples corresponded to both sequences respectively. About 10 different plasmids were taken from simulated samples of chilli: ‘choti ber’, six sequences showed 100% identity towards chilli and the four sequences corresponded to ‘choti ber’. The same was in case of chilli: beetroot simulation yielding sequences corresponding to both chilli and beetroot.

All the market samples yielded uniform sequences from different colonies confirming the absence of any plant based adulterants.

4.3.7. Construction of reference library and barcode generation

The sequences generated by amplification of barcoding loci viz., *psbA-trnH, rbcL* and *matK* were submitted with the NCBI. Barcodes were generated for all the reference sequence of *C. annuum*. Sequences were submitted to NCBI and Gen bank accessions ID were assigned (Table 15).

Table 15. *Capsicum* sequences submitted to NCBI

Capsicum varieties	**Locus**		
	matK	***rbcL***	***psbA-trnH***
Capsicum annuum variety PB7	KU936069	KU936073	KX010914
Vellakanthari	KU936070	KU936074	KX010915
PKM1	KU936071	KU936075	KX010916
Anugraha	KU936072	KU936076	KX010917

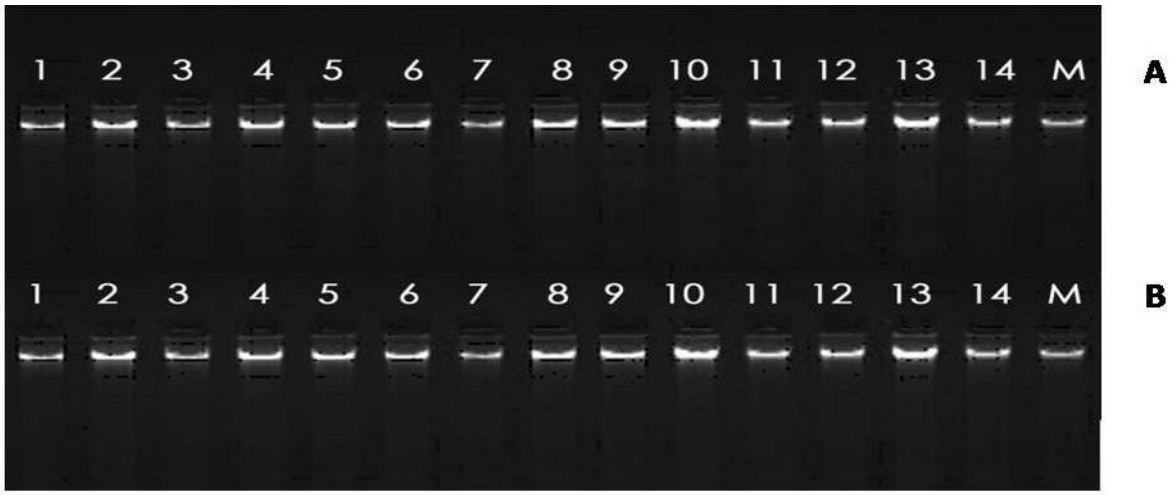


Figure 22 . Total genomic DNA isolated from chilli, probable adulterants and market samples. (A- Lanes1-4- chilli varieties, lanes 5-9 ' Choti ber', lane 10-11- beet root samples, lanes 12 -14 market samples (C1-C3),lane M- marker (Human genomic DNA, Genei). **B**- Lanes1-14 market samples (C4-C17), lane M- marker (Human genomic DNA, Genei).

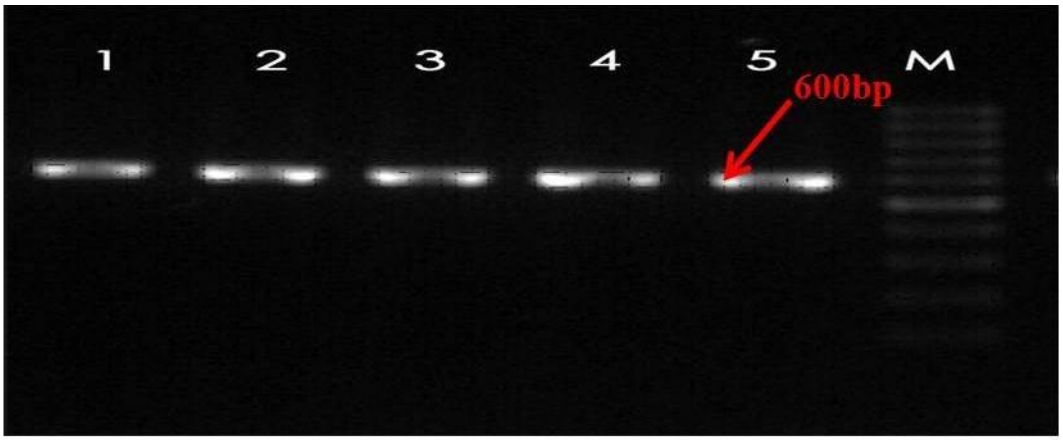


Figure 23a. Amplification of *rbcL* locus in chilli and its adulterants. (Lane 1- PB7 (chilli), lane 2- Vellakanthari (chilli), lane 3- PKM1 (chilli), lane 4- 'choti ber', lane 5- beetroot, lane M- marker (100bp ladder, Fermentas).

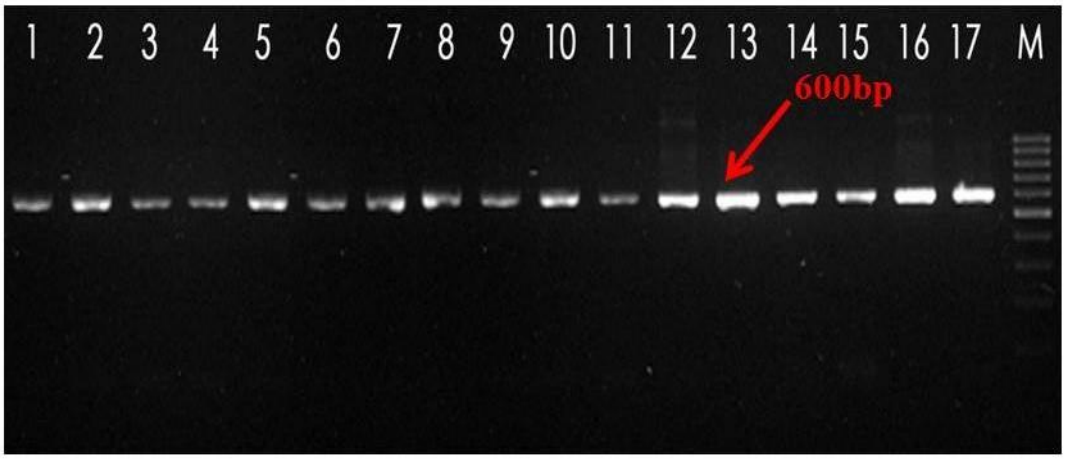


Figure 23b. Amplification of *rbcL* locus in chilli market samples. (Lane 1-17- market samples C1- C17, lane M-marker (100bp ladder, Fermentas).

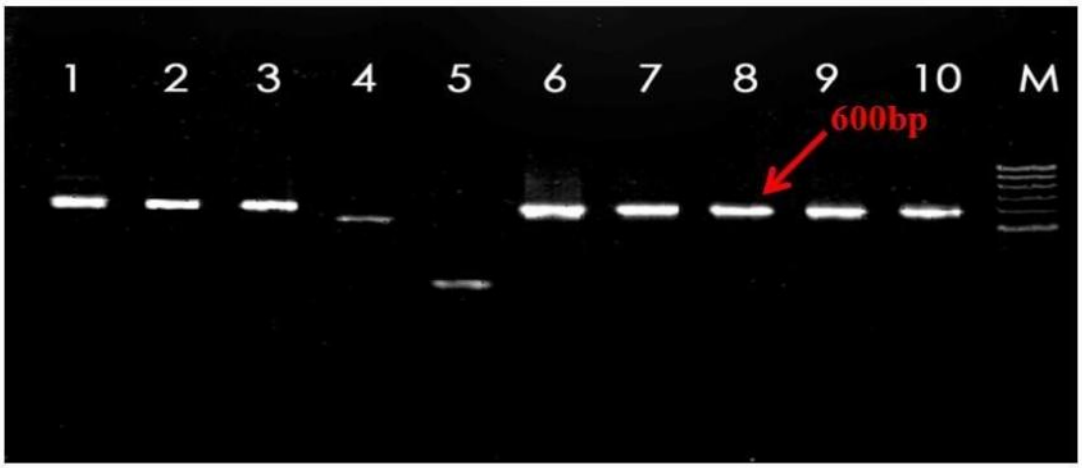


Figure 24a. Amplification of *psbA-trnH* locus in chilli, probable adulterants and market samples. (Lane1- PB7 (chilli), lane 2- Vellakanthari (chilli), lane 3- PKM1(chilli), lane 4- ' choti ber', lane 5- beetroot, lanes 6-10- market samples (C1-C5), lane M- 100bp ladder (Fermentas)

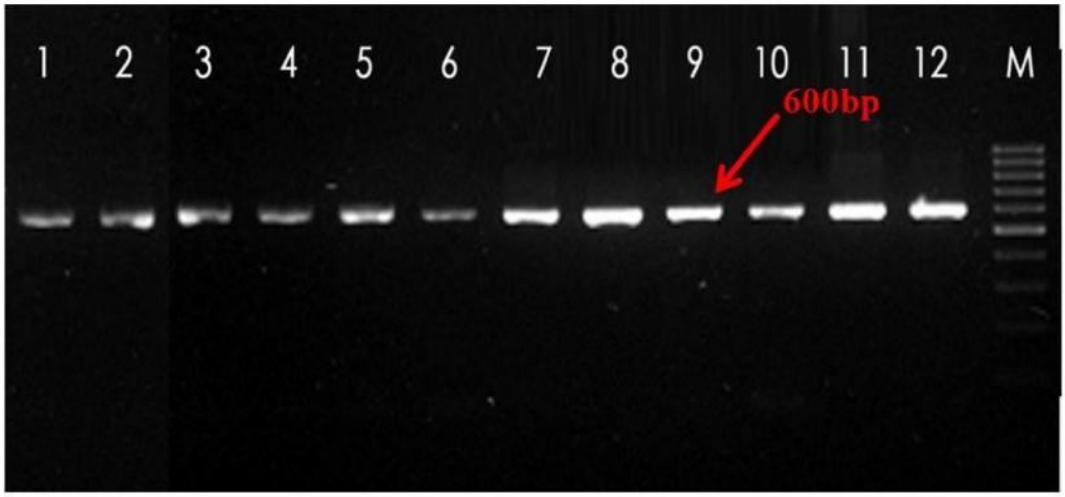


Figure 24b. Amplification of ***psbA-trnH*** locus in chilli market samples. (Lanes1-12- market samples C6-C17, lane M- marker (100bp ladder, Fermentas).

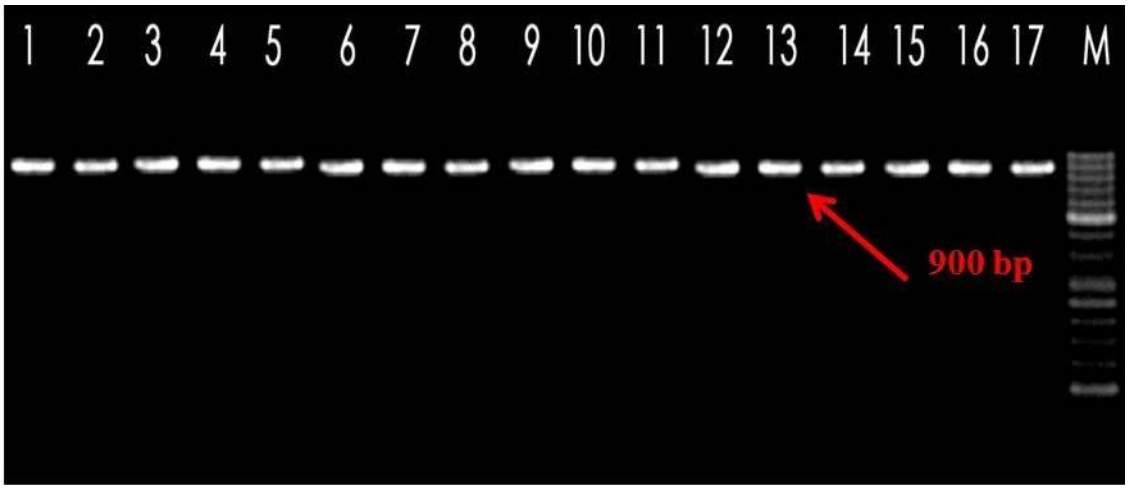


Figure 25a. Amplification of *matK* locus of group III). Samples. (Lane 1- PB7(chilli), lane 2- Vellakanthari (chilli), lane 3- PKM1(chilli), lane 4- choti ber, lane 5- beetroot, lanes 6-17- market samples C1-C12, lane M- marker (100bp ladder, Fermentas).

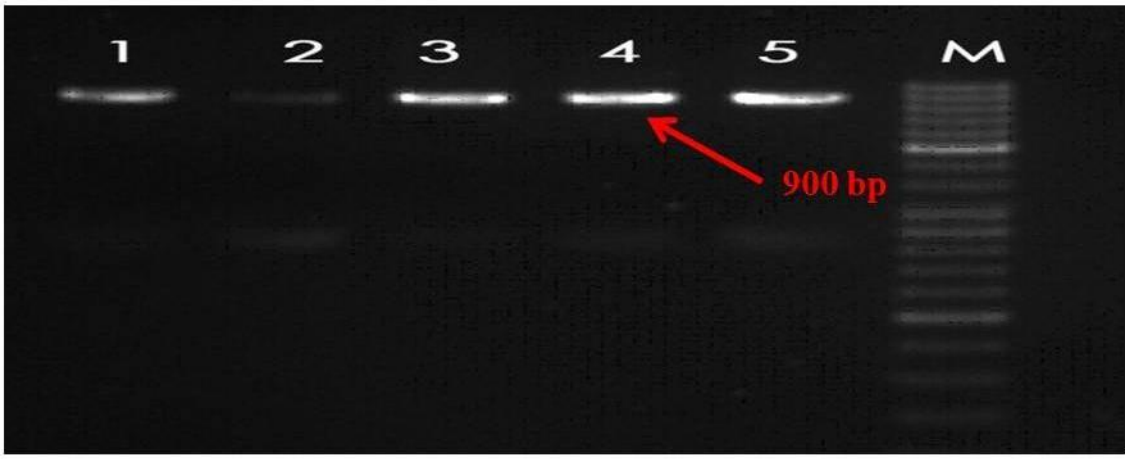


Figure 25b. Amplification of *matK* locus in chilli market samples. (lanes1-5- chilli market samples C13-C17, lane M- marker).

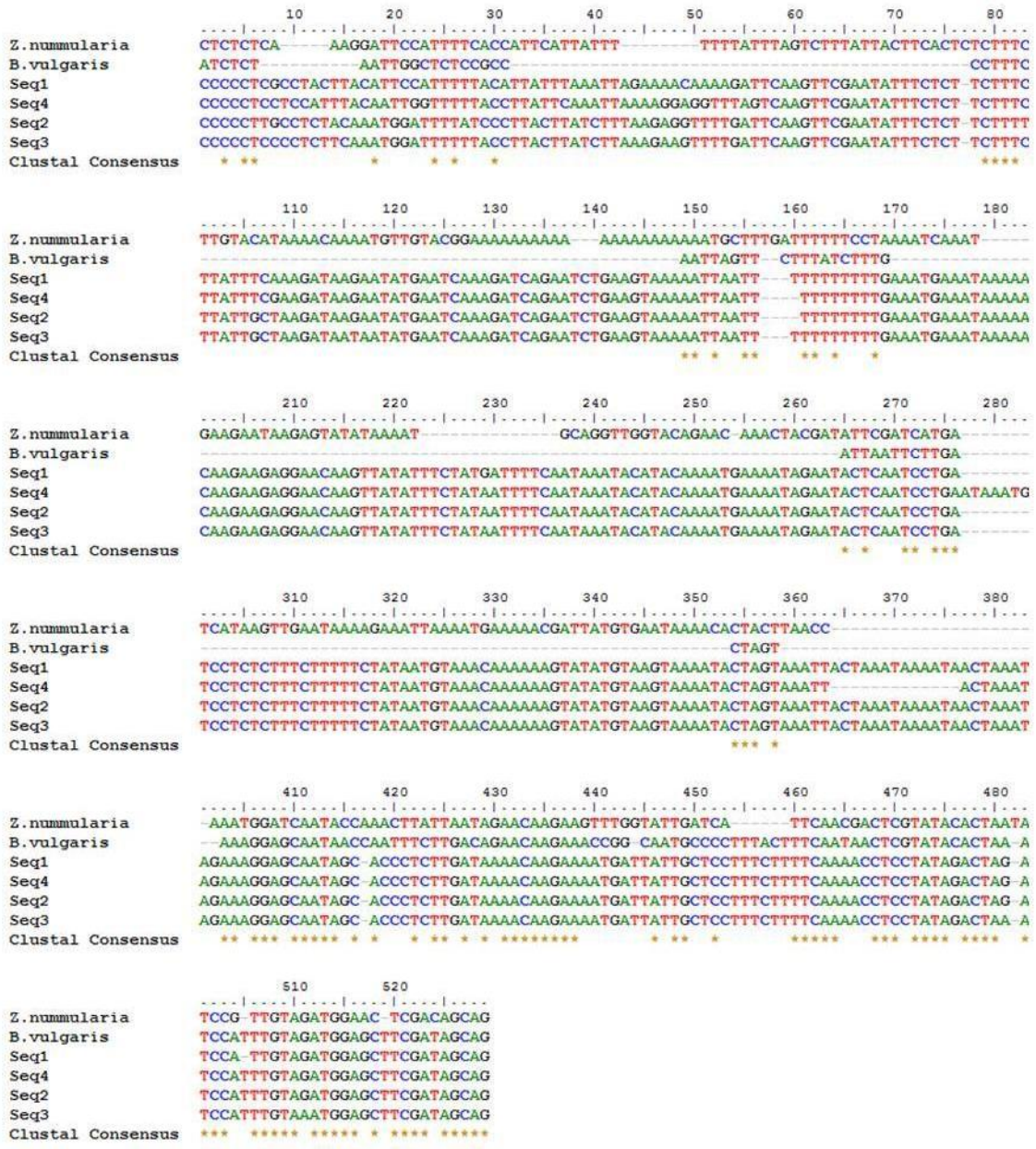


Figure 26. MUSCLE alignment of *psbA-trnH* sequences depicting the indels and polymorphic sites in *C. annuum* and their probable adulterants.

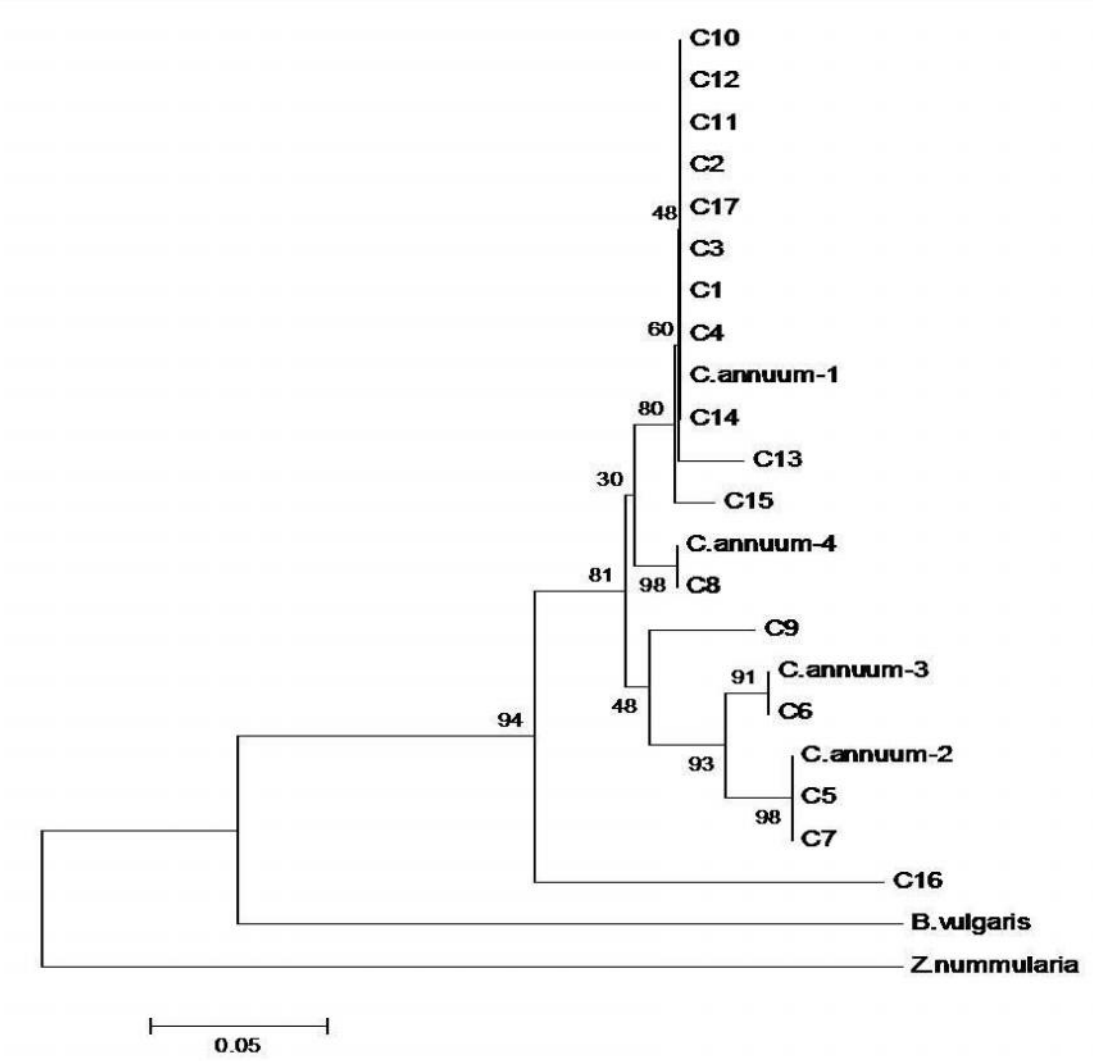


Figure 27. The N-J tree constructed from *psbA-trnH* sequences of group III samples based on the K-2-P distance

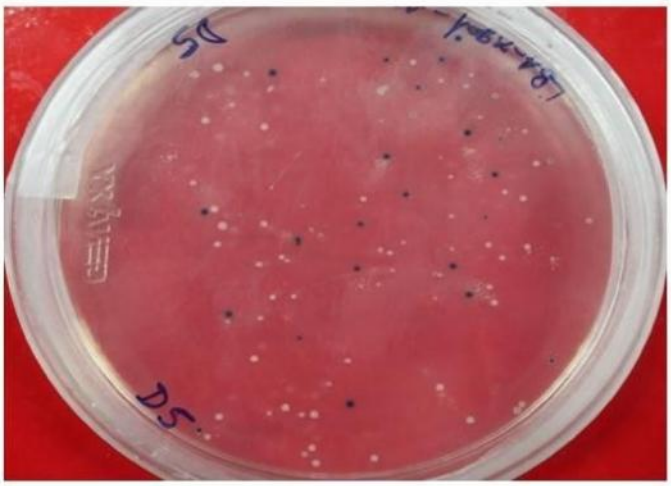


Figure 28. LB plates showing blue/white colonies after transformation of Group III samples.

DISCUSSION

Traded globally, spice powders are value added products of spices. They are of medicinal and culinary use internationally and so the product's authenticity is of great concern. Being a high value commodity, traded spices and its value added forms are adulterated mostly for economic gains. Competition among the different brands, lack of standard quick methods to ensure quality and authenticity checking leads to fraud practices of adulteration of spices and spice powders.

Powdered spices are more vulnerable to adulteration and are adulterated using artificial colors, dyes, earthy materials, powdered plant based materials of cheap origin and materials of morphological resemblance. The gravity of the issue has mandated in setting up different national and international bodies to check the illegal practices of adulteration in traded products. In addition to the WTO agreement concerning the Sanitary and Phytosanitary issues, the Food Safety and Standard Authority (FSSA) of India at national level and the Food Safety Commission (FSC) at state (regional) level are set up to address the issue. Quality specifications concerning the purity and authenticity of the traded spice commodity were laid down by International organizations like Codex Alimentarius, American Spice Trade Association (ASTA), European Spice Association (ESA), and International Organization for Standardization (ISO).

In spite of all these vigilance and caution concerning quality of the consignment, adulteration continues. About 12% of the spices exported from Mexico and India to USA were reported to be contaminated according to spice imports analysis by federal food authorities. The contaminants included insects, rodent hairs and other extraneous matter according to the US Food and Drug Administration (FDA) cited by the New York Times (New York Times,

Washington, May 14, 2014). Rejection of Indian shipments of black pepper and chilli by European countries like Britain, Germany, Netherlands and Spain due to adulteration/contamination has been a major concern on the Indian spices trade (Tamil Selvan and Manojkumar, 2002).

Improvement in the quality standards of the traded commodities, standardisation of new scientific methods of production and evaluation of the commodity etc. are of immediate requirement for boosting and sustaining India's share of global spice market. Though methods like morphological, physical, microscopic and analytical techniques are used in authentication of traded commodities, they are of limited utility for routine sample analysis because of being quite expensive, less precise, time consuming and complex to perform nature (Asensio *et al*., 2008). The use of DNA based biotechnological approaches has the potential to overcome these limitations. PCR based markers like RAPD, SSR, ISSR, SCAR etc. for adulteration detection of traded spice powders have proved to be successful (Sasikumar *et al*., 2004, Dhanya and Sasikumar, 2010).

However, some of these methods suffer from limitations. The limitations of RAPD include low reproducibility, variations across laboratories and limited application across wide genera. Low levels of polymorphism and high degree of skill and expertise are necessary for RFLP. ISSR and SSRs are characterised by species specificity which hinders its use in a broad range of plant groups. SCAR markers overcome these disadvantages, but the conversion of RAPD markers to SCAR needs the screening of a large number of primers which is tiresome and time consuming (Kumar *et al*., 2014). This disadvantage may be easily overcome by the use of universal DNA barcoding technique which is applicable to a broad range of plant taxa using universal barcode loci. DNA barcoding is highly reliable tool for authentication of species and enables the detection of known and unknown adulterants to be traced from mixed samples (Newmaster *et al.*, 2013).

5.1 Sample collection

For black pepper (group I), and turmeric (group II) the standard/ reference samples and the closely related adulterants (*Piper attenuatum, P. galeatum* and *C. zedoaria*) were collected from the germplasm pool of ICAR-Indian Institute of Spices Research (IISR). The germplasm collections maintained here comprised the collections of different species and cultivars of black pepper and turmeric from varied areas and geographical locations. The samples taken from this pool served best due to its rich geographical diversity. The power of barcode in species can be strengthened by sampling multiple individuals per populations and across geographical areas (De Salle *et al*., 2005).

Five different accessions of the samples in duplicates were collected to ensure the diversity and to avoid/ minimize sampling errors. Though Matz and Neilson, (2005) suggest taking 12 individuals per species as sampling size, it was not accepted by the barcode community. According to Hajibabaei *et al*., 2006 and the barcoding groups (http://www.barcodinglife.org/views/login.php) the samples typically included 5-10 individuals per species (some even containing only one or two). Guo *et al*., (2011) for his studies on adulteration detection in Lamiaceae used only 3-5 individuals from each population.

DNA barcoding mostly relies on publically available databases to obtain reference sequences, but may often lead to inaccurate identification due to the incorrect and missing sequences in the GenBank (Reynaud, 2015).Non availability of sequence of the candidate entity in the data base may also restrict the authentication as observed in case of *Ruta graveolens* (Al-Qurainy *et al.*, 2011). To overcome these drawbacks genuine and probable adulterant species were taken as authentic samples. The sequences from the test samples must be compared to the

known reference sequences for identification (Reynaud, 2015) and the same was adopted with the commercial samples taken in the present experiment.

Market competition is also a reason behind adulteration of a product. In certain cases the manufacturer may not be able to meet the quality criteria of the customer within the price limits. Thus adulteration is done as an attempt to meet a specification or to compete by offering an admittedly inferior product at a lower price. Powdered samples may be adulterated to meet a colour specification set by the customer or to allow the manufacturer to offer a lower priced product that allows him to compete with other available market brands. In other cases the adulterated product may be more visually appealing than the pure spice. To rule out the chances of missing any major competent brands, the test samples used in the present study were selected based on brand name and availability in the local markets. The price range was also taken into consideration for test sample selection.

Adulteration of a particular brand was established only after thorough analysis of three batches of the same product. Just a single batch if found to be adulterated cannot be labelled adulterated as it might be an error too. Costion *et al*., (2011) suggest taking two or three replicates of each sample to ensure accurate replication of results. Adulteration of a particular brand of the herbal samples could be confirmed only by validating triplicate samples of the same product (Newmaster *et al*., 2013). In the present study, to validate the authenticity of the product, three different batches of the same sample in duplicates were tested.

5.2 Isolation of genomic DNA from spice powders and their probable adulterants

Gismondi *et al*., 2012 in their study, prepared the samples for DNA extraction by grinding the material to fine powder using laboratory blender waring commercial grinder. The grinded powder was later made to a fine powder with liquid nitrogen using mortar and pestle. In the present study also the same method was adopted. The reference samples were made in the

laboratory by powdering the whole spice in a Cyclotech 1093 sample mill followed by grinding with liquid nitrogen to fine powder using mortar and pestle. Sealed labelled covers were used to avoid contamination and mishandling.

Genomic DNA isolation is an essential step in DNA barcoding technique. Taking samples in duplicate reduced the errors during DNA isolation and PCR amplification. Newmaster *et al*., 2013, reported taking multiple DNA extracts from samples, to ensure that no species DNA is missed from the market samples during the isolation process. To extract good quality DNA from dried and processed samples is a challenging task. During the harvest, storage, and processing, DNA degradation is inevitable (Techen *et al*., 2004). DNA isolation from the processed samples of black pepper, turmeric and chilli encounters different problems during the isolation process due to the difference in the constituents present in them. A single manual isolation protocol will not work for all the three spices. DNA isolation protocols needs adjustment/ variation in accordance with each plant species and even for different tissues depending on the primary and secondary metabolites present (Sangwan *et al*., 1998).

Commercial kits are available, but they are costly and also limit the applicability. DNA isolation from powdered market samples of turmeric was not possible using commercial kits. Only manual protocol was successful in isolating DNA from these samples. On the contrary, the simulated samples of black pepper: chilli could be isolated only using the commercial kit (Qiagen). Manual protocol of chilli and black pepper were tried separately but was unable to yield amplifiable DNA from simulated samples. As a means to cost reduction and for the ease of working, manual protocols were used for DNA isolation. The protocols followed for black pepper, turmeric and chilli are Dhanya *et al*., (2007), Remya *et al*., (2004) and Dhanya *et al*., (2008), respectively. These protocols were mainly the modified ones of Dellaporta *et al*., (1983), Doyle and Doyle, (1987), as well as Webb and Knapp, (1990). Extraction of DNA from recalcitrant plant tissues are reported to be tedious due to the presence of high amount

polysaccharides, proteins, polyphenols, alkaloids and other secondary metabolites. These polyphenols and polysaccharides bind with nucleic acids and interfere the further processes (Prittila *et al*., 2001). This activity may be the reason for lower yields and purity of the isolated DNA.

In general, CTAB method of DNA isolation was followed by varying the reagent composition to meet the needs of various tissues. The extraction buffer of the CTAB method mainly contains Tris (Tris-hydroxymethyl aminomethane) which buffers the pH of the cells. EDTA (Ethylene diamine tetraacetic acid) chelates the metal ions and weakens the cell membrane stability. Sodium chloride helps in the maintenance of osmoticum of the cells. Mercaptoethanol cleaves the disulphide bridges of proteins and causes denaturation of membrane and cytosolic proteins. DNA in the nucleoplasm is neutralized by the addition of Potassium acetate. The K^+ ions bind with the negative phosphate backbone of the DNA and shield them. This favours DNA precipitation from ethanol in cold condition. Addition of ice cold alcohol to the solution causes the DNA strands to come close together and coalesce. DNA is settled down as a pellet by centrifugation, purified by 70 % ethanol wash (Sambrook and Russell, 2001).

5.2.1 Group I-Black pepper and related samples

DNA isolation from group I (black pepper) samples were done using the manual protocol of Dhanya *et al*., 2007. Due to the presence of high amounts of polyphenols and polysaccharides in black pepper (Narayanan, 2000) and its adulterants like *P.attenuatum*, *P.galeatum* and papaya seeds, yielding good quality amplifiable DNA was a difficult task. The presence of polyphenols in the recalcitrant powders causes the covalent binding to the DNA in the oxidised form giving a brown colour, which makes the isolated DNA unusable for PCR amplification (Katterman and Shattuck, 1983). Dhanya *et al*., (2007), modified the Doyle and

Doyle, (1987) protocol by varying the components to yield good quality amplifiable DNA. The protocol was perfected by repeated trial and error methods.

The protocol of Dhanya *et al*., (2007) varied from the Doyle and Doyle, 1987 protocol mainly in the CTAB and NaCl concentrations. It was observed that increasing CTAB and NaCl concentrations improved the DNA yield from many plant species (Smith *et al*., 1991). Also the sample: extraction buffer ratio was increased. Addition of potassium acetate and precipitation of DNA using PEG 6000 were done. PEG is a poly phenol adsorbent and it removes the coloured pigment in the samples during DNA extraction (Syamkumar *et al.,* 2005). The DNA yield of black pepper, probable adulterants and test samples ranged between 18.6-34.2µg g^{-1} of dried tissue.

Nevertheless the DNA isolation from simulated samples of black pepper: chilli was not successful using this manual protocol. Manual isolation protocols of chilli (Dhanya *et al*., 2008) and black pepper (Dhanya *et al*., 2007) separately were tried but could not yield good quality DNA from the simulated sample. The presence of different components in varied levels might have made the manual isolation unsuccessful. The DNeasy plant mini kit (Qiagen, Germany) was opted for the DNA isolation from the simulated samples of black pepper: chilli which yielded good quality DNA. The yield of DNA ranged between 20.0-30.0µg g^{-1} of dried tissue.

5.2.2 Group II-Turmeric and related samples

Genomic isolation from turmeric and its adulterants were performed using the protocol of Remya *et al*., (2004). High amounts of polysaccharides, storage proteins, colouring pigments are reported to be present in *Curcuma* (Velayudhan *et al*., 1999). The same protocol worked for *C. longa, C.zedoaria* and cassava.

5.2.3 Group III-Chilli and related samples

Total genomic DNA extraction from group III, chilli and its adulterants (dried *Ziziphus nummularia* fruits and dried red beet pulp) was done using the protocol of Dhanya *et al.*, (2008). Chilli powder and its adulterants contains high levels of protein, cellulose, colouring pigments, pungent principals, sugars, resins and oils (Pruthi, 2003). Major modifications from the parent protocol were an initial ethanol wash of the powdered samples which helped to remove colouring pigments, increased sample: extraction buffer ratio and an initial precipitation using ethanol. DNA was spooled out and additional phenol: chloroform: isoamyl alcohol extractions and chloroform: isoamyl alcohol extractions were done. DNA yield was found to be between 5-15µ g per gram of dried tissue. The presence of RNA contamination may cause variability in PCR amplifications (Ellsworth *et al.*, 1993; Yoon, 1992). To avoid this, the DNA isolated using the manual protocol was subjected to RNase treatment using RNase enzyme.

5.3 Standardization of PCR parameters for the spices under study and their adulterants using barcoding primers

Barcoding loci for plants were selected on the basis of CBOL recommendations (CBOL, 2009). Eight loci and their universal primers were taken for the study. The universal primers were proven to be suitable for broad range of application for the authentication of land plants (Vijayan and Tsou, 2010). Primers were checked for the self complimentarity, melting temperature and GC content using Primer 3plus software. The primers were diluted and stock preparations were done in a universal manner as per Sambrook and Russell, (2001).

Attention was taken for standardisation of a uniform reaction mixture for all the spices and the adulterants, since the samples to be analysed may contain different known and unknown contaminants which need to be amplified for the adulteration detection from mixed samples.

Parameters like magnesium ion, template DNA, primer concentration and annealing temperatures influence the efficiency of a PCR reaction (Ellsworth *et al*., 1993). Standardisation of the reaction components of PCR is essential to avoid nonspecific amplification products such as primer-dimers or fragments of heterogeneous size (Rahman *et al*., 2000).The reaction mixture for PCR was optimized by varying one component and keeping the others constant. This method of PCR component standardisation ensured easy trouble shooting in case of PCR failures.

The kinetics of association and dissociation of PCR based enzyme reactions are altered by varying concentrations of dNTPs and $MgCl_2$. The kinetics is altered mainly by the primer-template rearrangements at the annealing and extension temperatures affecting the DNA polymerase activity (Huang *et al*., 1992, Wolff *et al*., 1993). Degradation of primer binding sites in certain genes in samples of degraded DNA, presence of complicated mixtures of organic extracts etc. may often influence amplification success of selected genes (Baker *et al*., 2012, Newmaster *et al*., 2013). Fazekas *et al*., 2009 reported the chemical and physical properties of certain DNA for selective amplification of selected sequences more than others. To avoid these irregularities, caution was exercised in standardization of the barcoding loci across the different spices and their adulterants in a uniform manner. Annealing temperature is the most important parameter that needs standardisation and it mainly depends on the primer sequences and salt concentrations (Innis *et al*., 1988, Rahman *et al*., 2000). Annealing temperature for each locus was standardised by gradient PCR. The denaturation profiles of different loci were standardised at a common temperature profile of 94-95^{o}C. The extension was performed around 72^{o} C with cycles ranging from 35 -40.

5.3.1 Group I –Black pepper

5.3.1.1 Selection of an ideal barcode locus

The first and foremost requirement towards adulteration detection of a commodity using DNA barcoding is to identify the ideal barcode locus for that particular species (Bruni *et al*., 2010). Barcoding loci may be considered ideal for a species, on the basis of consistent PCR amplification, successful sequencing and bi directional alignment of the sequences and most importantly the occurrence of variability (Kress and Erickson 2007, Hollingsworth *et al*., 2009). Eight barcoding loci were tested for the identification of the ideal locus. Out of the eight loci, *psbA-trnH, rbcL,* and *rpoC1* were short listed for further studies.

The *matK* locus failed to give consistent amplification and so was ruled out from further analysis. Two different primer sets (3F /1R and 390F/1326R) were tried using stringent conditions. Even though *matK* is considered an ideal locus in several plant species due to its high evolutionary significance (Lahaya *et al*., 2008), the locus is beset with consistent amplification failure in many other plant species (Kress and Erickson 2007, Ford *et al.,* 2009, Pettengill and Neel 2010, Wang *et al*., 2012). Previous studies on *Piper* species have revealed the poor resolution of *matK* gene (Wanke *et al*., 2007).

PCR amplification success of the three loci *psbA-trnH, rbcL,* and *rpoC1* were found to be 100% for *Piper* species and other related adulterants. Sequencing success of *rbcL* and *rpoC1* was 100% and that of *psbA-trnH* was 60%. The difficulty in aligning the bidirectional reads caused the reduced efficiency of *psbA-trnH*. Fazekas *et al*., 2008 reported the difficulty of sequence alignments in *psbA-trnH* locus, but the variability shown by this locus in discriminating species is far advantageous and makes it an ideal locus in barcoding studies (Kress *et al.,* 2005). The quality of the sequence reads was examined by checking the chromatogram which showed distinct non-overlapping and broad peaks with low background noise. These characteristics are considered as ideal and the sequences can be used for further analysis (Sanger *et al*., 1977). Sequencing failure has been reported in many market samples due to post harvest processing like drying at high temperatures, slow drying at moist

conditions, storage in alcohol etc. (Kool *et al*., 2012). In contrary to this all the commercial samples taken in our study gave good amplification and sequencing success.

Our results are in agreement with the studies by Guo *et al*., 2011 and Li *et al*., 2010. Their studies proved *rbcL* and *psbA-trnH* to be more efficient and to be considered ideal loci when compared to other loci. Li *et al.,* 2010 distinguished *Taxillus chinensis* from its adulterants and reported *psbA-trnH* to be more discriminatory than *rbcL*. The order of discrimination was *rpoC1<rpoB<atpF–atpH<rbcL<matK<psbK–psbI<trnH–psbA.* Among the non coding loci, *psbA-trnH* exhibits highest universality (CBOL, 2009*).* To identify the best locus in adulteration detection of *P. nigrum f*rom its adulterants, SNP analysis were done. No single locus could discriminate all the Piper species. *P. nigrum* was differentiated from other probable adulterant *Piper* species by *rpoC1* locus at position 11. *P. attenuatum* was discriminated from other *Piper* species by *rbcL* locus by a single SNP at position 234. The locus *psbA-trnH* discriminated *P. galeatum* from the other *Piper* species *(P. nigrum* and *P. attenuatum)* at position 48.

The low resolution of the locus may be overcome by using muti locus approaches. Fazekas *et al*., 2008 demonstrated the increased resolution of the species by combining two or more loci. Since no single locus could resolve the three *Piper* species, a two locus approach using *rbcL + psbA-trnH* and r*poC1 + psbA-trnH* was adopted which showed to increase the resolution of the species. The combination *rbcL + psbA-trnH* showed higher interspecific variation than the other combination. This combination was also found to be effective in discriminating other plant species (Kress and Erickson, 2007; CBOL, 2009). The *rbcL* locus offering high universality with phylogenetically conserved nature and *psbA-trnH* with high resolution and rapid evolution could be the reason for better resolution.

In earlier studies involving land plants across 48 genera, the combination of rbcL and psbA-trnH worked well due to its high discriminatory power. matK showed poor amplification success across the genera. Sequence quality especially to align bidirectional reads was found to be difficult with the trnH-psbA locus, but the limitation could be overcome by the high variability due to the presence of diagnostic insertion/mutations (Kress and Erickson, 2007). Calculation of intraspecific and interspecific distances based on the K2P parameter is another method to check the discriminatory power of a locus. The K2P parameter model of base substitution is the best accepted model for species level analysis with low distances (Hebert *et al.*, 2003). The non coding locus *psbA- trnH* exhibited higher inter and intra specific distance than the coding *rbcL* and *rpoC1* loci for *Piper* species.

5.3.1.2 Tracing out the plant based adulterants using DNA barcoding technique

Discriminating the *Piper* species was a collateral aim of the study, the major aim of the study being tracing out plant based adulterants from market samples of black pepper powder. The first step towards adulteration detection is the authentication of reference/ standard samples (Newmaster *et al*., 2013). The PCR amplification of *rbcL* and *rpoC1* loci yielded 600 bp and 500 bp sized fragments, respectively for all the genuine samples of *P. nigrum*, *P. attenuatum, P. galeatum,* papaya seeds, *Capsicum annuum* and market samples. The bands were unique in size for *rbcL* and *rpoC1* loci, whereas *psbA-trnH* yielded a 350 bp size fragment in all the *Piper* species and 600bp sized fragments for papaya seed and *C. annuum*. The variability in the nucleotide sequences for the *psbA-trnH* locus was greater, resulting in the length variation at DNA band level. Length variation for the locus was previously reported by Yang *et al*., 2011 and helped in adulteration detection and authentication of medicinal *Paris* from its adulterant *V. Jatamansi*.

The similarity/ BLAST searches are an essential requirement in authentication studies (de Boer *et al*., 2015). To authenticate the reference samples taken, the amplified products were custom sequenced and checked with the available sequences in the National Centre for Biotechnology Information (NCBI) and the Barcode of Life Data (BOLD) system using BLAST analysis. The analysis of reference/standard samples revealed maximum (100%) similarity to the respective sequences available in NCBI for all the three loci tested. This confirmed the authenticity of the reference sequences taken for the study.

Initial adulteration detection of the market/commercial samples was done by BLAST analysis. Out of twelve market samples taken for the study, ten gave 100% sequence similarity towards *P. nigrum* for all the three loci tested. Minimum BLAST cut off of 97% identity for top match is to be considered for identity searches (Wallace *et al*., 2012). The sequences of market samples did not correspond to the probable adulterants viz., *P.attenuatum, P. galeatum* and papaya seeds confirming the markets samples not adulterated with these adulterants.

The two market samples viz., P3 and P9 gave maximum identity hit to *C. annuum* sequences of NCBI database, implying the probable adulteration of the sample with chilli. Furthermore, the two market samples gave distinct band sizes 350 bp and 600bp sized fragments for the *psbA-trnH* locus amplification. The gel eluted products of these distinct bands when sequenced gave maximum identity towards *Piper* and *C. annuum*, respectively. Even though all the three loci could detect adulteration of the market samples, *psbA-trnH* yielded an adulterant specific band (600 bp) enabling easy detection. Band level comparison helped in detecting adulteration of a particular market sample but sequencing of the PCR products was essential to trace out the adulterant present. The higher inter specific divergence coupled with the greater ease of adulteration detection at the band level rendered the *psbA-trnH* an ideal locus for group I samples.

The adulteration detection of market samples was done by several methods including PCR amplification followed by sequencing, sequence alignment of the market samples along with the standard samples to detect SNPs. BLAST analysis of the market sample sequences against the common database and construction of the NJ tree on the basis of K2P parameter with 1000 boot strap values along with the reference samples helped in detecting adulteration. The locus *psbA-trnH* could cluster *Piper* species, *Capsicum* and papaya separately in distinct clusters and the market samples P3 and P9 clustered with *C. annuum*. All other market samples clustered with *Piper nigrum*.

None of the market samples showed sequence similarity with the wild *Piper* species viz., *P. attenuatum* and *P. galeatum*, but the chances of adulterating *P. nigrum* with these readily available entitites (Minor Forest Produces) cannot be ruled out. Papaya seeds having very close physical similarity with black pepper are more frequently used for black pepper adulteration. Papaya seed adulteration has been detected in market samples of black pepper using SCAR marker analysis (Dhanya *et al.*, 2009). However, in the present study papaya seed adulteration was not observed.

5.3.1.3 Validation studies

The validations of the results were done by cloning and by simulation studies. The *E. coli* strain variant JM109, was used in transformation experiments, due to its high copy number and ability to yield high plasmid DNA (Sambrook and Russell, 2001). Screening/selection of the recombinants was performed by blue white screening based on the principle of α-complementation. The Lac^+ bacteria that resulted from α-complementation could be easily recognized since they form blue colonies in the presence of X-gal (Horwitz *et al*., 1964). The white colonies represented transformed ones. Approximately 70-100 single blue white colonies were observed in each plate which is considered as successful cloning. Confirmation

of the recombinant strains was done by colony PCR (Gussow and Clackson, 1989). The size of the PCR product differentiated the recombinant and non recombinant ones. The simulated and putative market samples (P3 and P9) resulted in colonies of different sizes whereas *P.nigrum* samples yielded unique sized products.

HPLC has been used as an analytical tool in quality control and authentication of many agri-food commodities (El Hamdi and El Figza, 1995; di Anibal *et al*., 2009). Jain *et al*., 2007, reports the HPLC finger prints of black pepper along with two market samples used for adulteration detection of papaya seeds, *Embelia ribes* Burn. and *Lantana camara* L. Here we subjected the market samples and simulated samples to HPLC analysis to ensure the presence of capsaicin in the black pepper samples. The peaks of test samples were analysed against the standards viz., capsaicin and piperine. Analysis proved the presence of capsaicin in simulated samples as well as in the two market samples. The characteristic peak of capsaicin standard was found in the chromatogram of simulated and adulterated market samples. *P.nigrum* samples and non adulterated market sample tested negative for capsaicin. The characteristic peaks in chromatograph with respect to area and retention time proved the presence or absence of the components.

This is the first report on black pepper powder adulterated with chilli. Though ginger powder (*Zingiber officinale*) is reported to be adulterated with chilli (*Capsicum annuum*) (Dhanya and Sasikumar, 2010), there was no report of adulteration of black pepper with chilli hitherto. Adulteration of black pepper with chilli may be a malpractice to recycle the exhausted black pepper to a value added product by fortifying with pungent principles of chilli. Chilli being less expensive and easily available commodity, when compared to black pepper is used for adulteration. The exhausted black pepper after the extraction of oleoresins and other essential oils is of no use and form the waste products of the industry. These exhausted commodities are fortified with pungent principles of chilli and may be marketed as black pepper powder, a

fraudulent practice of marketing low/ inferior quality, morphologically non identifiable commodity in the name of genuine ones.

The barcode testing by Wallace *et al*., 2012, on product labelled "Black Cohosh" (*Actaea racemosa*), revealed presence of totally different *Acetea asiatica* species. Also the barcodes generated from a commercial sample labelled as Echinacea (*Echinacea purpurea*), produced sequences matching to sequence from walnut family (Juglandaceae). These adulterants were from totally different family and didn't have any morphological similarity to the genuine plant material but remained morphologically unnoticed. Mislabelling, misunderstanding and carelessness may be the due cause of these kinds of errors (Wallace *et al*., 2012).

In our study, the presence of chilli in black pepper cannot be a case of mislabelling and misunderstanding, but more of a known act of adulterating black pepper powder. However, there are chances of accidental mixing of chilli with black pepper by carelessness of the processing units where both the spices are processed in the same mill/ machine. Whatsoever, these practices reduce the quality and the effectiveness of the traded commodity. Also the contents present are in contrast to the label claims.

The utility of simulated samples in adulteration detection was suggested and demonstrated by Curl and Fenwick 1983, Paradkar *et al*., 2001, Paramita *et al*., 2003, Jain *et al*., 2007, Marieschi *et al*., 2009. Simulated samples of varying proportion were amplified to detect the sensitivity of the technique. Adulteration even at very low level (0.5% i.e.1:200 ratio) could be detected with this method. This precise level of adulteration detection is of paramount importance in the quality control parameters. The technique has far reaching advantage to be utilized by approved authorities/agencies for adulteration detection/authentication of spice powders. The technique can be routinely used for screening of batches, leading to immediate rejection of suspected samples or even in case of disputes arising from the quality of a lot.

5.3.1.4 Construction of reference library and barcode generation

A reference library is an essential part of barcoding studies. The sequenced samples are analysed against the common database. Larger database accommodating varied species is essential for accurate and correct identification of the samples. Absence of certain species in the common databases like NCBI, BOLD systems may result in incorrect identification of the test samples (Newmaster *et al*., 2013). BOLD (Barcode of Life Database) is a bioinformatics tool aiding the acquisition, storage, analysis and publication of DNA barcodes. The database approximately contains 370,000 plant barcodes representing 58,510 species. The barcodes submitted contains information regarding the locus, vouchers, images, maps, collection details etc. NCBI GenBank contains approximately over 3,00,000 formally described species

Most of the barcoding studies create databases for accurate identification of the commodity. A SRM (Standard Herbal Material) herbal barcode library was created using the 100 herbal species of known provenance and was identified by taxonomic experts. This library was used to identify the unknown herbal products and leaf samples (Newmaster *et al.*, 2013). To overcome the drawbacks of limited sequences in the common database, barcodes were generated for authenticated standard samples of the genuine *Piper* samples (*P.nigrum*, *P.attenuatum* and *P. galeatum*) for all the three loci. The barcoded sequences served as a reference library for the test samples. There were not many sequences available for *Piper* species in the common databases for these barcoding loci. The submission of these sequences helped to improve the database collection and further authentication studies. Submission of sequences to any one database is preferred because the sequences submitted to any one of the database, is commonly available to other databases.

5.4.1 Group II –Turmeric

5.4.1.1 Selection of an ideal barcode locus

For barcode locus selection in group II, amplification of the barcoding loci with the standard and probable adulterant species was done. *rbcL* and *ITS* were the final short listed candidate loci for the for group II on the basis of amplification and sequencing efficiency. Amplification and sequencing efficiency of *rbcL* and *ITS* were found to be 100% and yielded a sequence length of 600 bp and 500 bp, respectively for all the samples analyzed. PCR amplification success with two different primer sets of *matK* was 0% despite using different concentrations of the DNA and less stringent conditions, proving the locus not a suitable candidate for DNA barcoding for *Curcuma* species. Earlier the works of Kress *et al*., 2007 and Lahaya *et al*., 2008 reported the failure of *mat K l*ocus in different plant species. The locus *psbA-trnH* has failed with different plants due to its alignment difficulty (Sass *et al*., 2007, CBOL, 2009) and the same was observed in our study wherein the loci did not give consistent amplification for the standardized conditions and difficulty in sequence assemblies of the bi directional reads.

To identify the best loci among *rbcL* and *ITS*, the discriminatory power of the two loci to differentiate between the genuine *C. longa* and *C. zedoaria* was analysed. Costion *et al*., 2011, suggests the importance of interpretation of trace file data to correctly identify ambiguous sites. These sites if not correctly interpreted may cause misinterpretation by estimating additional species or diversity within species. *ITS* locus showed greater variability in the sequences between *C. longa* and their adulterants.

MUSCLE alignment of the *ITS* locus to detect the variable sites and SNPs revealed greater insertions and deletion for clear identification of *C. longa* and its common adulterant, *C. zedoaria*. *rbcL* locus did not show sequence variability in discriminating the two species of

Curcuma. The low resolution of the plastid *rbcL*, which has been ascribed to its low mutation rate when compared with that of the nuclear *ITS* was reported by Newmaster *et al*., 2013 , who also observed *ITS* to have greater sequence variability, allowing to differentiate between closely related species. The efficiency of *ITS* over *rbcL* in differentiating tropical tree species of India was reported by Tripathi *et al.*, 2013. The distance calculation by the K2P parameter using MEGA5 for the *ITS* and *rbcL* loci was done. *ITS* locus exhibited higher intra and inter specific distance than the coding *rbcL* which was unable to resolve *C. longa* from its adulterant species. The intra specific distance calculated for *rbcL* locus was zero for the two *Curcuma* species whereas for *ITS* locus it was 0.016± 0.005 and 0.026±0.005 for *C. longa* and *C. zedoaria*, respectively.

Slight sequence variations may be observed among individuals of same species due to geographical variation. Sequence variation in *C. zedoaria* from China and Japan was reported by Sasaki *et al*., 2002. This sequence variation may cause intra specific variation among different accessions of the same species, resulting increased intra specific variation in certain locus. In three species of Lauraceae and one species of Sapindaceae, *psbA-trnH* locus showed intraspecific variation due to geographical reasons (Costion *et al.*, 2011). The inter specific distance for the locus *rbcL* was zero and no sequence variability was observed to differentiate *C. longa* with *C. zedoaria*. For *ITS* it was 0.677± 0.066, showing variation to differentiate the two species of *Curcuma*. The inter specific distance calculated for *ITS* was greater than its intra specific distance, which is an important criteria for barcode selection.

5.4.1.2 Tracing out the plant based adulterants using DNA barcoding technique

The major aim of the study was to trace out the plant based adulterants of commercial samples. Band level detection of the adulterants was not possible with *ITS* and *rbcL*. The authenticities of market samples were checked using barcode databases in GenBank and

BOLD in addition to the reference database library developed using reference samples for the reported adulterants such as *C. zedoaria* and cassava starch. The authenticity of reference samples were further confirmed by 100% identity matches with the respective sequences in the common database. Minimum BLAST cut off of 97% identity for top match is taken for identity searches (Wallace *et al.,* 2012). Even though construction of a reference database is the most reliable approach for correct identification, it is also to be noted that an incomplete reference library may still allow correct identification by assigning best match within the classification tree (Newmaster *et al.,* 2013, Kool *et al*., 2012).

Out of ten market samples taken in the present study, four was found to be adulterated. Sequences of all the market samples except the four adulterated market samples showed 100% identity with *C. longa* sequences. Sequences of T5 and T8 revealed the presence of cassava starch, a probable adulterant. Maximum identity hit of market sample T4 was with *C. zedoaria* sequences. Presence of fillers like *Triticum aestivum* L. (wheat) and *Hordeum vulgare* L. (barley) was detected in the sequence of the market sample, T7. The results were validated using both the loci and also in different batches of the same brand. Newmaster *et al.,* 2013 reported the presence of fillers in the commercial herbal products and suggested the item to be contaminated/ substituted if it contains more than one barcodes other than the labeled ones. The studies of Srirama *et al*., 2010 on the species admixtures in raw drug trade of *Phyllanthus* using DNA barcoding confirmed the admixing using cheap, easily available and related species used in traded drug. Approximately 26% of the drug was found to be adulterated. In the present study too, the cheap and easily available fillers were found to be used for adulterating turmeric powder.

The phylogenetic N-J tree constructed on the basis of K2P with 1000 boot strap values clustered the T5 and T8 samples with the cassava sample while the market sampleT4 showed maximum affinity with the *C. zedoaria* samples. Market sample T7which contained fillers

grouped distinctly. The adulteration of the market samples were clearly visualised by the clustering of the market samples. A boot strap test with minimum of 1000 replicates is essential to access the reliability of phylogenetic trees (Anbazhagan *et al.*, 2014). Boot strapping is mainly performed to examine the reliability of the interior branches and the authenticity of the trees (Rai *et al.*, 2012).

MEGA (Molecular Evolutionary Genetic Algorithm) is an integrated tool for estimating the rates of molecular evolution, construction of phylogenetic trees and is mainly used to test evolutionary hypothesis. Both automatic and manual alignments of the sequences are possible with this software. BLAST and nearest distance method are mainly used to test the ability of different regions for authentication of species from the adulterants (Meizil *et al.*, 2012). The identification of 45 blind samples sold as palombo was done using BLAST searches against the common databases considering the closest matches up to 98% specimen similarity (Barbuto *et al*., 2010). The same similarity rules were adopted for the test sample analysis in the present experiment.

5.4.1.3 Validation of the results

Cloning experiments along with BLAST analysis ensured that none of the sequences of any of the species present in the product were missed. Reference sample gave maximum identity (100%) to the *C. longa* sequences of NCBI. Sequences of the clones picked from the simulated samples (*C. longa*: *C. zedoaria*, *C. longa*: cassava) corresponded to *C. longa, C. zedoaria* and cassava sequences, respectively. The clones selected from the simulated sample corresponded to the respective species, implying the accuracy of the technique to detect DNA barcodes even from mixed samples. Sequencing using cloned PCR products ensures all alleles of all species to be captured in the database. Missing of alleles may cause false identification as most of the identifications are based on single nucleotides (Sass *et al*., 2007).

Studies by Wallace *et al*., 2012 on authenticating ginseng using *ITS* proved not only the efficiency of the locus in discriminating between the two species of ginseng but also the effectiveness of barcoding in detecting adulterants and fillers. The SNPs detected using different accessions of *Curcuma* reference samples, proved useful in detecting adulteration of market sample T4 with *C. zedoaria*. These SNPs can be used in designing specific primers for developing diagnostic kits to detect adulteration of turmeric with *C. zedoaria.*

Manufactures of turmeric powder may be tempted to mix *C. zedoaria* with *C. longa* due to its cheap and easy availability. *C. zedoaria* has been reported to be toxic (Lakshmi *et al.*, 2011) and if mixed with turmeric powder, it lowers the biological value of turmeric thereby eroding consumer confidence and constituting a health hazard. Other adulterants found, namely cassava starch, wheat, rye, and barley, may have been added deliberately – despite the label's claim that the packet contains nothing but turmeric powder – to increase bulk so as to obtain higher profits. The presence/ consumption of adulterated products containing undeclared or unlabeled constituents may cause intoxication, allergies, or other undesirable side effects (Asensio *et al*., 2008).

Detecting adulteration is a prerequisite to food safety. Although authenticating value added herbal products such as powdered spices is a daunting task in the face of unethical practices including substitution, adulteration, use of fillers, and mislabeling, DNA barcoding may prove handy to meet these challenges in not only detecting the probable/known samples, but also for the detection of unknown adulterants and fillers.

5.4.1.4 Construction of reference library and barcode generation

The barcode sequences generated in this study were deposited in the NCBI database to contribute for the construction of universal barcode library and were assigned Gen bank accession IDs. This along with the other sequences in NCBI can act as a reference library for

further studies in *Curcuma.* The precision in identifying the adulterants increases with the increase in species diversity in the databases.

5.5.1 Group III. Chilli

The PCR performance and sequencing efficiency of *matK, rbcL* and *psbA-trnH* loci were found to be consistent in group III comprising chilli and its probable adulterants. Samples showed 100% PCR and sequencing successes for all the three loci. Sequences were considered good in quality with little back ground noise and non over lapping peaks. *psbA-trnH* proved to be the ideal locus to discriminate the *C. annuum* varieties from their probable adulterants by discriminating at the band level. The locus yielded 600bp for chilli varieties, 500pb for 'choti ber' and 350bp for beetroot. This enabled easy identification of the adulterants at the band level itself. *psbA-trnH* is one of the most variable locus exhibiting high interspecific divergence in authentication of species (Chase *et al*., 2007; Lahaye *et al*., 2008; CBOL, 2009).

The inter specific distance calculation was not possible with the group III samples as the probable adulterants belonged to different families. Coding of barcoding gaps is not very essential in identification/ authentication of specimen, as the identity searches are mainly based on BLAST analysis against the common databases (Kress and Erickson, 2007). An inter specific calculation becomes irrelevant in this case as there is a clear band level identification of the chilli with the probable adulterants. The MUSCLE alignment of *psbA-trnH* locus discriminated chilli varieties from 'choti ber' and beetroot.

5.5.1.1 Adulteration detection in commercial samples of chilli powder

Identity of a sample is authenticated mainly by GenBank BLAST search with atleast two barcoding loci. Accurate replication of the sequencing results using two or three replicates of

same samples is an alternate way (Costion *et al*., 2011). The authentication of the chilli genuine varieties was done by the BLAST analysis of the sequences from all the three loci (*rbcL*, *psbA-trnH* and *matK)*. The results of the analysis yielded 100% identity with the respective species, confirming the authenticity of the reference species taken for the study. These authenticated sequences formed the reference sample of the commercial market samples.

Out of seventeen market samples taken, none was found to be adulterated with plant based adulterants. Sequence analysis of all the market samples gave 100% identity to *C. annuum* sequences for all the three loci. The absence of plant based adulterants in the market samples was confirmed by cloning of the market samples along with reference sample and simulated samples. The phylogenetic tree (N-J tree) constructed on the basis of K2P parameter with 1000 boot strap values using *psbA-trnH* locus clustered all the market samples to *C. annuum*. The same method of phylogenetic N-J tree based on MEGA software method was followed by Sun *et al*., 2012 using *ITS2* locus to differentiate eight species of Cistanches herba from its adulterants.

5.5.1.2 Validation of the results

The absence of plant based adulterants in the 17 market samples were confirmed further using cloning and simulation studies. All the market samples yielded uniform sequences from different colonies confirming the absence of any plant based adulterants. However, the probability of adding *Z. nummularia* (‘choti ber’) to the chilli powder cannot be ruled out. Market sample analysis using SCAR marker has revealed the presence of ‘choti ber’ in chilli powder samples (Dhanya *et al.*, 2011). ‘Choti ber’ is commonly found in the north-western India and Andhra Pradesh and resembles chilli in colour. Once dried and powdered it can easily go visually undetected in chilli powder. Perhaps the non availability of the item while

processing the chilli, i.e. the seasonal effect, may be one of the reasons for the absence of this adulterant in the chilli powder. Alternately, the use of the synthetic adulterants might be more lucrative, easy and handy (Mitra *et al*., 1961; Banerjee *et al*., 1974; PFA, 2003).

5.5.1.3 Construction of reference library and barcode generation

The sequences generated by amplification of barcoding loci viz., *psbA-trnH, rbcL* and *matK* were submitted with the NCBI. Barcodes generated for all the reference sequences of C. *annuum* were submitted to NCBI and Gen bank accessions ID were assigned (Table 17). In general the barcoding of black pepper, turmeric, chilli and the related adulterants was successful. Out of the eight barcoding loci, *psbA-trnH* for black pepper and chilli; *ITS* for turmeric were found to be ideal in species discrimination and adulterant detection. Performance of *ITS* and *psbA-trnH* for the identification of Radix Astragali from its adulterants based on the genetic distance calculation and BLAST1 analysis was reported by Zheng *et al*., 2014.

The testing of black pepper powder in twelve different commercial brands revealed adulteration in two of them. Black pepper powder was adulterated using chilli. In case of turmeric, screening of ten different brands revealed six brands to be authentic. The remaining samples were found to be adulterated, two of the brands contained cassava starch as adulterant while one of the test samples contained *C. zedoaria* and another sample contained fillers. In chilli, seventeen brands were tested, of which none was found to contain plant based adulterants.

Performance of DNA barcoding technique is mainly depended on the molecular variability of the concerned organism. The successful use of DNA barcoding in the testing of exported item viz., roasted barley tea and its revertion to the port of origin in China due to adulteration by the importing country is a classic example of the application of DNAbarcoding in commodity

authentication. The presence of adulterants like *Morus* sp., *Triticum* sp., *Avena sterilis, Chenopodium ficifolium* and *Avena fatua* was detected in ten batches of roasted barley tea, resulting in its rejection. This case proved the effectiveness of the technique for screening and authenticating trade products (Jian *et al*., 2014). The barcoding technique using the common database is efficient to trace out unknown and known samples. Once the PCR standardisation of the loci followed by sequencing is done, presence of any adulterants can be detected. Galimberti *et al*., 2013 have established the usefulness of DNA barcoding for certifying food identity and also to track its origin and provenance of raw materials at different levels of transformation. The technique proved advantageous especially in case of shredded or powdered items, difficult in morphological identification due to the change of morphology and texture as in spice powders.

Epilogue

The study could contribute a new technique for routine screening and authentication of the traded commodities especially spices that would help raise quality, authenticity and accountability of the traded Indian spices. Creating links between academic, public and commercial sectors may hasten the technological advancement and may help in better exploitation of the method perfected for the welfare of mankind.

SUMMARY

Spices are high value, export-oriented commodities and are extensively used in varied sectors including medicine, food, culinary, cosmetics etc. Spices form expensive commodities playing an important role in agriculture and international trade. Accurate identification of plant commodity is a legal requirement in most countries and prerequisite for delivering a quality product that meets consumer expectations. Traceability is a major requirement of EU food legislation (Regulation (EC) No 178/2002) and Food Standard Agencies. The Sanitary and Phytosanitary regulations of the WTO make the issue more critical and significant.

The use of spices has increased globally in the recent times, leading to increased popularity of spices and its value added products. This has led to an increased demand for genuine and high quality raw materials. The increased demand for the products is being exploited by fraudsters based on the fact that the consumer cannot identify the fraud in ingredients or processes items. The presence of adulterants on retail markets of spices in the form of fraud such as contaminations and substitutions of cheaper or inferior species is evident. Spice adulteration is also a major economic fraud involving public health. Compared to the whole commodity the value added products like spice powders are more amenable to adulteration as the foreign matters go in to it visually undetected. Spice adulterants come in different forms. Apart from the addition of artificial colours, powdered plant based materials of cheap origin are currently on the rise as adulterants, especially in spice powders like black pepper, chilli and turmeric.

Traditional identifications include botanical taxonomy, physical, chemical and analytical methods. Advances in the identification of biological species using DNA-based techniques have led to the development of a DNA marker-based platform for authentication of plant

materials. DNA barcoding, in particular, has been proposed as a means to identify herbal ingredients and to detect adulteration of spices. The present study aimed at barcoding of spices (black pepper, turmeric and chilli), their probable adulterants and to trace out plant based adulterants in market samples of spices using DNA barcoding technique. Construction of barcode databases of genuine and adulterant species were also achieved. Spice samples of genuine and adulterant samples served as a reference library for the adulteration detection of the market samples. Simulated samples of the genuine and adulterants were also analysed to reconfirm the adulteration and to test the sensitivity of the technique.

The technique mainly involved the genomic DNA isolation, PCR amplification of the barcding loci, cloning, sequencing and analysis using Bioedit and MUSCLE softwares. The PCR reaction mixture were standardised for different barcoding loci and it contained 10-50 ng of DNA, 1X assay buffer with 1.5 mM $MgCl_2$, 1 mM dNTP, 1 picomol μl^{-1} each of forward and reverse primers and 1 U *Taq* DNA polymerase. The reaction was performed in an Eppendorf vapo protect thermal cycler. Eight barcoding loci were tested for each spice group and depending up on the amplification, sequencing success and discriminatory power, ideal locus were selected for each spice.

The entire results research work can be summarised as;

6.1 Group I- Black pepper

Total genomic DNA was isolated from five accessions of *Piper nigrum* L., *Piper attenuatum* Buch-Ham and *Piper galeatum* (Miq.) C. DC., papaya seeds and chilli varieties using manual protocol. These served as reference samples for the study. Twelve commonly available market samples of powdered back pepper were collected from the local market for analysis and the DNA was isolated using the same protocol. DNA yield ranged between 18.6-34.2µg g^{-1} of dried tissue and an absorbance ratio (A260/A280) between 1.8- 1.9 indicating high

purity and good quality of the isolated DNA. DNA isolation from the simulated samples of black pepper: chilli was done using the DNeasy plant mini kit (Qiagen, Germany) and the yield ranged between 20.0-30.0µg g^{-1} of dried tissue. Spectrophotometric and gel electrophoresis were done to confirm the integrity and purity of the isolated DNA.

Different barcoding loci viz., *psbA-trnH, rbcL, rpoB, rpoC1*, *matK, ITS, atpF- atpH* and *psbK-psbI* were amplified using different PCR annealing conditions. Of the different barcoding loci tested, *psbA-trnH, rbcL, rpoC1* and *matK* locus were shortlisted for further studies in the Group I, based on the recommendations of CBOL, review of literature studies, the amplification consistency and sequencing success. The PCR success rate of *psbA-trnH, rbcL,* and *rpoC1* were found to be 100% for *Piper* species and other adulterants. The *matK* primers (3F /1R and 390F/1326R) failed to give consistent amplification and so were ruled out from further studies. Sequencing success of *rbcL* and *rpoC1* was found to be 100% where as in case of *psbA-trnH* it was 60%, due to the difficulty in aligning the bidirectional reads.

The *rbcL* locus could discriminate *P. attenuatum* from other *Piper* species by the SNP at position 234. *rpoC1* locus were able to discriminate *P. nigrum* from the adulterants at position 11. The locus *psbA-trnH* discriminated *P. galeatum* from the other *Piper* species *(P. nigrum* and *P. attenuatum)* at position 48. The intraspecific and interspecific distances calculation based on the K2P parameter were done. The non coding locus *psbA- trnH* exhibited higher inter and intra specific distance than the coding *rbcL* and *rpoC1* loci. But no single locus was able to resolve all the three *Piper* species. So a two locus approach using *rbcL + psbA-trnH* and r*poC1 + psbA-trnH* was adopted. The combination of *rbcL + psbA-trnH* was found to be a better two locus barcode than *rpoC1 + psbA-trnH* resolving the probable adulterants of *Piper* species and collaterally discriminating the *Piper* species.

The PCR amplification of *rbcL* and *rpoC1* loci yielded 600 bp and 500 bp sized fragments respectively in all genuine samples of *P. nigrum, P. attenuatum, P. galeatum* and market samples. The locus *psbA-trnH* yielded a 350 bp size fragment in all the *Piper* species and 600bp sized fragments which corresponded to *C. annuum.* PCR amplification and sequencing of the twelve market samples except two gave 100% sequence similarity towards *P. nigrum* for all the three loci. The BLAST analysis of the sequences of the two putative market samples did not correspond to the probable adulterants viz., *P.attenuatum, P. galeatum* and papaya seeds. The two distinct market samples viz., P3 and P9 gave 600bp fragment and 350bp respectively for *psbA-trnH* locus. BLAST analysis of all the sequences from all the three barcoding loci proved P3 and P9 market samples are adulterated with chilli.

The phylogenetic tree constructed using K2P parameter with 1000 boot strap values clustered the P3 and P9 samples with *C. annuum* and the rest of the market samples along with *P.nigrum*. *psbA-trnH* locus was found to be the ideal locus for differentiating the chilli adulteration in black pepper due to its band level detection. Cloning and HPLC analysis re confirmed the adulteration of market samples. HPLC analysis gave distinct two peaks for capsicin and piperin standards for the adulterated and simulated samples and a single peak corresponding to piperin for the authentic samples. Simulations as low as 0.5% adulteration could be detected using the DNA barcoding technique using *psbA-trnH* primers at the band level itself. This is the first report on adulteration of black pepper with chilli.

The phylogenetic tree of market samples clustered the P3 and P9 samples with *C. annuum* and the rest market samples clustering together with *Piper nigrum*. Other *Piper* species were in separate clusters. Barcode sequences were generated for all the genuine *Piper* samples and probable adulterants for all the three barcode loci. The sequences generated were deposited in the National Centre for Biotechnology Information (NCBI) and are also available with the Barcode of Life Data (BOLD) system.

6.2 Group II. Turmeric

Whole genomic DNA from fresh rhizomes of five accessions of *C. longa* L., probable adulterants viz., four accessions of *C. zedoaria* Rosc. / *Curcuma xanthorrhiza* Roxb. and five samples of cassava was isolated using using the protocol of Remya *et al*., 2004. The same protocol was used in isolating DNA from the simulated samples of genuine and adulterant samples and from market samples representing ten different firms. The DNA yield was found to be between 10-12µg per gram of dried tissue and the market samples yielded 6-10µg DNA per gram of dried tissue. The PCR amplification and sequencing of the barcoding loci viz., *ITS, rbcL, psbA-trnH* and *matK* were done and the efficiency of *rbcL* and *ITS* were found to be 100% yeilding a sequence length of 600 bp and 500 bp, respectively for all the samples analyzed. PCR amplification success with two different primer sets of *matK* was 0% proving the locus not to be suitable candidate for DNA barcoding for *Curcuma* species.

The locus *psbA-trnH* did not show consistent amplification for the standardized PCR conditions with difficulty in aligning the bi directional reads. *rbcL* locus did not show variability in discriminating the two species of *Curcuma. ITS* locus showed greater variability in the sequences between *C. longa* and their adulterants with MUSCLE alignment showing greater number of variable sites and SNPs and higher inter and intra specific distance. The interspecific distance calculated for *ITS* was greater than its intra specific distance, which is an important criteria for barcode selection.

Market sample analysis of the ten different firms revealed the adulteration in four brands. Cloning and sequence analysis of market samples T5 and T8 revealed the presence of cassava starch and T4 sequences revealed maximum identity with *C. zedoaria* sequences. Presence of fillers like *Triticum aestivum* L. (wheat) and *Hordeum vulgare* L. (barley) was detected in sequence of the market sample T7. The sequence analysis of all the other market samples

except these four gave 100% identity to *C. longa* sequences for both the loci. The presence of these adulterants in the three market samples viz., T4, T5, T7 and T8 were reconfirmed by cloning and simulation studies. Adulteration as low as 1g in 200g of genuine powder could be detected using the technique. The N-J tree constructed on the basis of K2P with 1000 boot strap values clustered the T5 and T8 samples with the cassava sample while the market sampleT4 clustered with the *C. zedoaria* samples. Market sample T7which contained fillers grouped distinctly. All the other market samples clustered along with *C. longa* sequences.

The sequences generated by amplifying the barcoding loci viz., *ITS* and *rbcL* were submitted with the NCBI for all the reference sequence of *C. longa* and *C. zedoaria* and were assigned Gen bank accession ID.

6.3 Group III. Chilli

Whole genomic DNA from chilli, the probable adulterant samples, simulated samples and market samples was isolated using manual protocol and yielded 5-15µ g per gram of dried tissue. The PCR and sequencing efficiency of *matK, rbcL* and *psbA-trnH* loci was 100%. PCR amplification of capsicum varieties (reference samples) yielded 600bp for *rbcL* and *psbA-trnH* and 900bp for *matK* loci, respectively. *psbA-trnH* proved to be the ideal loci in discriminating *C. annuum* varieties from their adulterants. The band level identification of the adulterants was possible using this particular locus yielding 600bp for chilli varieties, 500pb for ‘choti ber’ and 350bp for beetroot.

Cloning, sequencing and BLAST analysis of all the seventeen market samples along with the reference samples gave 100% identity to *C. annuum* sequences for all the three loci. The phylogenetic tree constructed on the basis of K2P parameter with 1000 boot strap values using *psbA-trnH* locus grouped all the market samples to *C. annuum* cluster confirming the absence of any plant based adulterants in the chilli powder studied. The sequences generated

by amplification of barcoding loci viz., *psbA-trnH*, *rbcL* and *matK* were submitted with the NCBI. Barcodes were generated for all the reference sequence of *C. annuum* and were assigned Gen bank accessions ID.

In conclusion, using DNA barcoding technique, the detection of plant based adulterants in commercial market samples of powdered spices viz., black pepper, turmeric and chilli was achieved. The presence of chilli as an adulterant in commercial samples of black pepper powder was reported for the first time. Out of twelve market samples tested, two gave positive for the presence of chilli.

Among the barcoding loci *psbA-trnH* proved to be the ideal locus in detecting chilli adulteration in black pepper powder due to its capability to differentiate chilli and black pepper at band level itself. In case of turmeric out of ten different firms, plant based adulterants were found in four brands. Two market samples contained cassava starch, one was adulterated with *C. zedoaria* and another market sample contained undeclared fillers in it. Out of the eight barcoding loci *ITS* was found to be the ideal loci for turmeric samples.

In case of chilli powder samples, the technology confirmed the absence of any plant based adulterants from the seventeen different market brands. Among the loci tested *psbA-trnH* were found to be ideal in detecting the reported plant adulterants of chilli. The locus was able to differentiate among different adulterants by the band size discrimination. Simulation as low as 0.5 % adulteration was identifyable. The present study is the first report on the use of DNA barcoding technique in detection of adulterants in spice powders available in the Indian markets. The method proved to be simple, rapid, and highly sensitive. The cost of analysis of a single sample may be ₹. 3000/- (~40 US $) which includes cloning and validations.

The operating cost may further be reduced to half if an already standardised laboratory performs the initial screening by mere PCR amplification and sequencing followed by

BLAST analysis. In the present study all the adulterants could be detected by initial screening itself. The validation studies were further carried out to strengthen and authenticate the initial results. The cost may be further reduced when analysing more samples in a single batch.

The technology developed can be used by the regulatory authorities for large-scale screening of spice powders in authenticating genuine spice samples and to detect and prevent adulteration. The SNPs yielded could differentiate the genuine species from its closely related adulterants and can be further used for the development of species specific DNA barcoding kits. The development of diagnostic kits for adulteration detection of specific consignments can be future prospects of the present work.

The study proved the utility of DNA barcoding for routine screening of batches, which allows the immediate rejection of suspected samples or even in case of disputes arising from the quality of a lot. Purity of a natural product is the cornerstone of its perceived biological efficacy. Authentication of the value added products such as powdered spices is a daunting task in the face of unethical practices including substitution, adulteration, use of fillers, and mislabelling, DNA barcoding proved handy to meet these challenges.

Salient acheivements

- Standardised eight barcoding loci for black pepper, turmeric, chilli and their plant based adulterants
- Identified the ideal loci for each spice for the detection of adulterants in market samples of the spice.
- The technology could detect the presence of adulterant as low as 1g in 200 g of genuine powder
- Barcode database were generated and deposited with NCBI
- In black pepper, ***psbA-trnH*** proved to be the ideal locus with band level detection of the adulterants **Out of twelve b**lack pepper market samples analysed **two w**as found to be adulterated with chilli.

- In turmeric ***ITS*** locus was proved to be the best.
- **Out of 10** market samples, **four** was adulterated .The presence of *C. zedoaria* could be detected in one of the market samples whereas cassava starch was present in another two brands. Fillers like wheat, barley, and rye were present in one market sample.
- In chilli ***psbA-trnH*** was proved to be the best loci discriminating the adulterants at band level
- The seventeen market samples of chilli powder analysed were devoid of any plant based adulterants.

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ANNEXTURE

Annexture 1 Reagent preparation for manual DNA isolation protocol

Sl no.	Reagents	Preparation protocol
Stock Reagents		
1	1M Tris (pH 8)	Weighed and dissolved 121.1 g of Tris base (Sigma, USA) to 800ml of double distilled water and adjusted the pH to 8, using conc. HCl. The total volume was made up to 1000ml after adjusting the pH. The solution was sterilised by autoclaving.
2	0.5 M EDTA (pH 8)	186.1 g of Ethylene diamine tetra acetate. $2H_2O$ (Sigma, USA) was weighed and dissolved into 800ml of distilled water. A magnetic stirrer can be used intermediately for dissolving the salt. NaOH pellets were used to adjust the pH to 8.0, and the final volume was made up to 1000ml with double distilled water. The solution was sterilised by autoclaving
3	5M NaCl	292.2g of NaCl (Sigma, USA) was weighed and dissolved into 800ml of double distilled water. The final volume was adjusted to 1000ml after the salt completely dissolved. The solution was sterilised by autoclaving.
4	5M Potassium acetate	490.75g of Potassium acetate (Himedia, India) was weighed and dissolved into 800ml of double distilled water. The final volume was adjusted to 1000ml and the solution was sterilised by autoclaving.
Working Stock		

5	Extraction buffer/ CTAB buffer	100mM Tris HCl :100ml 20mm EDTA : 40ml 1.5M NaCl : 300ml 3% CTAB : 30g
6	Potassium acetate solution	60ml of 5M potassium acetate (Himedia, India) solution was mixed with 11.5ml of glacial acetic acid (SRL, India) and 28.5ml autoclaved double distilled water.
7	Poly ethylene glycol (PEG) -30%	Weighed and dissolved 30g of PEG 6000 (Himedia, India) to 1000ml of distilled water
8	Chloroform: isoamyl alcohol (24:1)	96ml of chloroform (Merck, India) was mixed with 4ml of isoamyl alcohol (Merck, India). The solution was stored in reagent bottles at 4˚C.
9	Phenol: Chloroform: isoamyl alcohol (25:24:1).	Mixed 25ml of Tris saturated phenol (pH>6.8) (Merck, India) with 25ml of Chloroform: isoamyl alcohol (24:1). The solution was stored in brown coloured reagent bottles at 4˚C.
10	1% PVP (Poly Vinyl Pyrrolidone)	1g of PVP was dissolved in 100ml of autoclaved double distilled water
11	70% ethanol(Sigma, USA)	70ml of 100% absolute ethanol was mixed with 30ml of autoclaved double distilled water.
12	β- mercaptoethanol (Sigma, USA)	0.3% β- mercaptoethanol was freshly added to the extraction buffer
13	RNase A(Genei, Bangalore, India)	Ready to use RNase A (10mg/ml) were used.

Annexture 2. Reagents used for electrophoresis

Sl no.	Reagents	Preparation protocol
1	10X TBE buffer (Tris borate buffer)	Dissolved 108g of Tris base (Sigma, USA), 55g of boric acid (Himedia, India) in 800ml of distilled water. 40ml of 0.5 M EDTA (pH-8) (Sigma, USA) was added to it. The final volume was made up to 1000ml. A working stock of 1X TBE buffer (1000ml) was made by mixing 100ml of 10X TBE with 900ml of water.
2	0.8% agarose	0.8g of agarose (Merck, India) was dissolved in 100ml of TBE buffer
3	6X gel loading buffer	Dissolved 25g of Bromophenol blue (Himedia, India) and 25 mg Xylene Cynol FF(Himedia, India) in 74ml of water and mixed with 26ml of 87% Glycerol (Himedia, India) using a magnetic stirrer. The solution was stored at 4°C.
4	Ethidium bromide	1g of ethidium bromide (Himedia, India) was added to 100ml of distilled water and stirred using a magnetic stirrer. The solution was stored in dark reagent bottle at room temperature.

Annexture 3. Reagents/media used for transformation experiments

Sl no.	Reagents	Preparation protocol
1	Luria-Bertani (LB)broth	25.0g of LB broth – Miller (Himedia, India) was dissolved in 800ml of distilled water. Made up the final volume to 1000ml using distilled water and sterilised by autoclaving
2	Luria-Bertani (LB) agar	30.0 g of LB agar-modified (Himedia, India) was dissolved in 800ml of distilled water. Made up the final volume to 1000ml using distilled water and sterilised by autoclaving.
3	X-Gal Stock solution (20mg/mL)	Dissolved 200mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in 10ml N, N-dimethylformamide. The solution was stored at -20 ^{o} C in the dark. 40 µl were used per plate
4	IPTG stock solution (100mM)	1.2g of IPTG (isopropyl- β- D- thiogalactopyranoside) was dissolved in 50ml deionised water. Filter sterilised and stored at 4 ^{o} C. 40 µl were used per plate
5	Ampicillin stock solution (50mg/mL)	Dissolved 2.5g of ampicillin sodium salt in 50ml of deionized water. Filter sterilised and stored at -20 ^{o} C. 1ml of ampicillin stock was added per plate to get a final concentration of 50µg/ml.