Study on Genetic Diversity in *Piper SPP*.using Inter Simple Sequence(ISSR),Random Amplified Polymorphic DNA(RAPD) AND Start Codon Target (SCoT) MARKERS

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CERTIFICATE-1

This is to certify that the thesis entitled **"*Study on Genetic Diversity in Piper spp. Using Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA(RAPD) and Start Codon Targeted (SCoT) markers.* "** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science in Agriculture (Agricultural Biotechnology)** to the Orissa University of Agriculture and Technology is an authentic record of *bona fide* and original research work carried out by ***Jadhav Akshay Ambadas*** **(Adm. No. 06ABT/14)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help obtained by him from various sources during the course of investigation has been duly acknowledged.

(Dr. G. R. Rout)

Chairman
Advisory Committee

CERTIFICATE-II

This is to certify that the thesis entitled **"Study on Genetic Diversity in Piper spp. using Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD) and Start Codon Targeted (SCoT) "**submitted by ***JADHAV AKSHAY AMBADAS*** **(Adm. No. 06ABT/14)** to Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture (Agricultural Biotechnology)** has been approved by the students' Advisory Committee and the external examiner.

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Abstract

Piper is an economically and ecologically important genus which belongs to the *Piperaceae* family. The genus *Piper* includes about 3000 diverse species of herbs, shrubs and climbers, a few of which are economically important as spice or medicinal plant. Taxonomically, *Piper* is a complex genus due to its greater range of variability and minute nature of flowers. Thus, efficient use of genetic resources in plant breeding programs requires knowledge about genetic diversity. Various molecular markers are used for analysis of genetic diversity like a RAPD, ISSR, SCoT, SCAR etc. In present study, total 31 *Piper* genotypes were collected from different geographical regions of Odisha state, India. All genotypes were subjected for exhaustive molecular characterization. Molecular markers such as ISSR, RAPD and SCoT were used to develop DNA fingerprints of 31 piper genotypes to evaluate the genetic variation of *Piper* accessions. ISSR and SCoT markers were generated distinct fingerprints with the highest polymorphic bands 98% and 100% respectively. The genetic similarity coefficient ranged from 0.25 to 0.93 within 31 *Piper* accessions on the basis ISSR profile. On the basis of cluster analysis by using ISSR we got greater similarity among *Piper longum* accession OP-15 and OP-16 (93%) respectively. One shows less similarity among the *Piper* accession i.e. *Piper longum* OP-9 (25%). SCoT markers were also employed to analyse the genetic diversity of 31 *Piper* accession. The genetic similarity coefficient ranged from 0 to 1 on the basis of SCoT profile. Cluster analysis were done on the basis of SCoT profile from this we got two *Piper* accession which having greater similarity. i.e. *Piper longum* accessions OP-26 & OP-28. From analysed data, we got one *Piper* accession (i.e. OP- 23) has greater diverse among the *Piper* accessions. In ISSR analysis we got highest band 1250 bp in ISSR (USB-835) primer and in SCoT (SCoT-5) analysis highest band were found 1000 bp. This investigation will help to develop new genotypes using breeding program.

ABBREVIATION

µg	=	Micro gram
µl	=	Micro litre
µM	=	Micro mole
Bp	=	Base pair
CTAB	=	N-Cetyl-N,N,N-trimethyl ammonium bromide
dNTP	=	Deoxy nucleotide triphophate
EDTA	=	Ethylene Diamine Tetra Acetate
et al.,	=	*et alia* (Latin : and others)
EtBr	=	Ethidium Bromide
ISSR	=	Inter-Simple Sequence Repeat
Mg	=	Milli gram
Min	=	Minute
Ng	=	Nano gram
PCA	=	Principal Component Analysis
PCR	=	Polymerase Chain Reaction
PIC	=	Polymorphism Information Content
RAPD	=	Randomly Amplified Polymorphic DNA
SCoT	=	Start Codon Targeted
RNase	=	Ribonuclease
RP	=	Resolving power
SSR	=	Simple Sequence Repeat
Taq	=	Thermos aquaticus
TBE	=	Tris boric acid EDTA buffer
TE	=	Tris EDTA
UV	=	Ultra violet
Nm	=	Nano meter

INTRODUCTION

The utility of remedial plants has long been practiced under the aboriginal systems of remedy like Ayurveda, Siddha and Unani for the reasons of availability, effectiveness, diversity and affordability. There is a universal as well national resurgence and revival of faith in drugs of plant rise including traditional medications. In spite of great advances of modern scientific remedy traditional or alternative medication is to stifle the primitive and trustworthy system than the modern remedial system, especially in developing countries like India and is gaining popularity and acceptance globally. The inventor of a potent natural product penicillin, Nobel-laureate Ernst Boris Chain wrote an inspiring article entitled "The quest for new biodynamic substances. The glory of Indian spices known throughout the world is 'The Land of Spices'. Standard ISO 676: 1995 of the International Organization for Standardization (ISO) defines spices and condiments as "vegetable products or mixtures thereof free from extraneous matter, used for flavouring, seasoning and imparting aroma in foods". In (1967) "In China and India there has been an extensive drive aimed at the systemic study of remedial plants traditionally used in these countries in folklore medicine. This has failed so far, to bring to light new classes of compounds with interesting pharmacologic activities.

Global estimates suggest that, over three-fourths of the total world population cannot afford the products of allopathic remedy and thus have to rely upon the use of traditional medications, which are largely derived from plants. Herbal remedy have been appliance for thousands of years in many parts of the world. Medical knowledge began with scattered references in the 'vedas'. Ayurveda is a branch of 'Atharva veda'. The self created Brahma before creating men, first formulated this science of life. Herbal remedy make up a major share of all the officially confession systems of health in India viz. Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy, except Allopathy. Peoples in developing countries still relay on traditional remedy based largely on classes of plants for their primary health care. Today estimate that, greater than 70% of India's 1.1 billion population still use these non-allopathic systems of remedy. Around 500 plants with remedial use are revealed in ancient literature and around 800 plants have been used in aboriginal systems of medicine. India is a vast repository of remedial plants that are used in traditional remedial treatments

(Chopra et al, 1956). Herbal remedies has effectiveness, easy availability, low cost and comparatively being devoid of serious toxic effects popularizes herbal remedies. The use of herbal remedy becoming popular due to non-toxicity and no side effects as compare with allopathic remedies. In India, about 20,000 remedial plant species have been recorded but more than 500 traditional communities use about 800 plant species for curing different diseases (Kamboj, 2000). Presently, 80% of the world population depends on plant-derived medicine for the first line of primary health care for human alleviation because it has no side effects. The remedial establishment has become a threat to health" wrote Ivan Illiach an eminent researcher in his book "Limits to Medicine". The modern allopathic system of medicine stakes no claim to its ability to fully cure several difficult to treat chronic diseases. The industrial petition for the remedial plant resources has been on rise due to the worldwide resilience in the herbal sector engaged in production of herbal health care formulations herbal based cosmetic products and herbal nutritional supplements. Many plant derived molecule have shown a promising effect in therapeutics (Lokhande et al., 2007). Spices and herbs are recognized as sources of natural antioxidants and thus play an important role in the chemoprevention of diseases and aging. It contains about 2,000 species of shrubs, herbs, and lianas, many of which are basis species in their native habitat. Amongst various remedial plants, one of the showing enormous potential is the pepper family otherwise known as *Piper*aceae (Dodson et al., 2000). It has play a significant role in human welfare.

The genus *Piper* the largest family of *Piperaceae*. These family contain more than 3,000 species reported from the tropical and subtropical regions all over the world (Rahiman and Nair,1983). Long Pepper is a climber, of South Asian origin (Deccan peninsula), cultivated for its fruit, which is usually dried and used as a spice and seasoning. *Long pepper* contain biological active component which is *Piper*ine. Plants play a major role in the discovery of new therapeutic agents. They have received a lot of attention during these days for the isolation of biologically active substances for the cure of diseases. Plant based medicinal compounds is long established to be used as traditional treatment for innumerable human diseases from time immemorial in many parts of the world. *Long pepper* is a close relative of the black pepper plant, and has a similar, though generally hotter, taste. The word *pepper* itself derived from the Sanskrit word for *long pepper* is pippali. It is a slender, aromatic, climber with perennial woody roots, creeping and jointed stems, and fleshy fruits embedded in the spikes. Leaves are numerous, 6.3 to 9.0 cm, broadly ovate or oblong-oval, dark green and shining above, pale and dull beneath. The older leaves are dentate, dark in

colour and heart shaped. The younger leaf is ovate in shape and contains 5 veins on them. Flowers are monoecious and male and female flowers are borne on different plants. Male flower stalk is about 1 to 3 inch long and female flower stalk is ½ to 1 inch long. Fruit is long. When it ripens it attains red colour and when it dries it attains black colour. It is one inch in diameter. The plant flowers in rains and fruits in early winters.

There are more than 2400 plant species belonging to 189 plant families these are said to be rich sources of bioactive organic compounds (Rao et al., 2005). Among those families of plants investigated as yet, one of the showing enormous potential is the *pepper* family, otherwise known as *Piperaceae* (Dodson et al., 2000). Though there is morphological variation among the gene pool of *Piper*, informative and neutral molecular markers, with a high degree of polymorphism, are important for evaluation of the variability existing in germplasm collections as they supplement the morphological variab ility, providing a betterfocus for conservation efforts, and generating guidelines or the development of cultivar improvement program.

Molecular tools hold the promise of allowing the identification of genes involved in a number of traits, adaptive traits, and polymorphisms causing functional genetic variation. DNA markers are useful in phylogenetic analysis and search for useful genes. Inter-simple sequence repeat (ISSR) was first employed by Zietkiewicz and Gupta et al. (1994) and has been proved to be a highly useful tool for estimating genetic diversity and assessing genetic relationships. It is simple, fast, cost-effective, reliable and highly discriminating (Ci et al., 2008; Crespe et al., 2009; Zhang and Dai, 2010; Uysal et al., 2010; Petros et al., 2008). New marker has been developed for the assessment of genetic diversity called Start Codon Targeted Polymorphism (SCoT) (Collared and Mackill 2009).It is type of targeted molecular marker technique. SCoT marker is based on the conserved regions which is surrounded by start codon ATG (Swant et al 1999). A few genetic studies using molecular marker RAPD have been conducted on analysis of genetic diversity within Indian cultivateros of *Piper spp.*

On the view of the above fact, the aim of present study is genetic diversity of *Piper* species by using molecular markers with the following objectives;

Objective:

- To collection of different germplasm of *Piper spp.*
- To isolate the genomic DNA from different *Piper* accession.
- To screen the germplasm through different molecular markers such as ISSR, RAPD and SCoT marker.
- To check the molecular diversity among different *Piper* accession.

REVIEW OF LITREATURE

Piper is an economically and ecologically important genus which belongs to the *Piperaceae* family. The genus *Piper* includes about 3000 diverse species of herbs, shrubs and climbers, a few of which are economically important as spice or medicinal plant. The *Piper* species, widely distributed in the tropical and subtropical regions of the world are used medicinally in various manners. *Piper* species are of high commercial and economical importance such as *Piper Nigrum* and it has worldwide spice market. *Piper* species have been also used in variety of traditional medicinal systems such as Traditional Chinese Medicine, Indian Ayurvedic System, Folklore Medicine of Latin America and West India. Medicinal plants play a vital role in the lives of rural people, in remote parts of developing countries with limited facilities for health care (Purohit and Prajapati, 2003). The plants of genus from *Piper* are also used for many other purposes such as foods and spices, fish poison, hallucinogens, insecticides, oils, ornaments, perfumes etc. The ripened fruit of *P. nigrum* is the source of white pepper, while the unripe fruit of the same species is the source of black pepper. Several species of *Piper* are grown domestically as house plants for their foliage. Plants belonging to the genus *Piper* are reputed in the Indian Ayurvedic system of medicine for their medicinal properties (The wealth of India, 1969). Chloroform extract of the stems of *P*. aborescens was found to display significant activity against a KB cell culture system and a P.388 lymphocytic leukaemia system in cell culture (Geran *et al,* 1972). *P.syfvaticum* roots are used as an effective antidote to snake poison in the indigenous system of Indian medicine. *P. chaba* roots and fruits find numerous applications in medicine. In particular they are useful in asthma, bronchitis, fever, pain in abdomen, as stimulant and in haemorrhoidal afflictions (Kirtikar *et al,* 1933). The *P. guineense* is a woody climber distributed throughout West Africa (West African black *pepper*). Its fruits have been used as a flavouring, while preparations of leaves, roots and seeds have been used internally as medicinal agents for the treatment of bronchitis, gastrointestinal diseases, venereal diseases and rheumatism. The preparations obtained from the seed kernels have

been used for their counter irritant and insecticidal properties (Irvine *et al*, 1961). Tremendous work is going on medicinal properties of *Piper* spp. and on its diversity.

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- **2.1 Taxonomy of *Piper spp*.**

Botanical classification

Kingdom	Plantae
Division	Magnoliophyte
Class	Mangnolipsida
Order	*Paperless*
Family	*Piper*aceae
Genus	*Piper*
Species	*longum*

The genus *Piper* was established by Linnaeus. The first report of *Piper* species of the Malabar region was by Van Rheeda in his 'Hortus Indicus Malabaricus' in which he describes five species of which four were illustrated. Linnaeus (1753) described 17 species and assigned five of them to Indian peninsula. Gamble (1925) reported the Flora of Presidency of Madras described 14 species. The hierarchical clustering is a visual representation of the closeness or the distance of the species. A dendogram scales the actual distance or the relativeness of the species. Ravindran (1997) had done the principal component analysis the genus *Piper* with the aim to group the related cultivars.

2.2 Distribution of *Piper Species*

Piper longum L. is the *pepper* plants consist of 2,000 species of shrubs, herbs, and lianas. *Piper longum L.* (Long *Piper*) is a native of the North East India. Its cultivate throughout the hotter parts of India such from Himalayas to Assam, Khasi & the Mikir hills, the lower hills of West Bengal and evergreen forests of the Western Ghats from Konkan to Travancore. Which is one of the most 20 species rich in genera of flowering plants and it is economically and ecologically important genus in the family *Piper*aceae.

Taxonomically *Piper* is a complex genus due to its greater range of variability and minute nature of flowers.

2.3 Medicinal property

Since ancient times, plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made a large contribution to human health and well-being. To promote the proper use of herbal medicine and to determine their potential as sources for new drugs. It is essential to study medicinal plants which have folklore reputation in a more intensified manner. More than 50000 species are used for medicinal purposes in worldwide from which almost 13% are flowering plants reported by Schippmann *et al,(* 2002). Above the past few years, many efforts have been made to discover new antimicrobial compounds from various kinds of natural resources such as microorganisms, animals and plants. In this regards Indian medicinal plants have been evaluated, a fair number of which possess potential antimicrobial activity and few natural products have been approved as new antibacterial drugs. Plants are known to produce some chemicals that are naturally toxic to bacteria. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct.

2.3.1 *Piper longum*

Piper longum L. is an aromatic climber with stout roots, jointed stems, and ovate leaves belongs to the family *Piperaceae*. P. longum is a native of Indo-Malaya region. Plants are monoicous in nature where male and female flowers are borne on different plants. These have eract, delicate with swollen nodes, roots clinching at nodes which help it to attach host trees. Arrangement of leaves is alternative like that shape ovate, apex acute to acuminate with entire margin. The *Piper* fruit which is a spikes long cylindrical, oblong, berries red or black when it is ripe and become smooth with aromatic odour and pungent taste. Which is very sparsely distributed in forests of the Western Ghats of India. *Piper longum Linn.(Piperaceae)* are accepted source of the drugs *Pippali* and *Pippalimulam* is roots of this plant (Sivarajan and Balachandran,1994). *Piper longum* is rich in two major

alkaloids, *Piperine* and *Piperidine* (Kanaki *et al*, 2008). The active compounds present in these natural products serve either as templates or as intermediates for synthetic drug ($C_{17}H_{19}NO_3$) is an alkaloid found in the fruits and roots of *Piper longum* species of *Piperaceae* family. Fruits of *Piper longum Lnn.* are very well known medicine for disease of respiratory tract, analgesic when applied locally for muscular pains and inflammation and general tonic and hematinic. This alkaloid is responsible for the pungency *long pepper (P.longum)* along with chavicine is an isomer of *Piper*ine. Fluckiger and Hanbury found that species of *"long pepper" P.longum* and *P.officinarum* also contain this alkaloid. The pungency of *Piperine* is caused due to the activation of the heat and acidity sensing Transient receptor potential vanilloid (TRPV) ion channel TRPV1 on nociceptors (pain sensing nerve cells). *Piperine* has diverse biological and therapeutic activities. It can dramatically increase the absorption of selenium, vitamin B and βcarotene as well as other nutrients. It can stimulate pancreatic and intestinal digestive enzymes and also increases biliary bile acid secretion when orally administrated. In addition to its involvement in increasing the absorption of other nutrients in the body, *Piperine* has other novel applications like helping to fight against colon cancer. It has anti-inflammatory, thermogenic, growth stimulatory, anti-thyroid and chemo preventive activities.

Piper longum is a highly valuable drug and is one of the essential ingredients in the most of the compound preparations included in Ayurvedic literature. In traditional and Ayurvedic medicines, mature spikes of the female plants *(long pepper)*, thick stems, roots and leaves are used against bronchial diseases, dyspepsia, worms and amoebiosis (Ghosal et al, 1996). The spikes have also been effectively used as an anti- inflammatory, memory enhancing and aphrodisiac agent (Mujumdar et al, 1999). When used in conjunction with a drug *Piperine* has been shown to improve the drug's bioavailability. Further, the use of the plant as a bioavailability enhancer has immensely increased its importance in the field of Ayurveda. Plant derived drugs secured importance in recent years because of their unrefuted efficacy as phytomedicines. *Piper longum* has various chemical component which aimed for develop biodegradable insecticidal component and anticancer drug. It has also gained significance in processing of essential oils and oleoresins that are used as food additives, in pharmaceuticals and as insecticide.

2.3.2 *Piper betel*

The leaves of *Piper betel* locally known as Pan. Leaves of *betel* vine are used with various condiments such as areca nut (kattha), cloves, cardamom, arecanut, candied rose and fennel for chewing purposes (Verma et al., 2004). It has long been in use in the Indian local system of medicine for its phytochemical properties such as antioxidant and antimicrobial. In the recent work, the antimicrobial activity present in *Piper betel* leaves (ethanol leaf extract) were evaluated against human pathogenic bacteria. The leaf powder was subjected to phytochemical screening and was found to contain carbohydrate, protein, polyphenolic compounds, flavonoid, alkaloids and total antioxidant.

- In Indian folkloric medicine, *betel* leaf is popular as an antiseptic and is commonly applied on wounds and lesions for its healing effects. This particular property has paved way for further experimental studies, which have established pan extract to have antimicrobial and antileshmian properties (Sarker et al., 2008).

Piper betel leaves contained caryophyllene, cadinene, γ-lactone, allyl catechol, p-cymene and eugenol methyl ether in varying amounts. The alcoholic extract of the leaf-stalk showed significant antifertility effects in both male and female rats. Recently, cepharadione dotriacontanoic acid and tritriacontane were reported to be isolated from the petrol extract of leaves; *Piper*ine and *Piper*longuminine from petrol + dichloromethane extract of stems and β-sitosteryl palmitate from petrol plus dichloromethane extract of root parts of *P. betel.*

2.3.2.1 Phyto-consitutions

Piper betel is blessed as evergreen and perennial plant, that has God created and have given the shape of his own heart. The leaf contains Water (85-90%), Proteins (33.5%), Carbohydrates (0.5-6.1%), Minerals (2.33.3%), Fat (0.4-1%), Fibre (2.3%),Essential oil (0.08 0.2%), Tannin (0.1 1.3%), Alkaloid (arakene). It also contains different vitamins like Vitamin C(0.0050.01%), Nicotinic acid(0.630.89mg/100gms), Vi tamin A (1.92.9mg/100gm)Thiamine (10-70µg/100gms), Riboflavin (1.9-30µg/100gms) beside this it contains minerals such as Calcium (0.2-0.5%), Iron (0.005-0.007), Iodine (3.4µg/100gms), Phosphorus (0.05-0.6%), Potassium (1.1-4.6%). It contain bitter compounds that are about (0.7-2.6%). The specific strong pungent aromatic flavour in leaves is due to phenol and terpene like bodies. The total phenol contain is vary on the gender. The male plant contains three fold higher total phenols content and two fold

higher thiocyanate content as compare to female plant. The quality of the leaf depends upon the phenolic content, i.e., more the phenolic content betters the leaf quality.

2.3.3 *Piper Nigrum*

Piper Nigrum Linn. are world's important and oldest spices, commonly known as *'Black pepper'*. It. is commonly known as Pepper or Kalimirch. *Piper nigrum L.* is used as drug in Ayurveda, Unani and Siddha system of medicine. *Piper*ine is a characterizing compound present in fruits of *Piper nigrum* used as bioavailability enhancer. *Piper*ine is an alkaloid found naturally in plants belonging to the pyridine group of *Piper*aceae family, such as *Piper nigrum* and *Piper lo ngum*. *Piper*ine is the Trans stereoisomer of 1 *Piper*oyl*Piper*idine. It is also known as (E, E)-1- *Piper*oyl*Piper*idine and (E, E)-1- [5-(1, 3- benzodioxol-5-yl)-1-oxo-2, 4-pentdienyl] *Piper*idine. *Piper*ine is the alkaloid responsible for the pungency of *black pepper* and *long pepper,* along with chavicine (an isomer of *Piper*ine). It has also been used in some forms of traditional medicine and as an insecticide. It is widely used as a pungent condiment. The fruit of the *Piper* plant is a common ingredient in many recipes. Dried fruit is employed commercially as a condiment. It is warming, drying and stimulating to the circulatory, digestive and respiratory system. The fruits have variety of activity including CNS depressants, antipyretic, analgesic, hepatoprotective (Gupta et al, 1986).Bioavailability enhancer (Mujumdar et al, 1999 ; Annamalai et al, 1989), Antioxidant (Khajuria et al, 1997), anti-inflammatory. The phytoconstitutes of *P.nigrum* and *P. longum* fruits include volatile oil, other minor alkaloids such as piplartine, *Piper*logumine, *Piper*idine, starch, resin. and pungent alkaloid *Piper*ine (Kokate et al, 1994; Evans et al, 2004; Khare et al, 2006). It has antimicrobial (Dorman and Deans, 2000), antimutagenic (El-Hamss et al., 2003), antioxidant and radical scavenging property (Gulcin, 2005) and inhalation of *black pepper* oil increase the reflexive swallowing movement (Vijayakumar et al., 2004). Cubeb (*Piper cubeba*), or tailed *pepper,* is a plant in genus *Piper,* cultivated for its fruit and essential oil. Majeed (1999) reported that *Piper*ine is widely used in various herbal cough syrups for its potent anti-tussive and bronchodilator properties. The chemistry of *Piper species* has been widely investigated and the phytochemical investigations from all parts of the World have led to the isolation of a number of physiologically active compounds viz. Alkaloids amides,

propenylphenols, lignin's, neolignans, terpenes, steroids, kawapyrones, *Piper*olides, chalcones, dihydrochalcones, flavones and flavanones. Lee et al. while doing a pharmacological study on *Piper*ine reported that it displayed central nervous system depressant (antagonism of electroshock induced seizures and muscle relaxation in mice), antipyretic (typhoid vaccinated rabbits), analgesic (taildip presence and writhing in mice) and anti-inflammatory (carrageenan-induced oedema in rats) activities. Miyakado et al. isolated *Piper*idine from *P*. nigrum which showed insecticidal activity against the adzuki bean weevil (Callosobruchus chinensis).

Amongst various remedial plants, *Piper* species under family *Piperaceae* has significant role in human welfare. Many plant derived molecule have shown a promising effect in therapeutics (Lokhande et al., 2007). In Jamaica, of the eleven *Piper* species known, *P.aduncum* and *P.hispidum* are listed as remedies for stomach aches and as insect repellents (Asprey et al, 1954). *P. amalago*, distributed from Mexico to Brazil is used to alleviate chest pains and as anti-inflammatory agent. *P.syfvaticum* roots are used as an effective antidote to snake poison in the indigenous system of Indian medicine. *P. chaba* roots and fruits find numerous applications in medicine. In particular they are useful in asthma, bronchitis, fever, pain in abdomen, as stimulant and in haemorrhoidal afflictions (Kirtikar *et al*, 1933). P. brachystachyum shows insecticidal properties (Jacobson *et al*, 1971), The stem of *P. futokadsura*, known as haifengteng is widely used in the Chinese herbal medicinal prescriptions for the treatment of asthma and arthritic conditions whereas *P. futokadsura* is a medicinal plant that grows in Fuchein and Taiwan Provinces; the benzene extract of its leaves showed anti-feed ant activity against the larvae of Spodoptera litura. An extract of the *black pepper* shows carcinogenesis in mice. The evidence of malignant tumours and of multiple tumours was greater in the *pepper* treated mice than in vehicle treated mice (Concon *et al*, 1979).

2.3.3.1 Chemical composition

Chemical studies carried out on Brazilian *Piperaceae* species have revealed the occurrence of pyrones, lignoids and chromenes besides various amides bearing isobutyl, pyrrolidine, dihydropyridone and *Piper*idine moieties. These amides have generated interest as a result of their potent insecticidal and antifungal properties. The structures of the several antifungal amides N-[7-(30, 40-methylenedioxyphenyl)-2(Z), 4(Z) heptadienoyl] pyrrolidine, (3Z, 5Z)-N-isobutyl- 8-(30, 40- ethylenedioxyphenyl) -

heptadienamide isolated from leaves of *Piper* hispidum and 8(Z)-N- (12, 13, 14-trimethoxycinnamoyl)-3-pyridin-2-one from *Piper* tuberculum besides eight known antifungal amides have already been reported. Two new amides which are N [10 (13,14 methylenedioxyphenyl) 7(E),9(Z) pentadienoyl] pyrrolidine,arbore mine and nine known antifungal amides N [10 (13,14 methylenedioxyphenyl) 7(E) pentaenoyl] pyrrolidine, its derivative N [10](13,14 methylenedioxyphenyl) pentanoyl] pyrrolidine and N [10 (13,1 4 methylenedioxyphenyl) 7(E),9(E) pentadienoyl] pyrrolidine; besides pellitorine, abdih ydro*Piper*ine , piplartine, dihydropiplartine, cis piplartine (or 8(Z) N (12,13,14 trimethox ycinnamoyl) 3 pyridin 2 one) and fagaramide have also been reported recently. In additio n to these amides two cinnamoyl derivatives, methyl 6, 7, 8 trimethoxydihydrocinnamate and methyl trans 6, 7, 8 trimethoxycinnamate have also been isolated (Bernard, et al 1999) he amides isolated from leaves of *Piper arboreum* and their hydrogenated derivatives are active against the fungus Cladosporium sphaerospermum, and the compounds isolated from seeds and leaves of *Piper tuberculatum* are also active against the fungi *Cladosporium sphaerospermum and C. Cladosporioides.*

2.4 *Piper*ine content

Biological source of *Piper*ine is dried unripe fruit of *Piper nigrum*, *Piper longum and Piper clusii* and also in the root of bark of *Piper geniculatum* belonging to family *Piper*aceae. *Piper*ine was the first amide to be isolated from *Piper* species. *Piper*ine is the main therapeutically active constituent of this plant. It has diverse activities such as biological and therapeutic. It can dramatically increase the absorption of selenium, vitamin B and βcarotene as well as other nutrients and it can also stimulate pancreatic and intestinal digestive enzymes. It has ability to increases biliary bile acid secretion when orally administrated. In addition to its involvement in increasing the absorption of other nutrients in the body, *Piper*ine has other novel applications like helping to fight against colon cancer. It has several activities like anti-inflammatory, thermogenic, growth stimulatory, anti-thyroid and chemo preventive etc. This also displays antipyretic, analgesic, insecticidal, immunomodulatory, antitumor, anti-depressant and anti-apoptotic activities.

- The dried unripe fruits are extracted with ethanol in a Soxhlet apparatus till extraction is complete. The solvent is evaporated under vacuum in a Rotary Thin

Film Evaporator. The residue of the alcoholic extract is digested with dilute alkali to affect saponification, when *Piper*ine remains unaffected. The residue, thus obtained is decanted and washed with distilled water several times. The resulting product is dissolved in hot ethanol and on cooling the crystalline *Piperine* separates out. It is obtained as monoclinic prisms from alcohol having melting point 130°C and is tasteless at first, but has a burning aftertaste. Its dissociation constant pK (18°C) is 12.22. It is soluble in ethanol, chloroform, ether; freely soluble in acetic acid and benzene; and almost insoluble in water (40 mg/l at 18°C), and petroleum ether.

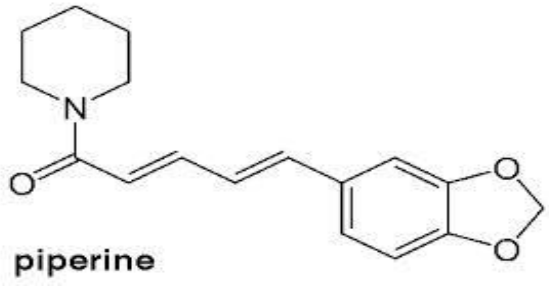
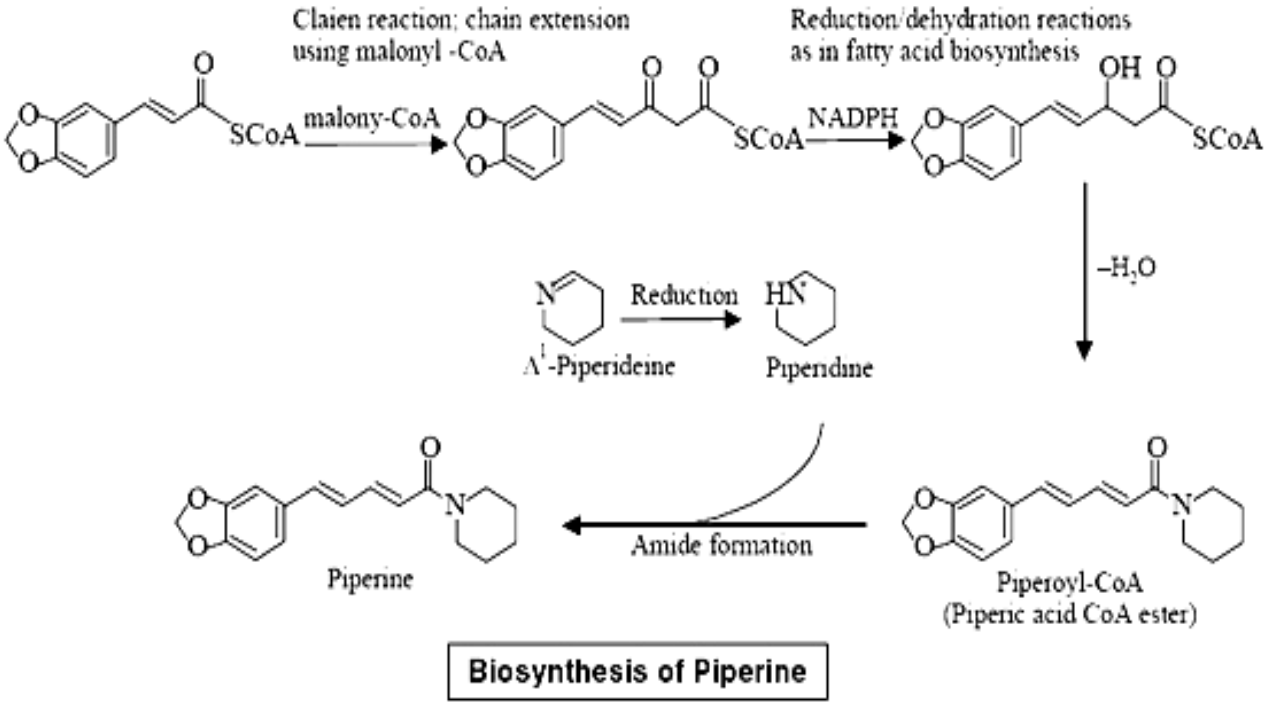


Fig: Chemical structure of *Piperine*

In biosynthesis of *Piper*ine, the *Piper*idine ring forms part of a tertiary amide moiety which is incorporated via *Piper*idine itself i.e., the reduction product of D1-*Piper*iedine as shown under. Interestingly, the pierce acid residue in obtained from a cinnamoyl-COA precursor. The extension of chain is caused by virtue of acetate/malonate and ultimately combines as its precursor with the previously obtained *Piper*idine nucleus.



Biosynthesis of Piperine

2.5 Genetic Diversity and Molecular Markers

Genetic diversity refers to variation of genes within species that is the heritable variation within and between population of an organisms. Most higher organisms (both plants and animals) reproduce sexually that is they produce offspring through the union of reproductive cells from two different parents. Sexual reproduction is critical for maintaining genetic diversity within a species because it combines the parents genetic material, resulting in offspring with unique genetic blueprints different from either parent.

Genetic diversity is important for two reasons. 1) when a population of an organism contains a large gene pool that is, if the genetic blueprints of individuals in the population vary significantly the group has a greater chance of surviving and flourishing than a population with limited genetic variability.

Because some of the individuals may have inherited traits making them particularly resistant to disease or tolerant of cold, or they may possess other traits that increase their chance for survival. In nature, the "fittest" individuals succeed and go on to reproduce

Darwin termed this process "natural selection." Suppose there's an outbreak of a disease that threatens to wipe out an entire species. The more genetic variability there is within that species, the higher the likelihood that at least some of the individuals will be resistant, and will survive. In the lab, plant breeders take advantage of these genetic variants to improve existing plants and create new varieties. Through cross breeding they strive to breed in disease resistance, superior fruit production, increased cold tolerance, or other desirable traits.

- 2) Genetic diversity also reduces the incidence of unfavourable inherited traits. In a small, isolated population of organisms, individuals may be forced to breed with close relatives. When this happens, the genetic makeup of the individuals becomes more and more uniform, and genetic flaws become increasingly more common. This phenomenon is called inbreeding. When closely related organisms interbreed, any genetic weaknesses that are hidden in the parents can be multiplied in the offspring. For example, animals can be carriers of a gene for an inherited disease, but not show any symptoms. If they mate with a partner who is also a carrier, then the offspring may exhibit symptoms of the disease. In an inbred population, chances are greater that carriers will interbreed. Over time, the entire population is weakened.

Germplasm improvement and genetic diversity is the key to durable and sustained production of *Dactylis glomerata*. There have been comparisons of molecular markers for estimating genetic diversity and also the combined analysis from all marker systems in different species. (Bhattacharyya P and Kumaria S 2015).

The intra-population genetic diversity is much higher than inter-population. Similar results were described by Madesis et al.(2013) in *Dactylis glomerata*

Manners et al.(2013) stated that, in *Vanda coerulea* showing that was higher genetic diversity within population that inter-population.

Taxonomically *Piper* is a complex genus due to its greater range of variability and minute nature of flowers. Several authors attempted to classify the *Piper* species based on morphological, cytological, biochemical, and yield potential. However differentiation of species only through morphological features are inefficient and even inaccurate. Thus,

efficient use of genetic resources in plant breeding programs requires knowledge about genetic diversity.

2.5 Molecular Markers

A genetic marker is a measurable character that can detect variation in either protein or DNA sequence. A difference whether phenotypic or genotypic may act as genetic marker if it identifies characteristics of an individual's genotype and phenotype and if its inheritance can follow through different generation.

2.5.1 Genetic markers types

Morphological Traits: Morphological differences assessing the diversity within and between populations

Biochemical Markers: Biochemical markers based on the migration properties of proteins which allow separation by electrophoresis.

- **Molecular Markers:** Molecular markers detected polymorphisms in the DNA sequence of nucleus and organelles. Molecular markers concern the DNA molecule itself and such as are considered to be objective measures of variation. A number of DNA based markers are now available for the effective quantification of genetic variation in plant species.

2.6 DNA markers

Markers	Codominant	Polymorphism
RAPD	NO	Medium
ISSR	NO	High
SCoT	NO	High
EST	Yes	Low/Medium

Table 2.1: List of DNA markers use for quantification of genetic variation

2.6.1 Random Amplify Polymorphic DNA (RAPD)

RAPD analysis is simple, less expensive, and rapid. It has the ability to detect extensive polymorphisms which require minute amounts of genomic DNA even without prior knowledge of DNA sequences. RAPD markers have been widely used in genetic diversity studies due to their great resolution power compared to morphological markers, the large number of DNA bands that can be obtained in relation to isoenzymes and because of their simplicity and practicality compared to other DNA markers (Bhat et al. 1999). Random amplified polymorphic DNA (RAPD) technique (Welsh and McClelland 1990; Williams et hybrid origin. al. 1990) has been widely used in the analysis of genetic diversity in plant species (Karp and Edwards 1995; Karp et al. 1998). In *Piper*, the technique has been successfully utilised in identifying soma clonal variants of P. longum L. (Parani et al. 1997). To estimate the genetic diversity in germplasm collections among individuals belonging to the same species or different species, one might choose to apply RAPD (Sen S, et al,2010)

- DNA fingerprinting techniques have been widely used to analyse the genetic variation and to differentiate of species or populations in plant conservation management. The random amplified polymorphic DNA (RAPD) fingerprinting method was first described by Welsh and McClelland and Williams et al.in which the amplification of random segments of DNA in the genome is carried out by polymerase chain reaction (PCR) using single primers of arbitrary nucleotide sequence typically the length of 10 nucleotides. Compare to other genetic markers, RAPD can provide the vital information for the development of genetic sampling, conservation, and improvement strategies.(Pradeep Kumar et al).
- **2.6.2 Inter Simple Sequence Repeat (ISSR)**
- Inter-simple sequence repeat (ISSR) was first employed by Zietkiewicz et al. (1994) and Gupta et al. (1994). It has been proved to be a highly useful tool for estimating genetic diversity and assessing genetic relationships. It is simple, fast, cost-effective, reliable and highly discriminating (Ci et al., 2008; Crespe et al., 2009; Zhang and Dai, 2010; Uysal et al., 2010; Petros et al., 2008). ISSR is found to be more robust and reliable even in case of closely related individuals (Martins Lopes et al. 2007; Christopoulos et al. 2010). ISSRs are highly variable, require less investment and generate high percentages of polymorphic loci (Jabbarzadeh et al. 2010; Li et al. 2010). The utility of ISSR markers for discriminating the accessions of P. nigrum from other *Piper* species was demonstrated by George et al. (2005).

More recently, the microsatellite sequences have been used as primers in polymerase chain reaction, where repeat motifs are anchored either at 5′ or 3′ end with one specific nucleotides and amplify the sequences between the two microsatellite loci referred to as inter simple sequence repeat (ISSR) markers. In addition, ISSRs can be targeted towards particular sequences, which are reported to be abundant in the genome and can overcome the technical difficulties of RFLP and RAPD (Rajesh et al. 2002). PCR analysis using ISSR primers has been acknowledged as a valuable technique in studies concerning genetic diversity, phylogenies and evolutionary biology (Pradeep Reddy et al., 2002). The information about genetic diversity within a species is helpful to come up with proper management programmes (Frankham, 1995). The leaves of P. betel along with other ingredients like areca nut and lime are used as "chewing gum" by local people and the leaves of P. sarmentosum are used as vegetable and spice (Liu, 2010). ISSR or Inter Simple Sequence Repeat marker is another PCR-based marker. This marker has wide application for all organisms, regardless of the availability of information about their genome sequence (Shi et al. 2010). ISSR markers are more reproducible than RAPD (Thimmappaiah et al. 2009) and have been proven to be a simple and reliable marker system for many organisms, especially plants, with highly reproducible results and abundant polymorphisms (Machkour-M'rabet et al. 2009). To estimate the genetic diversity in germplasm collections among individuals belonging to the same species or different species, one might choose to apply RAPD but to assess the relationships between individuals that are very close to each other it would be advisable to use the ISSR technique (Mao LH and Fang YM 2014)

- **2.6.3 Start Codon Targeted Polymorphism (SCoT)**
- New marker has been developed for the assessment of genetic diversity, called Start Codon Targeted (SCoT) marker (Collard & Mackill, 2009). SCoT marker is reproducible as compared to the RAPD as it has longer primer sequence and annealing temperature are not the sole factors determining reproducibility.
- It is a type of targeted molecular marker technique. Start codon (ATG) and flanking sequences are highly conserved in the plant genes. SCoT marker is based on the conserved regions which is surrounded by the start codon ATG (Sawant et al., 1999). However, SCoT markers can be developed from the transcribed regions and might be linked to the gene function as studied in Mangifera indica L., sugarcane etc. (Luo et al., 2014; Wu et al., 2013). Like RAPD and ISSR markers, SCoT marker is

developed using single primer in a PCR amplification reaction. It use a single 18-mer primer in the polymerase chain reaction (PCR) assays and higher annealing temperature (50 °C) For genetic diversity analysis has been successfully used in a potato by using SCoT marker (Amirmoradi et al.2012). Bhattacharyya et al.(2013) stated that by using SCoT marker we can analyse a genetic diversity in *Magnifera indica*. Shahlaei et al.(2014) reported that analysed genetic diversity in cicer plant. The SCoT is simple and reliable gene targeted marker. that has been well characterized (Joshi et al. 1997; Sawant et al. 1999).

Gillings and Holley (1997) suggest that primers between 18 to 24 nucleotides are preferable for producing reproducible markers. SCoT primer design was constrained by the number of highly conserved nucleotides within the conversed ATG region, and 18-mer primers were considered to be the maximum length.

MATERIALS AND METHODS

The contemporary study was carried out at Department of Agricultural Biotechnology, Orissa University of Agriculture and Technology, Bhubaneswar.

Materials

A total of thirty one elite genotypes of *Piper accession were* collected from different location of Orissa state and it was identified and authenticated by Dr G. Das, Associate Professor and officer in-charge, AICRP. These were used for diversity analysis in the present study

3.1: Selection of Plant:

Different *Piper accessions* were selected for study

Table 1: List of *Piper* accessions appliance in present study

Si. No.	*P.longum* Accession No.	Location	District	Lattiude N	Longitude E	Altitude (ft)
1	-	KAU	Thrissur	10˚ 54’.322’’	076˚28’.727	120
2	OP-1	Narayani	Ganjam			
3	OP-2	Chandragiri	Gajpati			
4	OP-3	G.P.Podagada	Koraput	18˚52’.161’ ,	082˚52’.568 ,,	2718
5	OP-4	Gupteswar	Koraput	18˚49’.218’ ,	082˚52’.698 ,,	1551
6	OP-5	Tunipur	Koraput	18˚58’.220	082˚59’.933 ,,	2864
7	OP-6	Bandhapada G.P.Godihanjar	Koraput	18˚32’.796	082˚028’.049’’	2834
8	OP-9	Akula	Telkoi	21˚15’.905’ ,	085˚24’.855 ,,	550
9	OP-10	Gonashika	Keonjhar	21˚30’.303’	085˚33’.105	2585

				’	’’	
10	Op-11	Gandhamardn	Keonjhar	21˚37’.257’	085˚30’.49’	2380
11	OP-12	Bantala	Nayagarh	20˚06’.982’	084˚56’.652	395
12	OP-13	Badasilinga	Nayagrah	20˚26’.631’ ’	084˚47’.950 ’’	663
13	OP-14	Balichatri, Janisahi	Nayagrah	20˚21’.224’ ’	084˚53’.028 ’’	401
14	OP-15	Kudoli, Purunakot (STR)	Anugul	20˚36’.250’ ’	085˚01’.743 ’’	1707
15	OP-16	Ambakhol, Purunakot (STR)	Anugul	20˚40’.343’ ’	084˚47’.955 ’’	735
16	OP-17	Kapilas hills	Dhenkanal	20˚40’.923’ ’	085˚45’.823 ’’	1574
17	OP-18	Ashok Jhara	Dhenkanal	20˚40’.548’ ’	085˚52’.508 ’’	563
18	OP-19	Samala	Anugul	21˚23’.028’ ’	085˚18’.008 ’’	883
19	OP-20	Khuludi, Jharbeda	Anugul	21˚22’.610’ ’	085˚18’.032	814
20	OP-21	Laxmipriyapur,Pechamunda	Anugul	21˚28’.642’ ’	085˚17’.685 ’’	785
21	OP-22	Gupteswaer	Koraput	18˚49’.321’ ’	082˚10’.036 ’’	1614
22	OP-23	Doraguda GP-Chandrapada	Koraput	18˚45’.353’ ’	082˚28’.328 ’’	1952
23	OP-24	G.Udayagiri	Koraput	20˚06’.951’ ’	84˚22’.315’ ’	2160
24	OP-25	Kalinga	Phulbani	20˚10’.015’ ’	84˚24’.108’ ’	2415
25	OP-26	Satapadar, Batada	Phulbani	20˚09’.133’ ’	083˚52’.358 ’’	1941
26	OP-27	Purunaguma	Kalahandi	19˚39’.468’ ’	083˚05’.535 ’’	2178
27	OP-28	Podapada Ratangi	Kandhamal	20˚18’.326’ ’	083˚08’.453 ’’	2321
28	OP-29	Suduli,Gudari	Boudh	20˚26’.883’	084˚017’.12	1704

				’	5’’	
29	OP-30	Kuchada,	Harabhanga	20˚25’.732’	084˚028’.14	1182
30	OP-31	Uchhatnagar	Keonjhar	21˚66’.154’	85˚95’.605’	785
31	OP-32	Barunei	Khurda	20˚16’.108’ ’	85˚64’.605’ ’	246

3.2 DNA extraction and purification

Extraction of total genomic DNA was carried out as described by Saghai-Maroof *et al.* (1984) with minor modifications to suit the material under consideration. CTAB (Cetyl Trimethyl Ammonium Bromide) is a powerful cationic detergent which aids in the lyses of cell membrane and will from insoluble complex with nucleic acid. Usually DNA is isolated in presence of salt concentrations. Sodium chloride aids in removing the polysaccharides and its break the CTAB nucleic acid complex. EDTA chelates the Mg2+ ions which is an essential cofactor for the DNase I enzyme to act and prevents the indigenous nuclease to act on nucleic acids. Mercaptoethanol is a reducing agent which protects the DNA against quinones, disulphides, peroxidases and polyphenol oxidases. Impurities such as RNA, protein, polysaccharides, pigments and tannins can be removed by treating extract with RNase, chloroform.

Protocol

- Extraction buffer (10 ml) (Annexure-II) maintained at 60ºC in a water bath was added to the centrifuge tube containing powder of crushed leaves.
- The contents were mixed gently and then it was incubated at 60ºC for 1 hour in hot water bath.
- In the meanwhile 6-7 intermittent shaking were given for complete mixing and formation of emulsion.
- After that equal volume of choloform : isoamyl alcohol (24:1) was added and mixed them by inverting for 7-8 minutes.
- After complete emulsion formation, centrifugation was done at 10,000 rpm for 20 min at 25ºC.

- The aqueous phase was transferred to a fresh centrifuge tube and then isopropanol (twice the volume of aliquot) and 300µl of 3M sodium acetate was added. DNA was allowed to settle down for overnight.
- Then DNA was spooled out and the excess chemicals were drained out with a pipette. To wash the salt impurities and some other residues present in the isolated DNA, 0.5 ml of 70% ethyl alcohol was added.
- The contents were mixed gently and incubated for 30 min. The contents were decanted by gentle spinning and then added 70% ethyl alcohol and kept for 2 hours.
- The alcohol was decanted and the pellets were dried under vacuum dryer (lyophilizer) at -40ºC for 45 min. The DNA was dissolved in 300 µl of TE (10:1) buffer (pH 8.0) and kept overnight for complete dissolution.

3.3 Purification

- Then 5µl RNase solution was added to the tube containing dissolved DNA and incubated at 37ºC for 1 hr. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed properly for 2 min and spun 5 min.
- The DNA supernatant was taken out and again added equal volume of chloroform: isoamyl alcohol and mixed well and centrifuged at 10 min. The aqueous layer was removed and repeated to produce creamy colour solution.
- Then $1/10^{th}$ amount of 3M Sodium acetate and 2.5 volume of absolute chilled alcohol were added to remove aqueous layer, mixed it gently so that DNA could precipitate down and kept overnight or for an hour at -20ºC when precipitation was not observed.
- The solution was then centrifuged at 8000 rpm for 5 min. and the supernatant was decanted off. Extra salts were removed by two washing with 70% ethanol. Then the DNA was dried under vacuum, dissolved in TE (10:1) buffer at room temperature and stored frozen at -20ºc

3.4 DNA quantification and purity checking

The concentration of DNA was estimated by the measurement of the UV irradiation absorbed by nucleic acid bases. First the spectrophotometer was calibrated using 2000 l of TE in a quartz cuvette at 260 nm and 280 nm. Then 5 l of DNA sample was added to 995 l of TE, mixed well and absorbance (OD) was taken at 260 nm and 280 nm. The concentration of the DNA in the sample was estimated as follows

Concentration of DNA (g/ml) = OD at 260 x Dilution factor x 50. The ratio between readings at 260 nm and 280 nm (OD_{260}/ OD_{280}) provided an estimate for the purity of nucleic acid. Any sample showing the ratio below 1.8 or above 2.0 was further subjected to purification.

Further, in order to know the concentration and intactness of genomic DNA, an aliquot (2µl) of each sample was subjected to agarose gel (0.8 % w/v) electrophoresis for about 2 hours along with 500 ng of molecular weight marker (Lambda / *Eco*RI digest). The gel was stained with ethidium bromide (0.5µg/ml), viewed under Gel-Doc system (UVITECH) and photographed immediately for further interpretation. By comparing the fluorescent intensity of the bands of genomic DNA with the standard molecular weight marker. DNA concentration was estimated following the method described by Sambrook *et al.* (1989).

3.5 Dilution of DNA

To yield a working concentration of 10ng/µl, part of the stock DNA samples were diluted with appropriate amount of TE buffer and stored at 4ºC.

3.6 Optimization of PCR condition for ISSR, RAPD and SCoT molecular marker

To determine optimal amplification reaction conditions for 31 *Piper* spp. germplasm, a factorial experiment was carried out at three concentrations of $MgCl_2$ (2.0mM, 2.5mM and 3.0mM), 1U of Taq DNA polymerase, two concentrations of template DNA (10ng, 20ng,) and 0.5µM primer to make a volume of 25µl at different annealing temperatures. The annealing temperatures tested were 5ºC above and below the melting temperature, Tm of the particular primer. Conditions were finally optimized as given in Table, under which clear and consistent bands were amplified. Available ISSR,RAPD and SCoT primers were tested for amplification and the ones that gave better resolution were chosen for the present study.

Table 2: Optimization of PCR conditions for ISSR, RAPD and SCoT markers

Component	**Stock**	**Final conc. per reaction**	**Quantity of PCR constituents for 31 reactions**
10X buffer	1x	3 µl	93.0µl
$MgCl_2$ (25mM)	25mM	3 µl	93.0µl
dNTPs (10mM)	200µM	2 µl	62.0µl
Primer	5µM	3 µl	93.0µl
Taq polymerase	1unit	1 µl	31.0µl
Deionised water Distilled	-	11µl	341µl
Total	-	23µl	713.0µl
DNA	10-50ng	2µl	-

3.6.1 Thermocycler conditions for ISSR

I. Pre-denaturation at 95ºC for 5 min.

II. Forty five cycles of denaturation; annealing and extension are as follows in the table.

III. Followed by final extension at 72ºC for 10 min.

Table 3.3: PCR conditions for I

45 cycles	Temp ºC	Duration
Denaturation	95ºC	1min
Annealing	Tmº	2min
Extension	72ºC	1min

Note: ATº= Annealing temperature which varies with primer to primer

3.6.2 Thermocycler conditions for RAPD

I. Pre-denaturation at 95ºC for 5 min.

II. Forty five cycles of denaturation, annealing and extension was as follows in the table.

III. Followed by final extension at 72ºC for 10 min.

45 cycles	Temp ºC	Duration
Denaturation	95ºC	1min
Annealing	37˚C	2min
Extension	72ºC	1min

Note: ATº= Annealing temperature which varies with primer to primer

3.6.3 Thermocycler conditions for SCoT

I. Pre-denaturation at 95ºC for 5 min.

II. Forty cycles of denaturation, annealing and extension was as follows in the table.

III. Followed by final extension at 72ºC for 10 min.

45 cycles	Temp ºC	Duration
Denaturation	95ºC	1min
Annealing	ATº	2min

Extension	72ºC	1min

Note: Tm and Ta represents the melting temperature and annealing temperature of the primers

3.6.3 PCR Reaction mix (Master mix)

Total volume of PCR reaction mix was made to 25µl of which 23.0µl was master remaining 2µl was the individual DNA of the genotype.

3.6.4 Primer Screening

Such as the primers RAPD,ISSR and SCoT used for present investigation were custom synthesized by Ahmadabad ,Thermo Fisher Scientific ,Ahmadabad, India. Initially, ISSR primers were screened for PCR reactions with the DNA of ten selected *Piper* germplasms. On the basis of screening, primers that resulted in discrete, reproducible well separated bands on agarose gel were selected for final amplification for all germplasm. Out of fifteen primers screened, five primers gave satisfactory amplification and used for ISSR analysis of 31 *Piper* germplasm of the present study.

3.6.5 Agarose gel Electrophoresis

Before agarose gel electrophoresis, reaction products were mixed with 2.0µl of 10x loading dye and spun for a while before loading.

Preparation of Agarose gel

- 1.5% agarose gel was prepared for ISSR. 3.0gm agarose was mixed well in 200ml of 0.5X TBE buffer and boiled in microwave oven till it completely dissolved.
- During warming intermittent shaking were done 3-4 times to prevent formation of clumps of agarose.
- The molten agarose was kept for cooling up to 50-60^{o}C and then ethidium bromide (1µg/ml) was added. The molten agarose was poured into the clean, levelled casting plate containing 26 well combs and was kept for solidification.
- The gel was transferred to the electrophoresis unit containing 0.5X TBE buffer. The PCR reaction products were first mixed with 2.0 µl of loading dye and spun for a while before loading into the wells of the gels. The medium range ruler plus was also loaded in first and/or last well of the gel to serve as

standard molecular weight marker for determining the size of the amplified DNA fragments.

- The gel was run at 75 volts for 2.50hr. The run was stopped when bromophenol blue dye had travelled 2/3rd length of the gel.

3.6.6 Gel photography

Gels were photographed under U.V. light using Gel Doc. System.

3.6.7 Scoring and Data Analysis

Scoring of amplification product was done by **'1'** if allele is present and **'0'** when allele is absent. Homology of alleles was based on the distance of migration of amplified DNA fragments according to their molecular weights in the gel.

3.6.8 Similarity coefficient

For similarity coefficient selected genotype was compared with the rest of genotypes. Greater the value of coefficient, compared variety will be more similar to selected variety. In addition to this the value of coefficient also signifies about the extent of similarity between the two varieties. Jaccard's similarity coefficient was calculated according to Jaccard (1908) as follows

Where,

n_{xy} = Number of bands common in sample a and b

n_1 = Total number of bands present in all samples

n_2 = Number of bands not present in sample a or b but found in other samples

The similarity matrix was subjected to generate a dendogram using software programme NTSYS pc Ver 2.1. Exeter Software, N.Y. (Rohlf, 2005).

3.6.9 Principle Component Analysis (PCA)

The Jaccard's similarity matrix was subjected to principle component analysis. This coordination method makes use of multidimensional solution of the observed relationship.

PCA resolves complex relationships into a function of fewer and simpler factor. In this, data matrix is derived from the distance or similarities between the operational taxonomic units.

3.6.10 Percent polymorphic Loci

A locus is defined as polymorphic when the frequency of marker (allele) is <1.0.

3.6.11 Primer Efficiency

Primer Resolving power (Prevost and Wilkinson, 1999) was used to identity the primer that would distinguish cultivars most efficiently. Resolving power is calculated to find the best informative primer

RP =Sum of Band in formativeness (Ib)**:**

$$Ib = [1-2x|0.5-Pi|]$$

Where Pi is the proportion of accession containing band

Primer efficiency

Marker index (MI)*:* It was used to assess the utility of a marker for detecting genetic variation. It was calculated according to Powell *et al.* (1996) as follows

MI = EMR X DI ,

where EMR (Effective Multiplex Ratio) was no. of polymorphic markers generated per assay. Diversity Index (DI) was calculated, which is equivalent to the average PIC value.

Polymorphic information content (PIC): Most informative primers were selected based on the extent of polymorphism. The polymorphic information content (PIC) was calculated by applying the formula given by Powell *et al.* (1997).

$$PIC = 1 - \sum_{i=1}^{n} fi^2$$

Where fi is the frequency of the i^{th} allele and the summation extends over n alleles

Table 3: Details of the ISSR primer present study

Sr.No	Primer	Sequence 5'-3'	Tm	Ta
1	USB-835	AGAGAGAGAGAGAGAGYC	54˚C	51˚C
2	USB-836	AGAGAGAGAGAGAGAGC	52˚C	48˚C
3	USB-841	AGAGAGAGAGAGAGAGYC	52˚C	50˚C

4	ISSR-8	CACACACACACACACAA	50˚C	47˚C
5	ISSR-11	GAGAGAGAGAGAGAGAC	52˚C	48˚C
6	AM-1	GGCGGCGGCGGCGGCAT	64.0˚C	58˚C
7	AM-2	AAGAAGAAGAAGAAGGC	50.5˚C	38˚C
8	AM-4	AAGAAGAAGAAGAAGCC	69.6˚C	51˚C
9	UBC-807	AGAGAGAGAGAGAGAGT	42.5˚C	34˚C
10	UBC-811	GAGAGAGAGAGAGAGAC	43.3˚C	45˚C
11	UBC-813	CTCTCTCTCTCTCTCTT	43.5˚C	36˚C
12	UBC-818	CACACACACACACACAG	52.1˚C	36˚C
13	UBC-854	TCTCTCTCTCTCTCTCRG	54.0˚C	50.1 ˚C
14	UBC-857	ACACACACACACACACYG	54.0˚C	50.1˚C
15	UBC-864	ATGATGATGATGATGATG	48.0˚C	50.1˚C

Table 4: Details of the SCoT primer present study

Sr.No	Primer	Sequence 5'-3'	Tm	Ta
1	SCoT-1	CAACAATGGCTACCACCA	54 ˚C	50 ˚C
2	SCoT-2	CAACAATGGCTACCACCC	56 ˚C	52 ˚C
3	SCoT-13	ACGACATGGCGACCATCG	58 ˚C	53 ˚C
4	SCoT-33	CCATGGCTACCACCGCAG	60 ˚C	57 ˚C
5	SCoT-70	ACCATGGCTACCAGCGCG	60 ˚C	57 ˚C
6	SCoT-19	ACCATGCCTACCACCGCG	60 ˚C	55 ˚C
7	SCoT-25	ACCATGGCTACCACCGGG	60 ˚C	56 ˚C
8	SCoT-55	ACAATGGCTACCACTACC	54 ˚C	50 ˚C
9	SCoT-8	CAACAATGGCTACCACGT	54 ˚C	52 ˚C
10	SCoT-20	ACCATGGCTACCACCGCG	60 ˚C	58 ˚C
11	SCoT-24	CACCATGGCTACCACCAT	60 ˚C	58 ˚C
12	SCoT-30	CCATGGCTACCACCGGCG	62 ˚C	60 ˚C
13	SCoT-31	CCATGGCTACCACCGCCT	60 ˚C	58 ˚C
14	SCoT-36	GCAACAATGGCTACCACC	56 ˚C	52 ˚C
15	SCoT-35	CATGGCTACCACCGGCCC	62 ˚C	58 ˚C

Table 5: Details of the RAPD primer present study

Sr. No.	Primer	Sequence 5'-3'	Tm	Ta
1	OPC-20	ACTTCGCCAC	37	35
2	OPN-11	TCGCCGCAAA	37	34
3	OPC-7	GTCCCTACGA	37	35
4	OPL-11	ACGATGAGCC	37	33
5	OPAN-5	GGGTGCAGTT	37	34
6	OPAA-1	AGACGGCTAG	37	33
7	OPA-8	GTGACGTAGG	37	36

RESULTS

4.1 Molecular Characterization of various *Piper accession*

DNA extraction and purification described in materials and methods section yielded a good quality of DNA which was applicable for PCR amplification.

4.1.2 Primer Screening

Thirty one elite *Piper* accessions were used for screening the primers in order to determine the suitable ones that produce a clear, various and reproducible bands. Of the fifteen SCoT, fifteen ISSR and seven RAPD primers that were screened, eight SCoT, five ISSR and two RAPD were finally chosen as that gave the uniform amplification and band resolution.

ISSR analysis

Five primers produced a total of 15 scorable markers among the genotypes. The size of amplified products ranged from 300 bp to 1250 bp. The number of scorable markers produced per primer ranged from 3 to 26. The total number of polymorphic markers and the percentage of polymorphism were 98% respectively. ISSR marker profile produced by the primer UBC835 in agarose gel is shown. In the case of ISSR analysis, the mean PIC value was 0.179 and the highest and lowest PIC values were 0.231 (UBC835) and 0.132 (USB-841) respectively. Similarity coefficients for the *Piper* accession based on 5 ISSR markers ranged between 0.21 and 0.93. Of the 31 genotypes among the accession, similarity index was highest between *Piper longum* accession OP-15 and *Piper longum* accession OP-16 (93%) and among *Piper longum* accession and lowest similarity was observed in *Piper longum* OP-9 and *Piper longum* accession OP-8 (25%). The mean similarity index was 0.49.

The cluster analysis of ISSR markers separated the *Piper* accession into five distinct clusters and also it contains one outer group. The first cluster included *Piper longum* OP-1 to *Piper longum* OP-3 while the second cluster included the *Piper longum* OP-24, *Piper longum* OP-25 and *Piper longum* OP-26. Third cluster containing *Piper longum* OP-10 to *Piper longum* OP-13. Fourth cluster containing remaining five *Piper* accession such as *Piper longum* OP-21 to *Piper longum* OP-23.In fifth cluster containing *Piper longum* OP-12 to *Piper longum* OP-18. An outer group has formed having in two *Piper* accession i.e.

Piper longum OP-8 and *Piper longum* OP-9. The first cluster two *Piper* accession had similarity (93%) among *Piper longum* OP-15 and *Piper longum* OP-16.

Table: 6 DNA profile and polymorphism generated in 31 *Piper* accession using 5 ISSR Primers

Primer Code	Total No. of bands (bp)	Monomorphic band (bp	Polymophic band (bp)	%age Polymorphism (bp)	Size Range(bp)	Average PIC value
USB-835	15	3	12	79.0	300-1250	0.231
USB-836	13	0	13	100	300-1300	0.132
USB-841	14	2	12	93	300-1250	0.174
Total	42	5	37	--	--	0.537
Mean	14	1.6	11.3			0.179

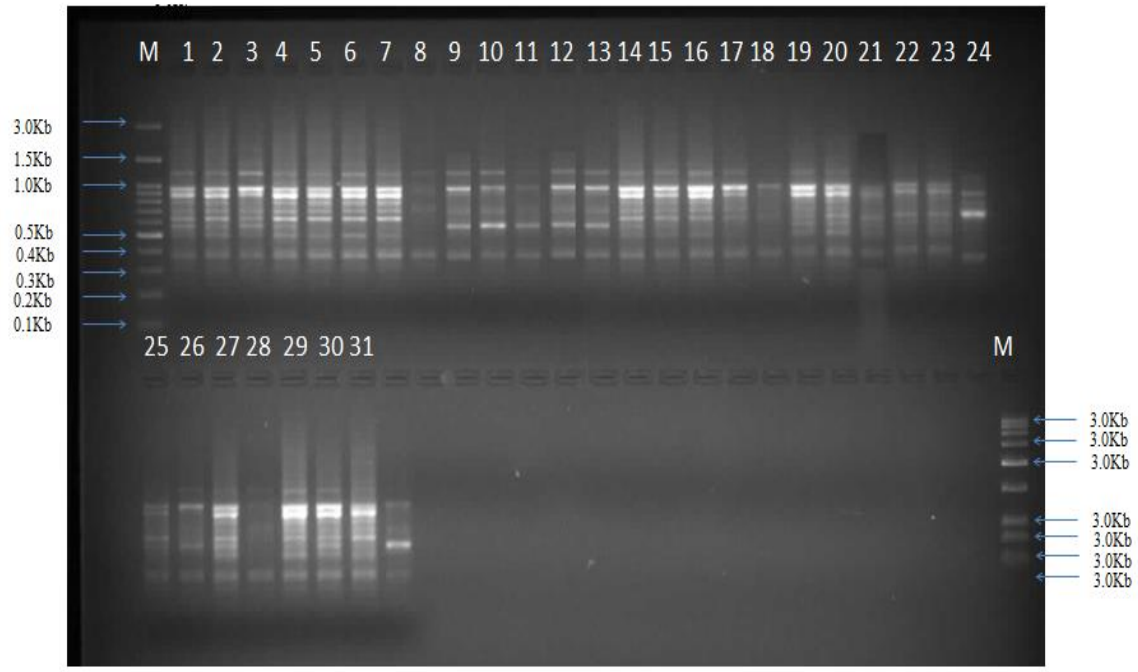


Fig: 1 Amplification profile of 31 Piper accession employing of ISSR (USB-835)

M= Low range DNA ruler plus; Number on the top of the lane represents Piper accession as given (Table: 3.1); Numbers on right and left side margin represent molecular weight marker DNA in base pair (bp)

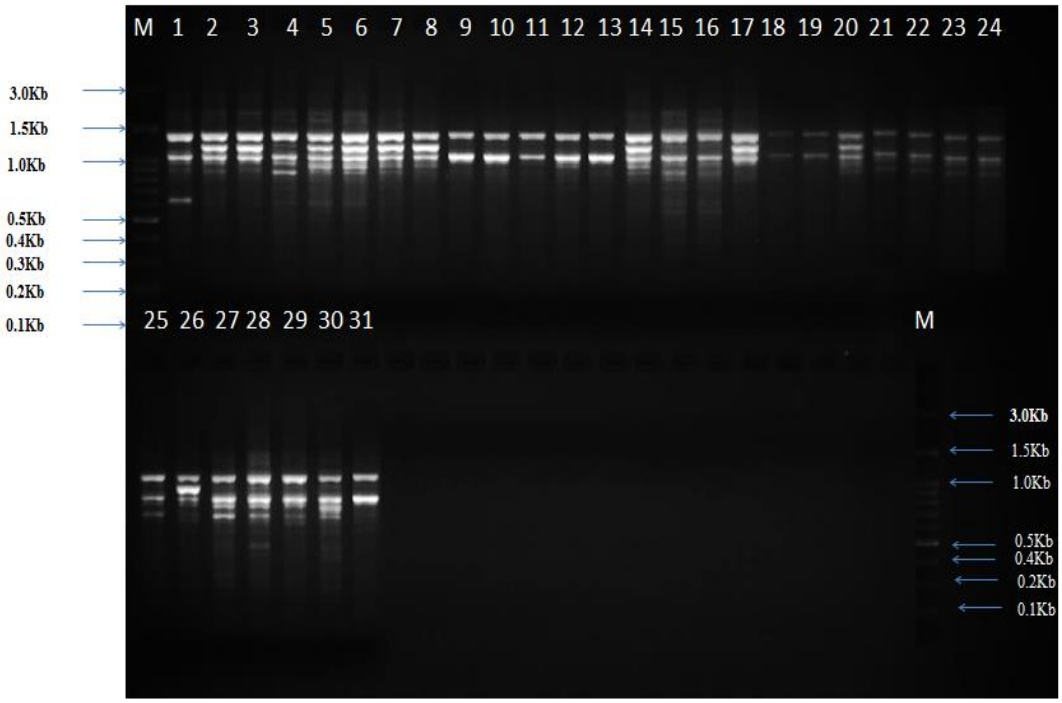


Fig: 2 Amplification profile of 31 *Piper* accession employing of ISSR (USB-836)

M= Low range DNA ruler plus; Number on the top of the lane represents *Piper* accession as given (Table: 3.1); Numbers on right and left side margin represent molecular weight marker DNA in base pair (bp)

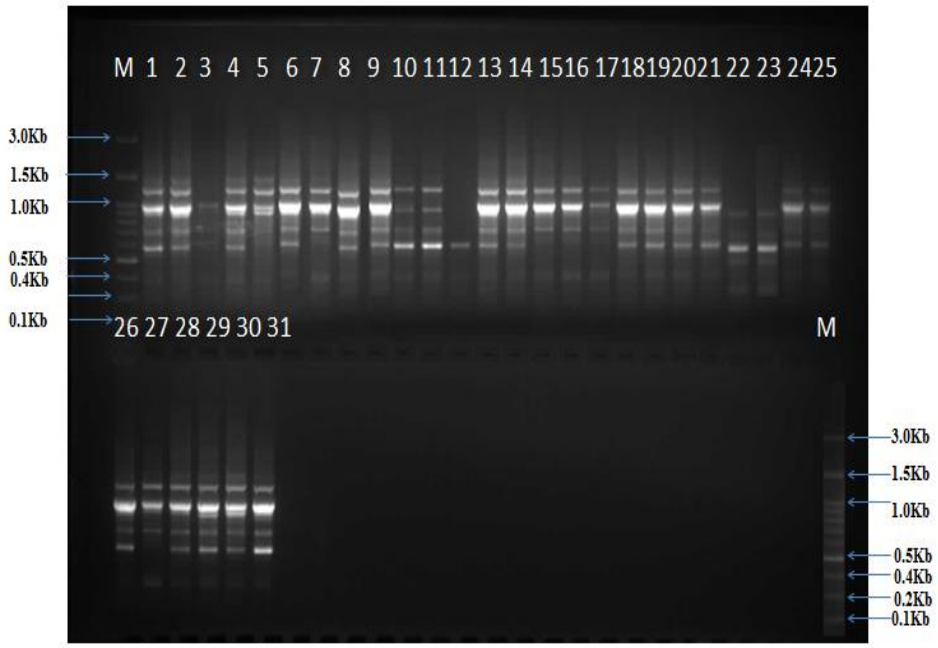


Fig: 3 Amplification profile of 31 *Piper* accession employing of ISSR (USB-841)

M= Low range DNA ruler plus; Number on the top of the lane represents *Piper* accession as given (Table: 3.1); Numbers on right and left side margin represent molecular weight marker DNA in base pair (bp)

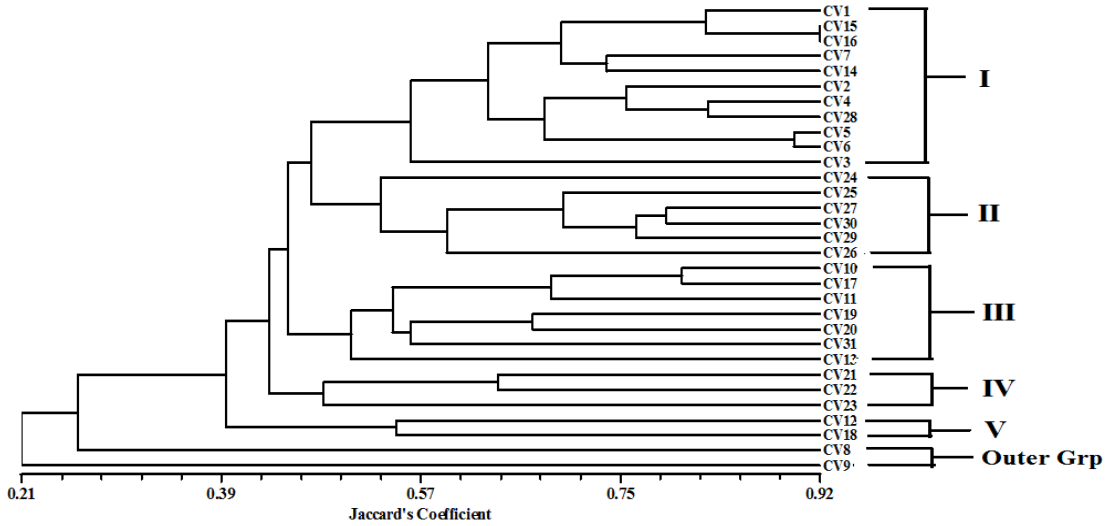


Fig: 4 Dendrogram depicting genetic relationship among 31 *Piper* accession based on ISSR profiles

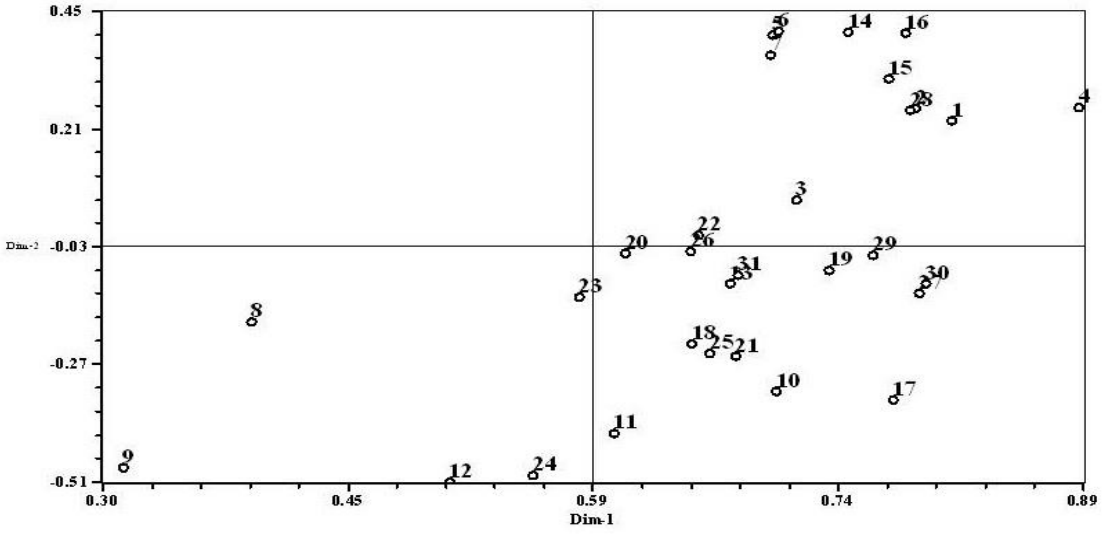


Fig: 5 Two Dimensional scaling by Principal Component Analysis (PCA) of 31 *Piper* accession using Jaccards Similarity Coefficient of ISSR profile data.

(Number denoting the plotted data points represent the respective *Piper* accession as listed in Table 3.1)

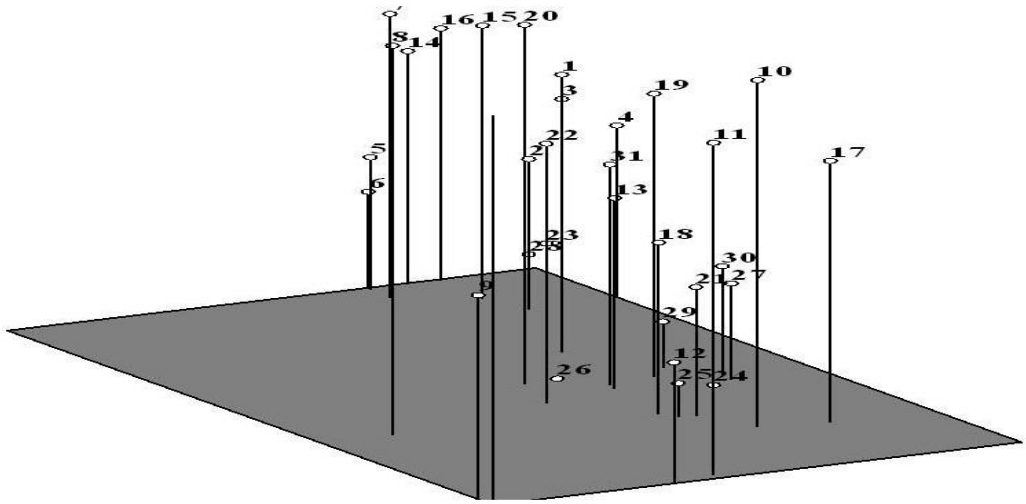


Fig: 6 Three Dimensional scaling by Principal Component Analysis (PCA) of 31 *Piper* accession using Jaccards Similarity Coefficient of ISSR profile data.

(Number denotating the plotted data points represents the respecting *Piper* accession listed in Table 3.1)

	CV1	CV2	CV3	CV4	CV5	CV6	CV7	CV8	CV9	CV10	CV11	CV12	CV13	CV14	CV15	CV16	CV17	CV18	CV19	CV20	CV21	CV22	CV23	CV24	CV25	CV26	CV27	CV28	CV29	CV30	CV31
CV1	1.00																														
CV2	0.61	1.00																													
CV3	0.60	0.59	1.00																												
CV4	0.81	0.78	0.69	1.00																											
CV5	0.52	0.67	0.50	0.67	1.00																										
CV6	0.52	0.75	0.43	0.67	0.90	1.00																									
CV7	0.71	0.59	0.69	0.69	0.58	0.50	1.00																								
CV8	0.31	0.18	0.36	0.25	0.21	0.15	0.36	1.00																							
CV9	0.23	0.12	0.17	0.19	0.05	0.05	0.08	0.17	1.00																						
CV10	0.62	0.41	0.46	0.50	0.29	0.29	0.46	0.50	0.38	1.00																					
CV11	0.43	0.35	0.50	0.44	0.30	0.24	0.38	0.57	0.25	0.67	1.00																				
CV12	0.25	0.35	0.38	0.35	0.24	0.24	0.20	0.22	0.43	0.36	0.40	1.00																			
CV13	0.44	0.61	0.50	0.53	0.52	0.52	0.41	0.21	0.23	0.50	0.43	0.43	1.00																		
CV14	0.65	0.72	0.53	0.72	0.70	0.70	0.73	0.27	0.13	0.44	0.29	0.22	0.56	1.00																	
CV15	0.79	0.56	0.53	0.75	0.55	0.55	0.64	0.33	0.15	0.54	0.36	0.19	0.39	0.69	1.00																
CV16	0.86	0.61	0.50	0.81	0.60	0.60	0.71	0.31	0.14	0.50	0.33	0.18	0.37	0.75	0.92	1.00															
CV17	0.53	0.53	0.62	0.63	0.38	0.38	0.50	0.40	0.30	0.80	0.70	0.55	0.64	0.47	0.47	0.44	1.00														
CV18	0.35	0.63	0.40	0.53	0.38	0.45	0.31	0.17	0.18	0.50	0.42	0.55	0.53	0.39	0.38	0.35	0.67	1.00													
CV19	0.56	0.65	0.44	0.56	0.48	0.48	0.44	0.33	0.25	0.67	0.46	0.36	0.56	0.59	0.60	0.56	0.57	0.57	1.00												
CV20	0.50	0.41	0.46	0.50	0.35	0.29	0.46	0.33	0.22	0.60	0.50	0.15	0.40	0.44	0.54	0.50	0.50	0.38	0.67	1.00											
CV21	0.47	0.47	0.43	0.56	0.33	0.33	0.33	0.18	0.33	0.42	0.45	0.45	0.38	0.41	0.50	0.47	0.46	0.46	0.50	0.42	1.00										
CV22	0.47	0.47	0.54	0.56	0.40	0.40	0.43	0.30	0.20	0.42	0.33	0.33	0.38	0.50	0.62	0.57	0.46	0.46	0.50	0.42	0.64	1.00									
CV23	0.50	0.50	0.46	0.50	0.29	0.35	0.36	0.20	0.22	0.45	0.36	0.36	0.40	0.35	0.33	0.40	0.50	0.50	0.33	0.23	0.42	0.55	1.00								
CV24	0.31	0.33	0.27	0.33	0.29	0.29	0.19	0.20	0.38	0.45	0.50	0.50	0.40	0.28	0.25	0.24	0.50	0.38	0.43	0.23	0.55	0.31	0.33	1.00							
CV25	0.50	0.42	0.38	0.50	0.43	0.43	0.29	0.15	0.27	0.36	0.38	0.50	0.41	0.30	0.44	0.41	0.40	0.40	0.44	0.27	0.67	0.43	0.36	0.58	1.00						
CV26	0.47	0.56	0.35	0.56	0.48	0.55	0.35	0.07	0.15	0.33	0.27	0.36	0.47	0.42	0.33	0.39	0.47	0.47	0.33	0.18	0.40	0.31	0.54	0.54	0.53	1.00					
CV27	0.67	0.47	0.53	0.65	0.48	0.48	0.44	0.23	0.25	0.54	0.46	0.36	0.47	0.42	0.60	0.56	0.57	0.38	0.50	0.43	0.62	0.50	0.43	0.54	0.77	0.60	1.00				
CV28	0.65	0.72	0.53	0.82	0.62	0.70	0.53	0.12	0.13	0.35	0.29	0.29	0.40	0.58	0.59	0.65	0.47	0.47	0.42	0.35	0.50	0.41	0.44	0.35	0.53	0.69	0.69	1.00			
CV29	0.63	0.53	0.50	0.71	0.52	0.52	0.50	0.21	0.23	0.40	0.43	0.43	0.44	0.47	0.47	0.53	0.53	0.35	0.39	0.31	0.57	0.38	0.40	0.50	0.71	0.67	0.79	0.75	1.00		
CV30	0.53	0.53	0.50	0.71	0.52	0.52	0.41	0.21	0.23	0.50	0.43	0.43	0.44	0.47	0.56	0.53	0.64	0.53	0.56	0.50	0.57	0.47	0.31	0.50	0.60	0.56	0.79	0.75	0.73	1.00	
CV31	0.50	0.42	0.38	0.59	0.43	0.43	0.38	0.25	0.27	0.58	0.38	0.29	0.50	0.44	0.53	0.50	0.62	0.50	0.53	0.58	0.33	0.33	0.27	0.36	0.38	0.44	0.53	0.53	0.50	0.71	1.00

Table: 7 Similarity Coefficient Matrix for ISSR primer in 31 *Piper* accession

Scot Analysis

Out of the 15 markers analysed over all the accessions, 8 were found scorable. Amplified fragments were ranged from 150 bp to 1000 bp among 31 *Piper* accessions. The numbers of bands were ranged from 3 to 12 (SCoT-5) with an average 7.0 per primers. The primer SCoT-5 produced maximum 12 numbers of bands followed by SCoT-6 primer which produced 10 numbers of bands. The polymorphisms percentage was 100% across all accessions. The PIC values for SCoT primers were ranged from 0.142 to 0.366 with an average PIC value 0.234 for 31 *Piper* accession.

Cluster analysis for SCoT primers showed two main groups (i.e. I and II) along with an outer group. First major cluster consisted of *Piper longum* accession OP-1 to *Piper longum* accession OP-11. Further, cluster I was sub divided into three sub clusters viz. I-A, I-B & I-C. In these sub-clusters, *Piper longum* accession OP-1 to *Piper longum* accession OP-9 was placed in sub cluster I-A. Among these three sub clusters; sub-cluster I-A showed maximum 80% similarity between two *Piper* accessions i.e. OP-7 and OP-9. Whereas, Sub-cluster I-B and Sub-cluster I-C includes *Piper longum* accession OP-8 to *Piper longum* accession OP-24 and *Piper longum* accession OP-3 to *Piper longum* accession OP-11 respectively. Meanwhile, Second group confined more numbers of *Piper longum* accessions as compared to first major group which includes *Piper longum* accessions OP-2 to OP-29. Further, group second was sub divided into six sub clusters viz. IIA, IIB, IIC, IID, IIE and IIF. Sub-cluster II-A, mainly confined two *Piper longum* accessions such as OP-2 and OP-30. Similarly, sub cluster II-F also includes two *Piper longum* accessions namely OP-6 and OP-29. Whereas, sub clusters II-D and II-E comprises in total three *Piper longum* accessions each namely OP-16, OP-18, OP-25 and OP-4, OP-21, OP-31 respectively. Sub cluster II-C comprises maximum numbers of five *Piper longum* accessions from OP-5 to OP-15. *Piper longum* accessions such as OP-17 to OP-19 was placed in cluster II-B among them the accessions OP-26 and OP-28 showed highest Jaccard's similarity coefficient i.e. 100 per cent. Whereas, *Piper longum* OP-10 and OP-23 was depicted as a variant of which *Piper longum* accession OP-23 was the outermost accession. Pair-wise estimates of similarity matrix ranged from 0.00 to 0.100 and average similarity among all 31 *Piper longum* accessions was 0.65. Among the 31 accession, similarity index was recorded highest between *Piper longum* accession OP-26 and OP-28 (100%). The lowest similarity coefficient was observed in *Piper longum* OP-23 (0%) and OP-10 (29%) and the mean similarity index was 0.49.

Table:8 DNA profile and polymorphism generated in 31 *Piper* accession using 8 SCoT primers

Primer Code	Total No. of bands (bp)	Monomorphic band (bp	Polymophic band (bp)	%age Polymorphism (bp)	Size Range(bp)	Average PIC value
SCoT-5	12	0	15	100	150-1000	0.336
SCoT-6	10	0	14	99	350-1500	0.132
Total	22	0	29			0.468
Mean	11	0	14.5			0.234

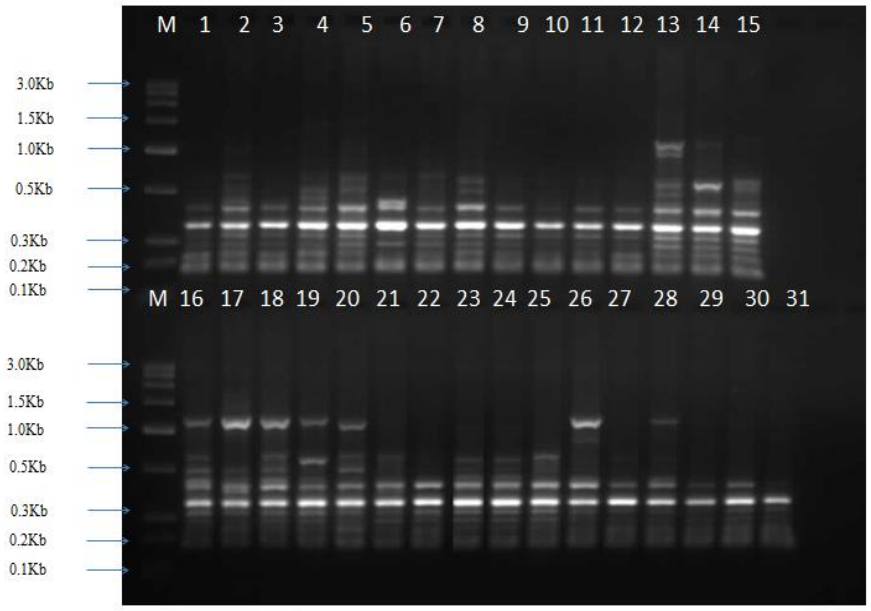


Fig: 7 Amplification profile of 31 *Piper* accession employing of SCoT-5

M= Low range DNA ruler plus; Number on the top of the lane represents *Piper* accession as given (Table: 3.1); Numbers on right and left side margin represent molecular weight marker DNA in base pair (bp)

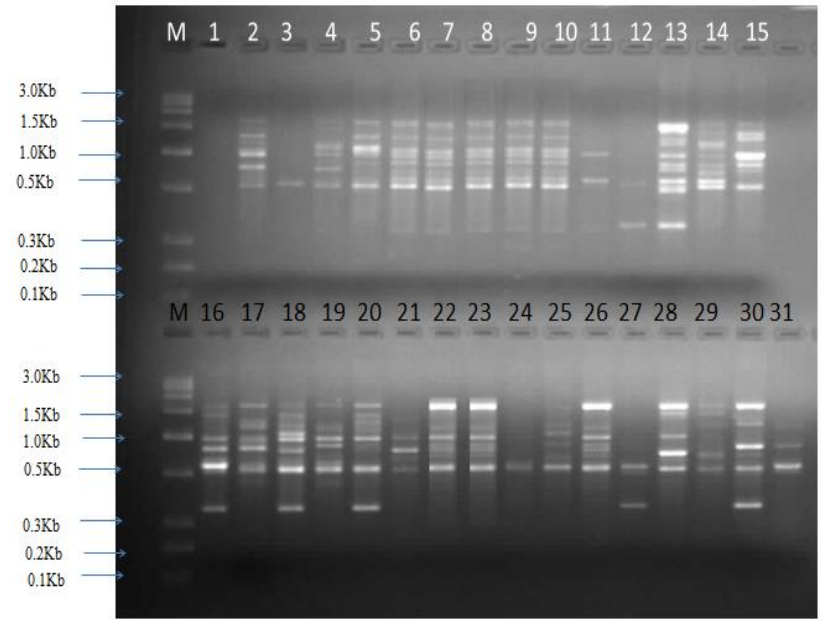


Fig: 8 Amplification profile of 31 *Piper* accession employing of SCoT-6

M= Low range DNA ruler plus; Number on the top of the lane represents *Piper* accession as given (Table: 3.1); Numbers on right and left side margin represent molecular weight marker DNA in base pair (bp)

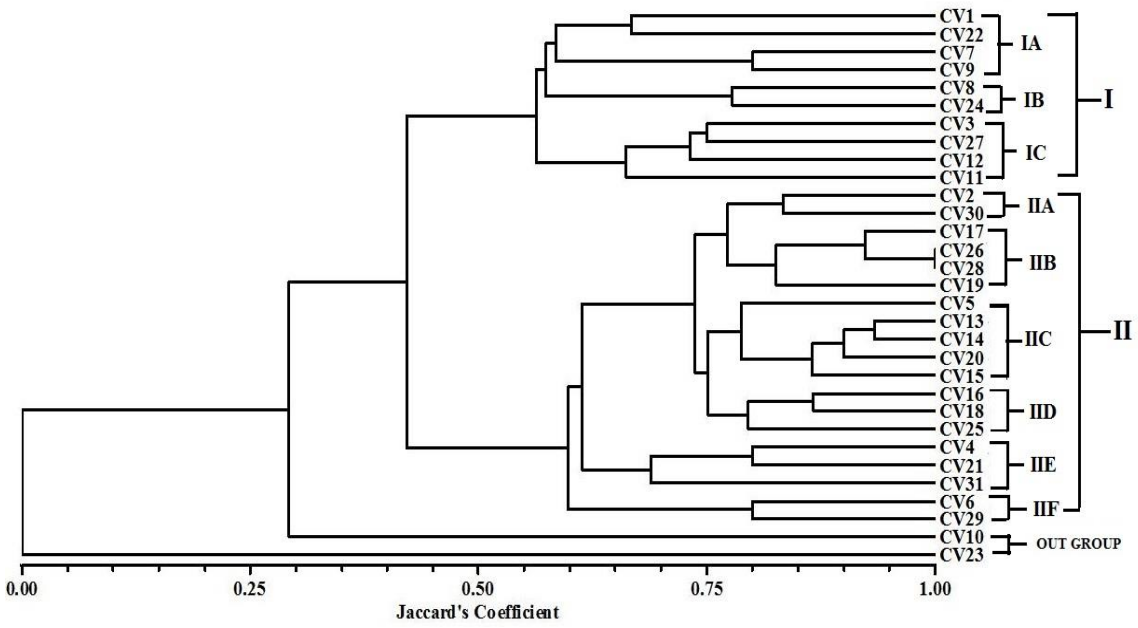


Fig: 8 Dendrogram depicting genetic relationship among 31 *Piper* accession based on SCoT profiles

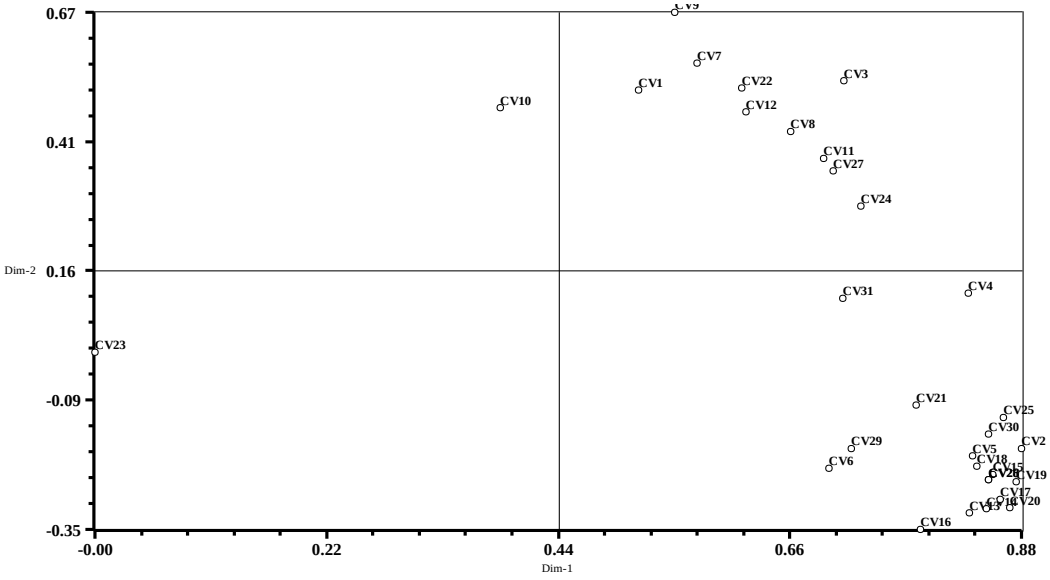


Fig: 9 Two Dimensional scaling by Principal Component Analysis (PCA) of 31 *Piper* accession using Jaccards Similarity Coefficient of SCoT profile data.

(Number denoting the plotted data points represent the respective *Piper* accession as listed in Table 3.1)

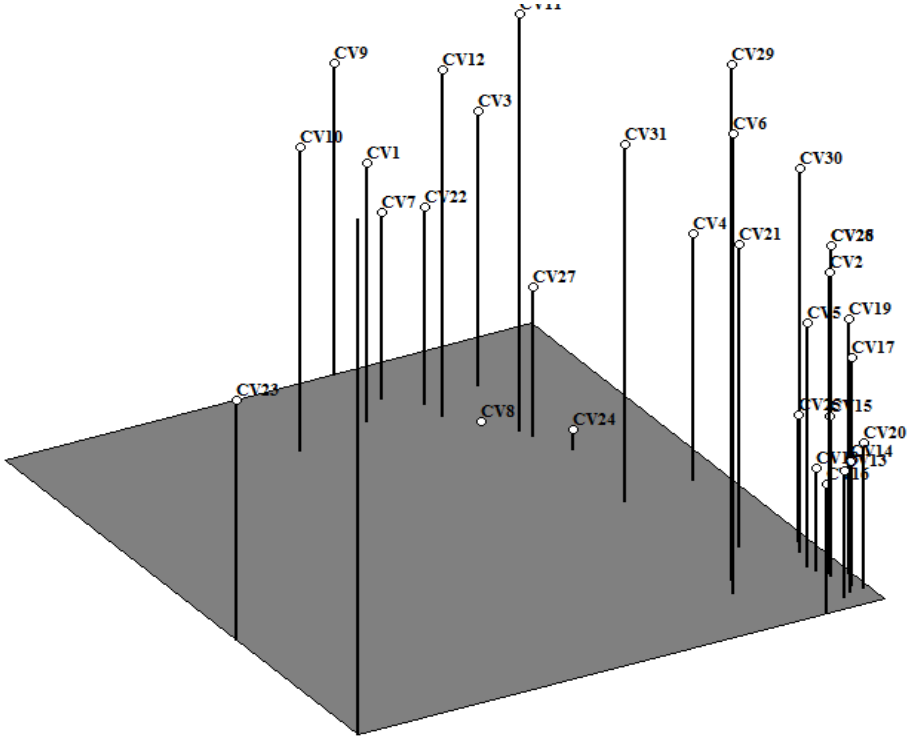


Fig: 10 Three Dimensional scaling by Principal Component Analysis (PCA) of 31 *Piper* accession using Jaccards Similarity Coefficient of SCoT profile data.

(Number denotating the plotted data points represents the respecting *Piper* accession listed in Table 3.1)

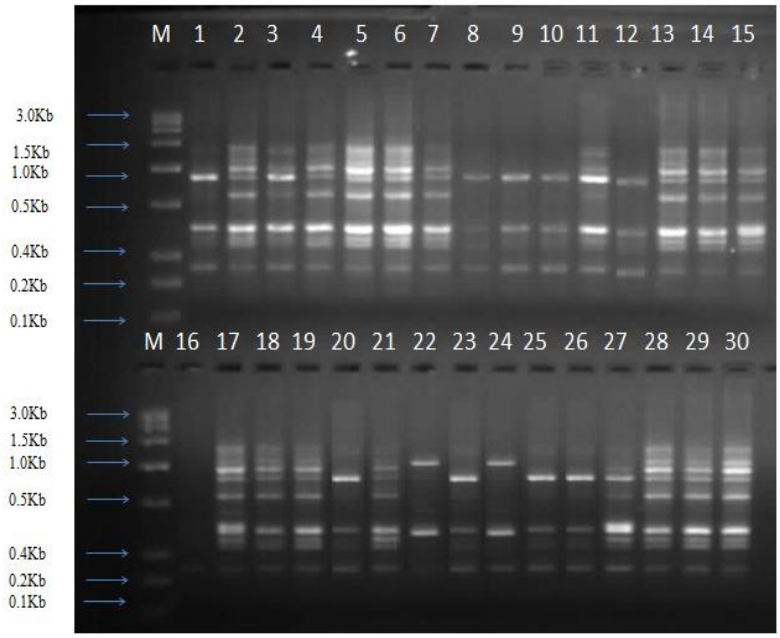


Fig: 12 Amplification profile of 31 *Piper* accession employing of RAPD OPC-20

M= Low range DNA ruler plus; Number on the top of the lane represents *Piper* accession as given (Table: 3.1); Numbers on right and left side margin represent molecular weight marker DNA in base pair (bp)

RAPD, ISSR and SCOT markers Analysis

In the present study, three molecular markers (RAPD, ISSR and SCOT) were used in combination for genetic diversity assessment. Both RAPD and ISSR showed same patterns of clustering of genotypes. Seven RAPD primers were screened against 31 *Piper longum* accessions DNA to identify potential primers producing higher number of polymorphic and repeatable fragments. RAPD analysis revealed a good polymorphism among *Piper longum* accessions. Of the 7 primers used 5 primers did not give satisfactory

results. This causes majority of the amplicons were found to be monomorphic. The remaining 2 primers generated reproducible RAPD patterns were used for subsequent analysis in combinations with ISSR and SCOT markers. Each primer-template yielded distinct, easily detectable bands of variable intensities. Light, dense and sharp bands are considered for the consideration of genetic diversity. The monomorphic bands of low degree of similarity indicated high divergence between the genotypes evaluated. Assaying RAPD variation in 31 accession of *Piper longum* with these primers yielded total of 79 bands ranging molecular weight between 150 - 1350 bp. Number of bands produced ranged from 2 to 9 with an average of 3.50 bands per genotype. The total polymorphic bands in 2 primers are 60 and therefore, reproducible polymorphic band are 3 averages per primer and percentage polymorphism are 70.25%. Maximum number of 9 bands was shown by primer OPC 20, while least number of single bands was shown by OPN 11. Out of 7 potential primers, 2 RAPD primers were thus chosen for fingerprinting logically based on the high number of polymorphic fragments generated and then concomitant reproducibly.

The dendogram was generated based on combination of three molecular markers such as RAPD, ISSR and SCOT separated the genotypes into three major clusters. Second major cluster comprises maximum number of 18 *Piper longum* accessions and third major cluster represents the least number of genotypes i.e. 3 *Piper longum* accessions. First major cluster consisted of *Piper longum* accession OP-1 to OP-23. Further, cluster I was sub divided into three sub clusters viz. I-A, I-B and I-C. Sub-cluster I-A and I-B represents 4 *Piper longum* accession each, OP-1 to OP-11 and OP-8 to OP-10 respectively. Whereas, sub cluster I-C comprises two *Piper longum* accessions namely OP-22 and OP-23. The second major cluster represents maximum number of five sub clusters. Among them Sub cluster II-A includes maximum number of six *Piper longum* accessions from OP-2 to OP-19 which also represents closely related genotypes (OP-14 and OP-15) at 90 per cent similarity index. On the other hand, Sub-cluster II-B and II-C comprises three accessions each, OP-13, OP-17, OP-18 and OP-6, OP-29, OP-30 respectively. OP-26 and PO-28 represented by the sub cluster II-D. Whereas, Sub-cluster II-E includes *Piper longum* accession OP-21 to OP-27. Meanwhile, third group confined only one sub cluster which represents *Piper longum* accessions OP-16 and OP-20 as well as OP-31 as an out group. Pair-wise estimates of similarity matrix for all three molecular markers ranged from 0.54 to 0.90 and average similarity among all 31 *Piper longum* accessions was 0.77.

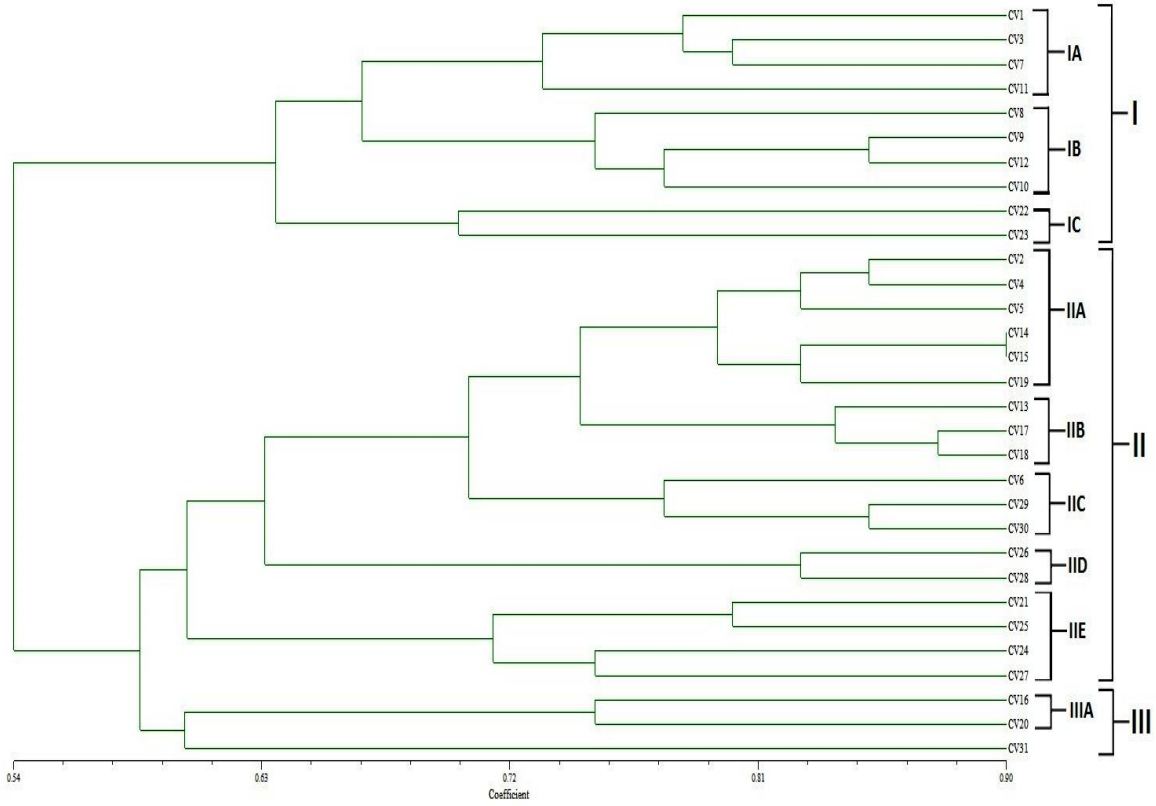


Fig: 13 Dendrogram depicting genetic relationship among 31 *Piper* accession based on SCoT, ISSR and RAPD profiles

Discussion

The present investigation is intended to observe genetic diversity in the *Piper* accession gene pool. The experimental findings presented in the preceding chapter revealed some useful information. A large varietal diversity is observed in *Piper* accession due to long period of cultivation, Therefore, it is paramount importance to collect, characterize and conserve land races that are regionally and locally important.

Characterization of plant genetic resources is vital for the identification of species and cultivars and for efficient management of germplasm resources and also breeding program. Moreover, the accurate and unambiguous identification of plant material is essential for effective germplasm characterization, which is helpful for plant breeders in selecting material for development of new crosses, in solving disputes related to patenting and intellectual property rights and to check bio-privacy, adulteration etc.

5.1 Molecular characterization

Molecular marker techniques have various applications in almost all the fields of life science and thus have great importance in biology. Molecular characterization of plants, animals, microbes etc. helps to differentiate the origin/source of evolution. Characterization of plant genetic resources and quantification of diversity is essential for identification of species and cultivars, deciphering genetic relationships including parentages and for efficient management of germplasm. (Blair *et al.*, 1999; Joshi *et al.,* 2000), proper characterization helps in explicit discrimination between cultivars, identification of economically important plant genetic resources, detection of redundancies and in monitoring genetic changes during maintenance due to environmental or genetically.

The producing of molecular markers is based on the analysis at the DNA level, because the DNA of a particular plant is identical in all the cells. DNA may be obtained from any kind of plant tissues. DNA characteristics are not influenced by the environmental or nutritional status or developmental stage or cultural practices followed; thus DNA based analysis are free from various kind of external limitations.

Molecular markers which are the PCR base those have proven quite useful for genome analysis, gene tagging, QTL mapping, DNA fingerprinting and marker assisted

selection (MAS). Genetic diversity analysis grater be subject to the type of molecular markers employed, distribution of markers in the genome, type of loci they amplify, level of polymorphism and its reproducibility of products. By using various molecular marker which ensures a good coverage of plant genome, is likely to be more authentic and reliable(Kantety et al; 1995).

5.1.1 ISSR characterization

In the present investigation thirty one *Piper* accession used and it could be differentiated from one another on the basis of their ISSR profiles. ISSR analysis can be undertaken for any species that contains a sufficient number of well distributed ISSR motifs.

ISSR (Inter Simple Sequence Repeat) is a novel technique and has proved to be powerful, rapid, simple, reproducible and inexpensive way of asses of genetic diversity and to identify the closely related accession in many species. (Blair *et al.*, 1999; Joshi *et al.*, 2000).

Inter-simple sequence repeat (ISSR) was first employed by Zietkiewicz et al. (1994) and Gupta et al. (1994). It has been proved to be a highly useful tool for estimating genetic diversity and assessing genetic relationships. To estimate the genetic diversity in germplasm collections among individuals belonging to the same species or different species, one might choose to apply RAPD but to assess the relationships between individuals that are very close to each other it would be advisable to use the ISSR technique (Mao LH and Fang YM 2014).

5.1.1.1 Genetic diversity and cluster analysis on ISSR profile

First we tested 15 primers, five ISSR primers produced clear banding pattern in some selected *Piper* accessions. By using these primers generate DNA profile all of the 31 *Piper* accession. These five ISSR primers yielded 70 bands with an average of 4.66 bands per primer ranging the size of band from 300 to 1250 bp. The ISSR primer USB-835 produced maximum 7 bands followed by ISSR USB-836(6 bands). The lowest number of bands was observed in ISSR USB-841 (4 bands).The maximum PIC value was obatained by using ISSR primer (USB-835).

The ISSR amplification pattern was used to establish the genetic variability among the *Piper* accession by cluster analysis and to detect the various markers. In first cluster

containing 11 *Piper* accession in which *Piper longum* OP-15 and *Piper longum* OP-16 has shown 93% similarity

Followed by *Piper longum* OP-5 and *Piper longum* OP-6 among them has observed 85% similarity. Second cluster containing 6 *Piper* accession. Third cluster group containg 7 accession. In fourth and fifth cluster 3 & 2 accession respectively. In these cluster one outer group has observed and having 25% similarity among other *Piper* accession.

Yan Jiang et al; (2011). Inter-Simple Sequence Repeat (ISSR) analysis was used to evaluate the genetic variation of *Piper* spp. from Hainan, China. 247 polymorphic bands out of a total of 248 (99.60%) were generated from 74 individual plants of *Piper* spp. Bhuyan *et al* (2007) illustrated the genetic diversity in traditional lowland rice grown in Assam using both RAPD and ISSR markers. Youssef *et al* (2010) used both RAPD and ISSR markers to identify the new promising drought tolerant lines of rice under drought stresses. Wang et al (2014) reported that, *Rheum officinale* Baill., an important but endangered medicinal herb, is endemic to China. Inter-simple sequence repeat (ISSR) markers were employed to investigate the genetic diversity and differentiation of 12 populations of R. officinale. Thirteen selected primers yielded 189 bright and discernible bands, with an average of 14.54 per primer. Ranjan Kumar Shaw(2008) stated that four ISSR primers amplified 31 loci out of which 17 were polymorphic and 14 are monomorphic. The ISSR protocol can be readily used in breeding activities for registration and characterization of even closely related *Piper* accessions, and for cataloguing collections.

5.1.2 SCoT and RAPD Characterization

The SCoT marker is based on the single primer amplified region principle since it uses a single primer as a forward and reverse primer, like RAPD and ISSR technique. It is likely that some SCoT markers would be codominant due to insertion–deletion mutations; these would be the minority like codominant RAPDs (Davis et al. 1995). Due to the basis of SCoT primer design, we expect SCoT markers to be distributed within gene regions that contain genes on both plus and minus DNA strands. It is also possible that pseudogenes and (genes within) transposable elements may be used as primer binding sites by SCoT polymorphism technique. An important factor is the distance in base pairs between primer

binding sites of the template.In the present investigation thirty one *Piper* accession used and it could be differentiated from one another on the profiles of SCoT marker. The SCoT is simple and reliable gene targeted marker.

RAPD reveal similar patterns of genetic diversity when compared with other marker types and car be performed more rapidly than most other methods (Morcll et al. 1995) and can provide vital information for the development of genetic sampling, conservation and improvement strategies (waugh and powell 1992, chalmers el al. 1994).

5.1.2.1 Genetic diversity and cluster analysis on SCoT and RAPD profile

Primer screening was done for selection of scorable markers and clear banding pattern on selected *Piper* accessions from that eight primers were selected for genetic diversity assessment. These eight primers were used to produce DNA profile of 31 *Piper* accessions. These eight SCoT primers yielded 90 bands with an average 7.0 bands per primer. Amplified product band size ranging from 150 bp to 1000 bp. SCoT-5 has produced maximum 12 bands followed by SCoT-6 which gives 10 bands. The highest PIC value was estimated for SCoT-5 primer i.e. 0.336.

The SCoT amplification pattern was used to establish the genetic variability among the *Piper* accession by cluster analysis and to detect the various markers. On the basis of Jaccard coefficient the genotypes were grouped into two major clusters and two out group. In first cluster, *Piper longum* accession OP-1 to *Piper longum* accession OP-11were placed. Second cluster consisted *Piper longum* accession OP-2 to *Piper longum* accession OP-29. These two main clusters was sub divided into sub clusters, first main cluster was divided into three sub clusters and second cluster was divided into six sub clusters. From the basis of the cluster analysis, we concluded that *Piper longum* accession OP-26 and *Piper longum* accession OP-28 has greater similarity among the 31 accessions which indicates the close relatedness of these two accessions. *Piper longum* accession OP-23 (0%) and *Piper longum* accession OP-10 (29%) has placed as an out group in dendrogram that means these are more diverse over other *Piper* accessions.

Assaying RAPD variation in 31 accession of *Piper longum* yielded total 79 bands ranging molecular weight between 150 and 1350 bp. Number of bands produced ranged from 2 to 9 with an average of 3.50 bands per genotype. The total polymorphic bands in 2 primers are 60 and therefore, reproducible polymorphic band are 3 averages per primer

and percentage polymorphism are 70.25%. Maximum number of 9 bands was shown by primer OPC 20, while least number of single bands was shown by OPN 11. Similarly, Salim Khan et.al. (2010) and Sandeep sen et.al (2010) showed the discriminatory power of RAPD (Random Amplified Polymorphic DNA) technique which was employed for authentication of *Piper* nigrum (black pepper) from its adulterant Carica papaya. The genetic diversity of eight species of *Piper* (*Piper*aceae) viz., P. nigrum, P. *longum*, P. betle, P. chaba, P. argyrophyllum, P. trichostachyon, P. galeatum, and P. hymenophyllum from Kerala state, India were also analysed for assessment of *Piper* species by Random amplified polymorphic DNA (RAPD).

Some of the SCOT, ISSR and RAPD primers resulted in polymorphic bands which were amplified from different alleles of a given locus. As the shared absences represent the inverses of the shared presences for all individuals, we anticipate that they were different alleles of one locus and should not be scored as different characters. Scoring of different alleles of a given locus is problematic for dominant markers when plants having three or more alleles per locus are tested (Albert 2005; Strong and Lipscomb 1999). The average polymorphism of SCOT, ISSR and RAPD markers was 100, 98 and 70 per cent. Similarly Gorji et al. (2011) showed that maximum percentage of average polymorphism among varieties was higher than that of genotypes and it was 61, 47 and 31 for SCOT, ISSR and RAPD, respectively. This suggested that the relative genetic diversity of the genotypes is more when SCOT markers are used but it is fairly similar when ISSR and RAPD markers are used.

SUMMARY AND CONCLUSION

The genus *Piper* includes about 3000 diverse species of herbs, shrubs and climbers, a few of which are economically important as spice or medicinal plant. *Piper* is an economically and ecologically important genus which belongs to the *Piperaceae* family. The *Piper* species, widely distributed in the tropical and subtropical regions of the world are used medicinally in various manners. *Piper* species are of high commercial and economical importance such as *Piper nigrum* and it has worldwide spice market. *Piper longum* is a highly valuable drug and is one of the essential ingredients in the most of the compound preparations included in Ayurveda literature. In the present investigation molecular characterization of 31 *Piper* accession were under taken. ISSR, SCoT and RAPD markers were used for genetic diversity assessment of different *Piper* accession. Out of fifteen ISSR, fifteen SCoT and seven RAPD primers that were screened, five ISSR, eight SCoT and three RAPD markers gave the satisfactory amplification and better band resolution in PCR amplification for all 31 *Piper* accession.

Five primers produced a total of 15 scorable markers among 31 *Piper* genotypes. The size of amplified products ranged from 300 bp to 1250 bp. The number of scorable markers produced per primer ranged from 3 to 26. The total number of polymorphic markers and the percentage of polymorphism were 15 and 98% respectively. ISSR marker profile produced by the primer UBC835 in agarose gel is shown in figure 1. In the case of ISSR analysis, highest and lowest PIC values were 0.231 (UBC835) and 0.132 (USB-841) respectively and the mean PIC value was 0.179. Similarity coefficients for the *Piper* accession based on 5 ISSR markers ranged from 0.21 to 0.93. Out of 31 genotypes evaluated, similarity index was highest between *Piper longum* accession OP-15 and *Piper longum* accession OP-16 at 93% and lowest similarity was observed in *Piper longum* OP-9 and *Piper longum* accession OP-8 at 25%. In total, all 31 *Piper* accessions were divided into five clusters and its mean similarity index was 0.49.

Among the 15 markers analysed over all the accessions, 8 were found scorable. An amplified product has ranged from 150 bp to 1000 bp for 31 *Piper* accessions. The number of bands ranged from 3 to 12 (SCoT-5) with an average 7.0 per primers. The primer SCoT-5 produced the maximum 12 numbers of bands followed by SCoT-6 primer which

produced 10 numbers of bands. The per cent of polymorphism were 100% across all accessions and PIC value ranged from 0.142 to 0.366. The average PIC value content of SCoT primers among the 31 *Piper* accession were found 0.234. Cluster analysis was done using SCoT primers which clustered into two main groups (i.e. I and II) along with two outer group accessions. *Piper longum* accession OP-26 and *Piper longum* accession OP-28 has greater similarity 100%. *Piper longum* accession OP-10 and *Piper longum* accession OP-23 had diverse group.

The dendogram was generated based on combination of three molecular markers such as RAPD, ISSR and SCOT separated the genotypes into three major clusters. Second major cluster comprises maximum number of 18 *Piper longum* accessions and third major cluster represents the least number of genotypes i.e. 3 *Piper longum* accessions. First major cluster consisted of *Piper longum* accession OP-1 to OP-23. Further, cluster I was sub divided into three sub clusters viz. I-A, I-B and I-C. Sub-cluster I-A and I-B represents 4 *Piper longum* accession each, OP-1 to OP-11 and OP-8 to OP-10 respectively. Whereas, sub cluster I-C comprises two *Piper longum* accessions namely OP-22 and OP-23. The second major cluster represents maximum number of five sub clusters. Among them Sub cluster II-A includes maximum number of six *Piper longum* accessions from OP-2 to OP-19 which also represents closely related genotypes (OP-14 and OP-15) at 90 per cent similarity index. On the other hand, Sub-cluster II-B and II-C comprises three accessions each, OP-13, OP-17, OP-18 and OP-6, OP-29, OP-30 respectively. OP-26 and PO-28 represented by the sub cluster II-D. Whereas, Sub-cluster II-E includes *Piper longum* accession OP-21 to OP-27. Meanwhile, third group confined only one sub cluster which represents *Piper longum* accessions OP-16 and OP-20 as well as OP-31 as an out group. Pair-wise estimates of similarity matrix for all three molecular markers ranged from 0.54 to 0.90 and average similarity among all 31 *Piper longum* accessions was 0.77.

The genetic analysis based on PCR amplifications by employing ISSR, SCoT and RAPD markers were used to evaluate the genetic relationships. SCOT, ISSR and RAPD generated high number of polymorphic markers which can be used in genetic assessment of *Piper longum* species. Based on the average percentage polymorphism, PIC, diversity index and marker index the efficiency of SCOT for fingerprinting of *Piper* genotypes was more than other markers. In these terms ISSRs also had superiority over RAPD markers. The efficiency of SCOT, ISSR and RAPD markers for fingerprinting of *Piper longum* genotypes is relatively

the same. In general, these three markers could be used in conjunction with each other for evaluation of genetic diversity of *Piper longum* species. Nevertheless, this molecular method is very useful for germplasm characterization of even closely related *Piper* accessions. The approach involving molecular markers can have an important role in securing plant variety rights as the need to protect proprietary germplasm is likely to increase in the future. This finding will considerably facilitate marker validation for agronomical important characters, genome mapping and recombination breeding programme for development of new cultivars. This will also provide a powerful tool for the generation of potential fingerprinting diagnostic markers for genomes/species/cultivars. Also, phylogenetic analysis on the basis of ISSR, SCoT and RAPD derived phonogram supports polyphyletic evolution in the *Piper* accession multiple lineages underwent independent divergence after separation from a common ancestor.

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APPENDIX

Extraction buffer

1M Tris–HCl (pH 8.0) = 19.8 ml
0.5 M EDTA (pH 8.0) = 8 ml
3M NaCl = 46.6 ml
2% CTAB(w/v) = 2gms

Dissolve, make up to 100 ml with de-ionized water and autoclave at 15 psi For 20 min.

10% working C-TAB

10% CTAB = 10 gm

5 M NaCl = 14 ml

Dissolve in water, make up to 100 ml and autoclave at 15 psi for 20min.

3M NaOAC (pH 6.8)

Sodium Acetate = 40.83 gm

Dissolve, adjust pH to with glacial acetic acid, make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

Choloroform : Iso-amyl alcohol Mixture (24:1)

Choloroform = 96 ml

Iso-amyl alcohol = 4 ml

70% Ethanol (100ml)

Absolute alcohol = 70 ml

Double distilled water = 30 ml

RNase stock

1 M Tris- HCL (pH 8.0) = 100 µl

5 M NaCl = 300 µl

RNase = 10 mg

Adjust volume to 1 ml with de-ionized water, boil for 15 minutes and allow to cool slowly and stored at -20ºC

TE (10:1)

1 M tris-HCL (pH 8.0) = 1 ml

0.25 M EDTA (pH 8.0) = 0.4 ml

Dissolve, make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min

10X TBE (pH 8.0)

Tris base = 108 gm

Boric acid = 55 gm

EDTA = 9.3 gm

Dissolve and make up volume to 100 ml with double distilled water.

DNA Quatification

1. DNA Standard Assay solution

Component	**Low range DNA assay (10-50ng/ml find DNA conc.)**
HOECHST 33258 Stock soln	10µl
10XTNE	10µl
Distilled Water	90ml

above solutions were mixed and volume made upto 100ml.

The solution was prepared afresh as and when necessary and stored in amber coloured bottle.

2. Hoechst Dye (H-33258)10X solution

Add 10ml distilled water to 10mg of Hoechst 33258 and stored at 4°C for upto 6 months in an amber coloured bottle.

3. 10X TNE buffer stock solution (100:10mM EDTA:2M NaCl, pH-7.4)

Component	**Concentration**	**Amount taken**
Tris	100mM	12.11g
EDTA	10mM	3.72g
NaCl	2mM	116.89g

4. DNA standard – Low range Standard 1 mg/ml

Component	**Volume(µl)**
Calf Thymus DNA(mg/ml)	100
TNE buffer (10X)	100
Sterile water	800

APPENDIX-II

1. LIST OF CHEMICALS AND THEIR MANUFACTURES:

	Chemical	Manufacture
1.	**DNA extraction**	
	(a)CTAB buffer	
	100mM Tris (pH 8.0)	Qualigens
	1.4% M NaCl	Qualigens
	2% CTAB	Qualigens
	2%ß-Mercaptoethanol	Amresco
	(b)Choloform:Isoamyl alcohol(24:1)	Qualigens
	(c)Isopropanol	Qualigens
	(d)Tris EDTA(T.E)in 10:1 ratio	
	10mM Tris (pH 8.0)	Qualigens
	1 mM EDTA	Sigma
	(e)Cold Ethanol	Merk
2.	**DNA Purification**	Qualigens
	(a)Phenol:Chloroform:Isoamyl alcohol	
	(b)RNase solution	Bangalore Genei
	Rnase	
	Tris HCL (pH 7.5)	Bangalore Genei
	NaCl	Sigma
	(c)Sodium Acetate	Qualigens

3. DNA Quantification

(a)10X TNE Buffer

100mM Tris Bangalore Genei

2M NaCl Bangalore Genei

10mM EDTA Sigma

(b)Hoechst 33258

(c)Calf Thymus DNA Bangalore Genei

(d)Assay Buffer(in dark bottle) Bangalore Genei

H33258

10X TNE buffer(10µl)

Double distilled water(90 ml)

4. SSR analysis

(a)Taq DNA polymerase(1U/reaction) Bangalore Genei

(b)Buffer (10X) (2µl/reaction) Bangalore Genei

(c)$MgCl_2$ (25mM) (1.2µl/reaction) Bangalore Genei

(d)dNTP mixture (2µl/reaction) Bangalore Genei

(e)Primer (2µl/reaction) Bangalore Genei

5. ISSR analysis

(a)Taq DNA polymerase(1U/reaction) Bangalore Genei

(b)Buffer (10X) (2µl/reaction) Bangalore Genei

(c)$MgCl_2$(25mM) (1.2µl/reaction) Bangalore Genei

(d)dNTP mixture (2µl/reaction) Bangalore Genei

(e)Primer (2µl/reaction) Bangalore Genei

6. Agarose gel electrophoresis

(a)Agarose	Bangalore Genei
(b)1X TBE buffer	Bangalore Genei
(c)Ethidium bromide solution	Sigma
(d)Loading dye(10X)	Bangalore Genei
(e)DNA Ladder (20bp and 100bp)	Bangalore Genei