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Phylogenetic analysis of *Piper* species of Assam (India) using molecular markers

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Abstract

Phylogenetic analysis of 18 species belonging to the genus *Piper* were conducted in order to bring out their genetic relationships present at molecular level using molecular markers. Molecular information derived from the *mat*K gene and consequent phylogeny reconstructions based on maximum likelihood (ML), maximum parsimony (MP) and neighbor joining (NJ) methods revealed considerable diversity among the different *Piper* species.

Keywords: *Piper*, *mat*K, phylogenetic, diversity

Introduction

Piper happens to be a large genus prevailing throughout the tropics and sub-tropics and primarily distributed in the Central and Northern South America in the new world and in India, Malaysia, Indonesia and Sri Lanka in the old world [1]. *Piper* bears great economic importance. Many species belonging to this genus are already well explored. Plants belonging to this genus bear immense ethnobotanical significance [2]. Medicinal values of *Piper* are attributed mainly due to the prevalence of alkaloids and amides that are secondarily produced in the plants [3]. *Piper nigrum* L. and *Piper betle* L. are two intensively cultivated forms of the genus. Besides enormous medicinal usefulness, *Piper nigrum* L. happens to be the oldest known spice that is most widely utilized throughout the world [4]. Because of large-scale utility as spice, *Piper nigrum* L. has come to be known as the "King of Spices". *Piper betle* L. finds extensive application in traditional systems of medicine [5-8]. The most reputed use of *Piper betle* L. is the use of leaves for chewing purpose along with betel nut in order to maintain oral freshness. In India, the plant is famed as "Green Gold" that is chewed regularly after every meal by a large section of the population. Large number of wild *Piper* species also reportedly possesses important economic and medicinal properties [9]. There are several significant reports on biological activities concerning different species of the genus *Piper* with therapeutic as well as preventive potentiality against various ailments [10-15].

Molecular marker-based characterization upholds great significance for the elucidation of genetic diversity and consequent relationships. Molecular characterization for the purpose of diversity analysis had received the upper hand over conventional approach that is based on morphological traits [16]. SSR markers adopted for molecular characterization are considered advantageous because of several counts. They are reliable, co-dominant, variable, highly reproducible, present in large numbers throughout the genome, easily scorable and multi-allelic in nature [17]. SSR markers have been successfully adopted for molecular characterization of economically important genus such as *Piper* [18-25]. Genetic diversity of *Piper* species of North-East India had received inadequate attention [26]. That the North eastern *Piper* species are distinct from their counterparts growing in the Western Ghats of India [27], makes molecular characterization of the genus of special significance.

Materials and Methods

Plant materials

18 *Piper* species, which includes two cultivated forms were collected from within the geographic boundaries of the state of Assam, India, that were subjected to molecular characterization. The *Piper* species considered are *P thomsonii*, *P sarmentosum*, *Pattenuatum*, *P hymenophyllum*, *P nigrum*, *P rhytidocarpum*, *P mullesua*, *P peepuloides*, *P griffithii*, *P nepalense*, *P lonchites*, *P betleoides*, *P longum*, *P betle*, *P pedicellosum*, *P brachystachyum* and *P sylvaticum*. Collected plant materials were identified in consultation with various literature and monographs [28-32]. Also, for identification voucher specimens were compared with preserved specimens that are available in Herbarium like GUBH, CAL and ASSAM.

Extraction of genomic DNA and PCR amplification

Cryogenic grinding using liquid nitrogen was carried out and DNA was extracted from leaf tissues using HiPurATM Plant Genomic DNA Miniprep Purification Kit (MB507-50PR, Himedia, India) by following the recommended protocol provided by the manufacturer. 2 µL of extracted genomic DNA from each plant samples were run against uncut λ DNA on 0.8% Agarose gel electrophoresis in 1× TAE buffer containing 0.5 mg/mL of (EtBr). DNA bands thus obtained were visualized under UV light.

SSR primer adopted for PCR amplification was obtained from Operon Technologies Inc., CA, USA (Table 1). PCR was performed using a SimpliAmp™ Thermal cycler (Applied Biosystem).

The reaction started with DNA denaturation at 95°C for 5 min followed by 35 cycles of exposure at 95°C for 1 min, annealing at 50 °C temperature for 1 min, elongation at72°C for 1 min, final extension at 72°C for 5 min and the reaction was stopped at 4°C. Amplified products were analysed by electrophoresis on 2% agarose (Hi Media) gel alongside 100 bp molecular marker.

Gel photograph was scanned by a gel documentation system (Thermo Fisher Scientific, USA).

Table 1: List of primers adopted for phylogenetic analysis.

Locus	Primer	Sequence (5′ -3′)	Tm (°C)
*mat*K	matK390f	CGATCTATTCATTCAATATTTC	50
	matK1326r	TCTAGCACACGAAAGTCGAAGT	

Sequencing of *mat*K region of the experimental plants

Amplified *mat*K region upon purification were resorted to sequencing by Automated DNA Sequencer (Applied Biosystems, ABI3730 *xl*). Bidirectionally sequencing was performed with respective forward and reverse primers.

Construction of phylogenetic tree

Phylogenetic tree was constructed using the MP (maximum parsimony), ML (maximum likelihood) and NJ (neighbor joining) methods with the adoption of MEGA 7.0 software. For bootstrap analysis, multiple data sets were obtained by seqboot in 1000 replicates. Sequenced data obtained from the experimental plants were used to derive ortholog sequences from NCBI nucleotide database using nBLAST. ClustalW computer program was adopted in order to find out multiple sequence alignment among the derived and downloaded sequences and a global phylogenetic reconstruction was made.

Results and Discussion

Extracted genomic DNA derived out of the experimental plants exhibited almost identical bands of high molecular weight. PCR amplifications of the targeted *mat*K gene yielded bands of approximately ~950 bp compared to 100 bp DNA ladder at 1.5% (w/v) agarose gel (Plate 1). Sequencing reactions performed by automated DNA sequencer yielded ~ 950 bp sized amplicons for the PCR products. 26 homologous sequences pertaining to related species of *Piper* were retrieved from the DNA databank. Retrieved sequences showed very high similarity with the targeted sequences of the experimental plants. Multiple sequence alignment among derived and retrieved DNA sequences was carried out using ClustalW. Accession numbers, E-value, query coverage and per identity of *mat*K gene sequences of different *Piper* species that were retrieved from DNA databank are shown in Table 2.

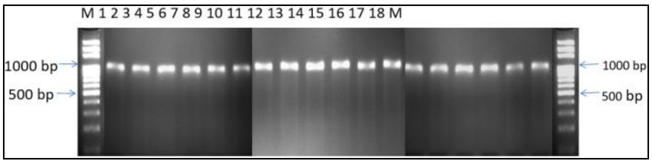

Plate 1: PCR amplified *mat*K gene of the 18 *Piper* spp. at 1.5% (w/v) agarose gel. Lane M: Molar mass marker (100 bp DNA ladder); Lane 1: *P thomsonii*; Lane 2: *P sarmentosum*; Lane 3: *P attenuatum*; Lane 4: *P hymenophyllum*; Lane 5: *P nigrum*; Lane 6: *P rhytidocarpum*; Lane 7: *P mullesua*; Lane 8: *P peepuloides*; Lane 9: *P griffithii*; Lane 10: *P nepalense*; Lane 11: *P lonchites*; Lane 13: *P betleoides*; Lane 14: *P longum*; Lane 15: *P betle*; Lane 16: *P pedicellosum*; Lane 17: *P brachystachyum* and Lane 18: *P sylvaticum*

Table 2: Retrieved data profile of *Piper* species available in DNA databank.

Species	Country	E- value	Per Identity	Query coverage	Accession No.
Piper chinense	China	0.0	98.98%	96%	EF450303.1
Piper sarmentosum	China	0.0	98.98 %	96%	EF450302.1
Piper hancei	China	0.0	98.87%	96%	EF450296.1
Piper hancei	China	0.0	98.64%	96%	EF450301.1
Piper hancei	China	0.0	98.53%	96%	EF450294.1
Piper hancei	China	0.0	98.53%	96%	EF450295.1
Piper laetispicum	China	0.0	98.75%	96%	EF450300.1
Piper nigrum	China	0.0	98.75%	96%	EF450299.1
Piper hainanense	China	0.0	98.75%	96%	EF450297.1
Piper austrosinense	China	0.0	98.64%	96%	EF450293.1
Piper betle	China	0.0	98.64%	96%	EF450298.1
Piper longum	China	0.0	98.30%	96%	EF450292.1
Piper nigrum	Japan	0.0	98.53%	96%	AB040153.2
Piper longum	Pakistan	0.0	98.29%	96%	MH287271.1
Piper penninerve	Germany	0.0	98.75%	96%	DQ882214.1
Piper crocatum	Germany	0.0	98.64%	96%	AF543745.1
Piper crocatum	Germany	0.0	98.64%	96%	DQ212714.1
Piper ornatum	Germany	0.0	98.64%	96%	DQ882211.1
Piper bavinum	Germany	0.0	98.53%	96%	DQ882210.1
Piper decumanum	Germany	0.0	98.19%	96%	DQ882212.1
Piper caninum	Germany	0.0	98.07%	96%	DQ882213.1
Piper cinereum	Germany	0.0	97.51%	96%	DQ882216.1
Piper hispidum	Germany	0.0	97.62%	96%	DQ882219.1
Piper munchanum	Germany	0.0	97.28%	96%	DQ882207.1
Piper arieianum	Germany	0.0	97.17%	96%	DQ882204.1
Piper reticulatum	Germany	0.0	97.05%	96%	DQ882221.1

Phylogenetic relationships among the 18 experimental *Piper* species as per MP (maximum parsimony), ML (maximum likelihood) and NJ (neighbour joining) methods based on the derived *mat*K datasets are shown in Figure 1. *Piper sarmentosum* showed maximum proximity towards the cultivated *Piper nigrum* as per ML and MP methods. Similar closeness between the two species was also revealed in the NJ analysis even though the two species did not share the same most recent common ancestors (MRCAs). *Piper lonchites* was found closest to *Piper diffusum* in the MP and NJ analysis. ML phylogenetic tree also revealed closer relationship between between the twospecies although not sharing the same most recent common ancestors (MRCAs). *P. rhytidocarpum* revealed closest relationship towards *P. nepalense* in the ML and NJ analysis. MP analysis also revealed close proximity between the two species. Rest of the experimental species formed part of different clades in the three *mat*K phylogenetic trees revealing their distant relationships. *Piper betleoides* and *Piper longum* are clubbed together as a monophyletic clad as per ML analysis and be considered as sister species. However, *Piper betleoides* and *Piper longum* are apart from rest of the experimental *Piper* species being positioned as outgroup species in accordance with the MP, ML and NJ phylogenetic trees. Genetic variations among the *Piper* species estimated during the present investigation with the adoption of SSR markers are identical with results obtained by earlier workers [33-44].

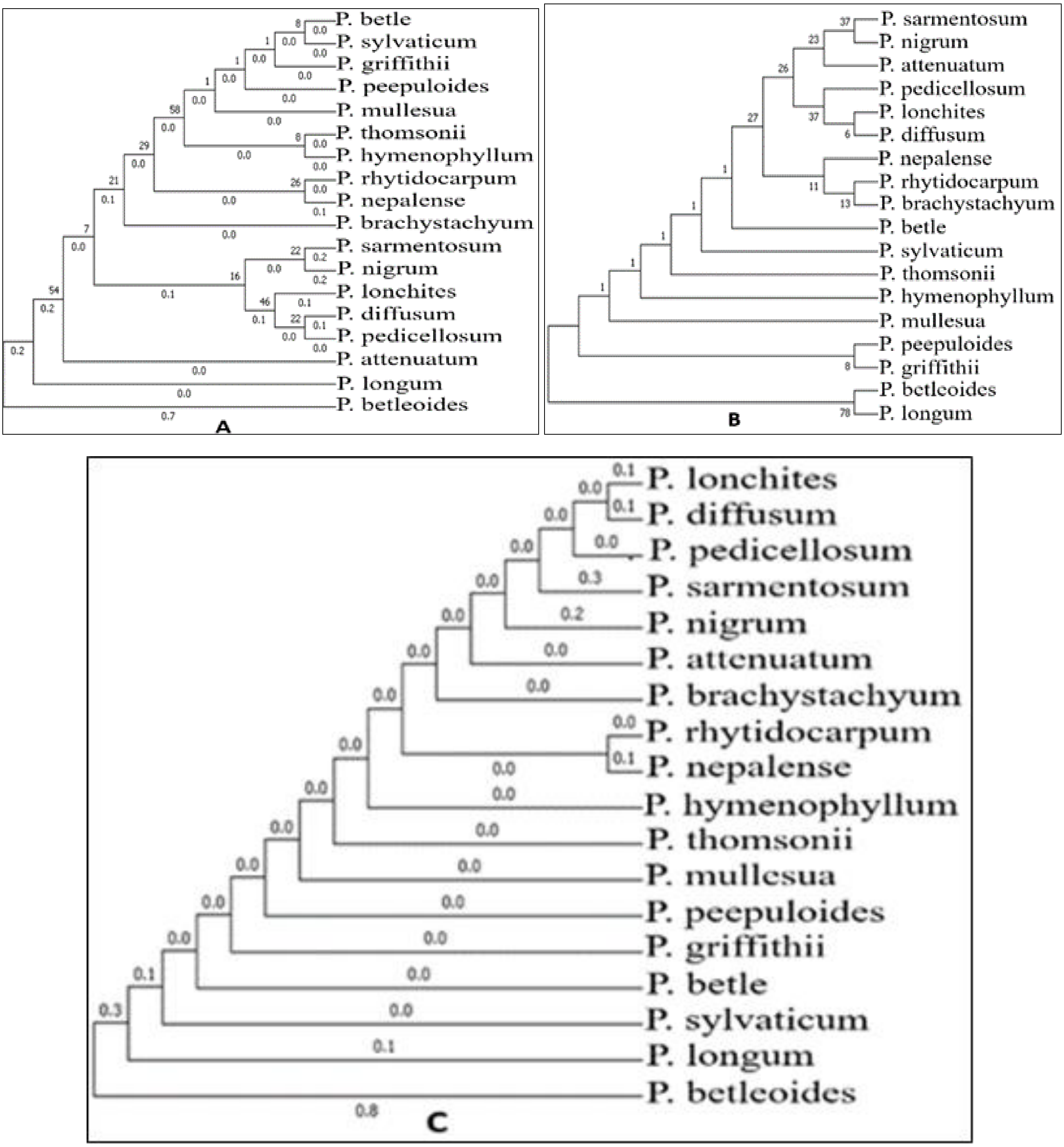

Fig 1: Phylogenetic tree constructed on the basis of *mat*K sequences of *Piper* spp. using MEGA 7 package. A-ML, B-MP & C-NJ.

Global phylogenetic reconstruction using partial-length *mat*K sequences of *Piper* plants are shown in Figure 2-4. MP, ML and NJ phylogenetic trees revealed distinct major clusters with different clades in each cluster. Most of *Piper* species of Assam state were included in the same clade as per global ML and MP phylogenetic trees. In the NJ phylogenetic tree made on global scale, the experimental *Piper* species were forming 2 main groups. That the retrieved sequences are closer to them and distanced from the derived *mat*K sequences is evident by their clustering behaviour. Phylogenetic tree constructed on the basis of the *mat*K sequences of the experimental *Piper* species in conjunction with the retrieved sequences obtained from NCBI clearly revealed divergences of different clades. The phylogenetic analysis showed a paraphyletic lineage.

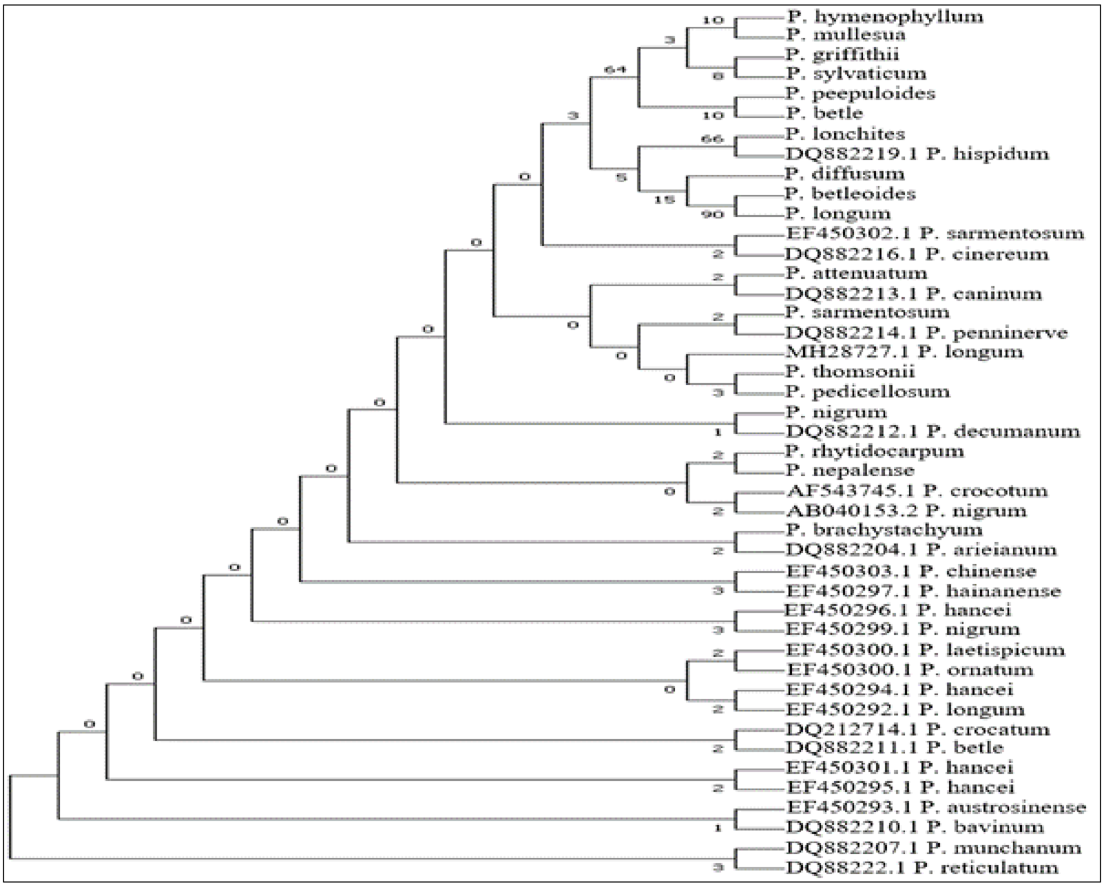

Fig 2: Phylogenetic tree based on the *mat*K sequences of experimental *Piper* spp. and homologous downloaded sequences of related *Piper* spp.using MEGA 7 package adopting ML (Maximum Likelihood) method.

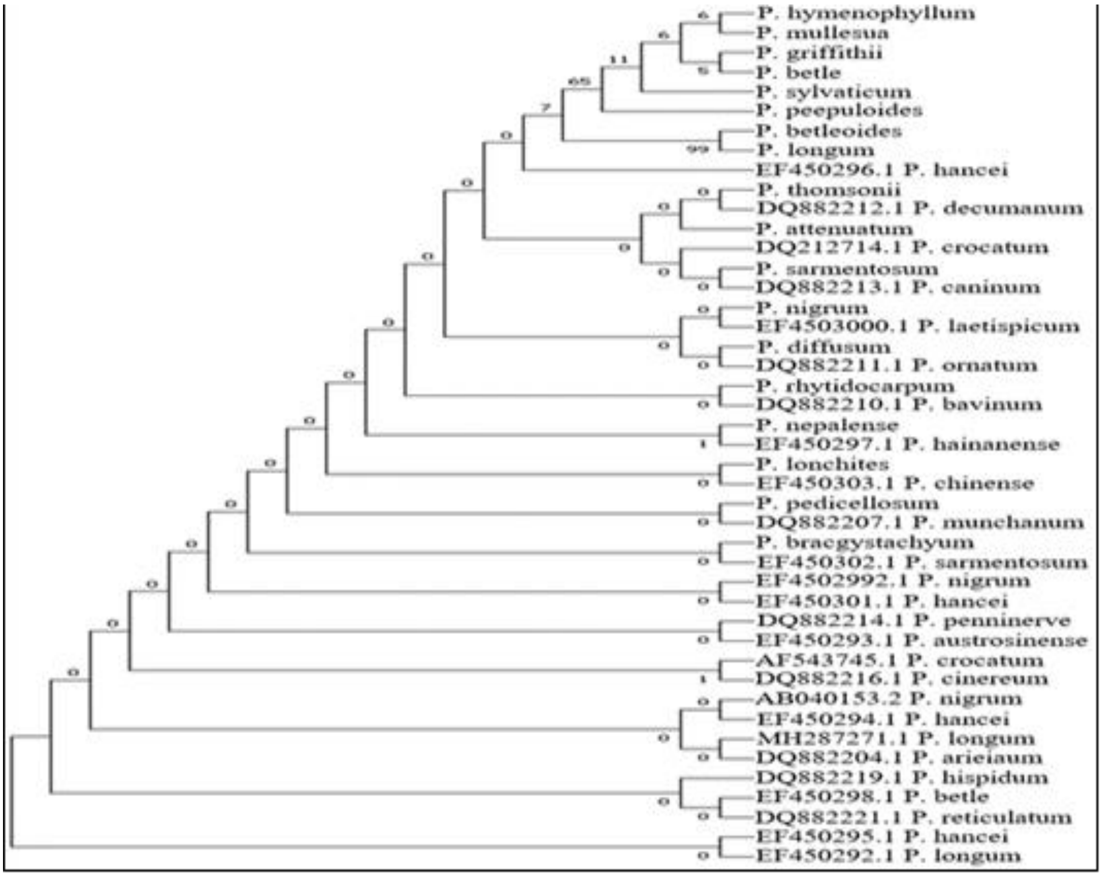

Fig 3: Phylogenetic tree based on the *mat*K sequences of experimental *Piper* spp. and homologous downloaded sequences of related *Piper* spp. using MEGA 7 package adopting MP (Maximum Parsimony) method.

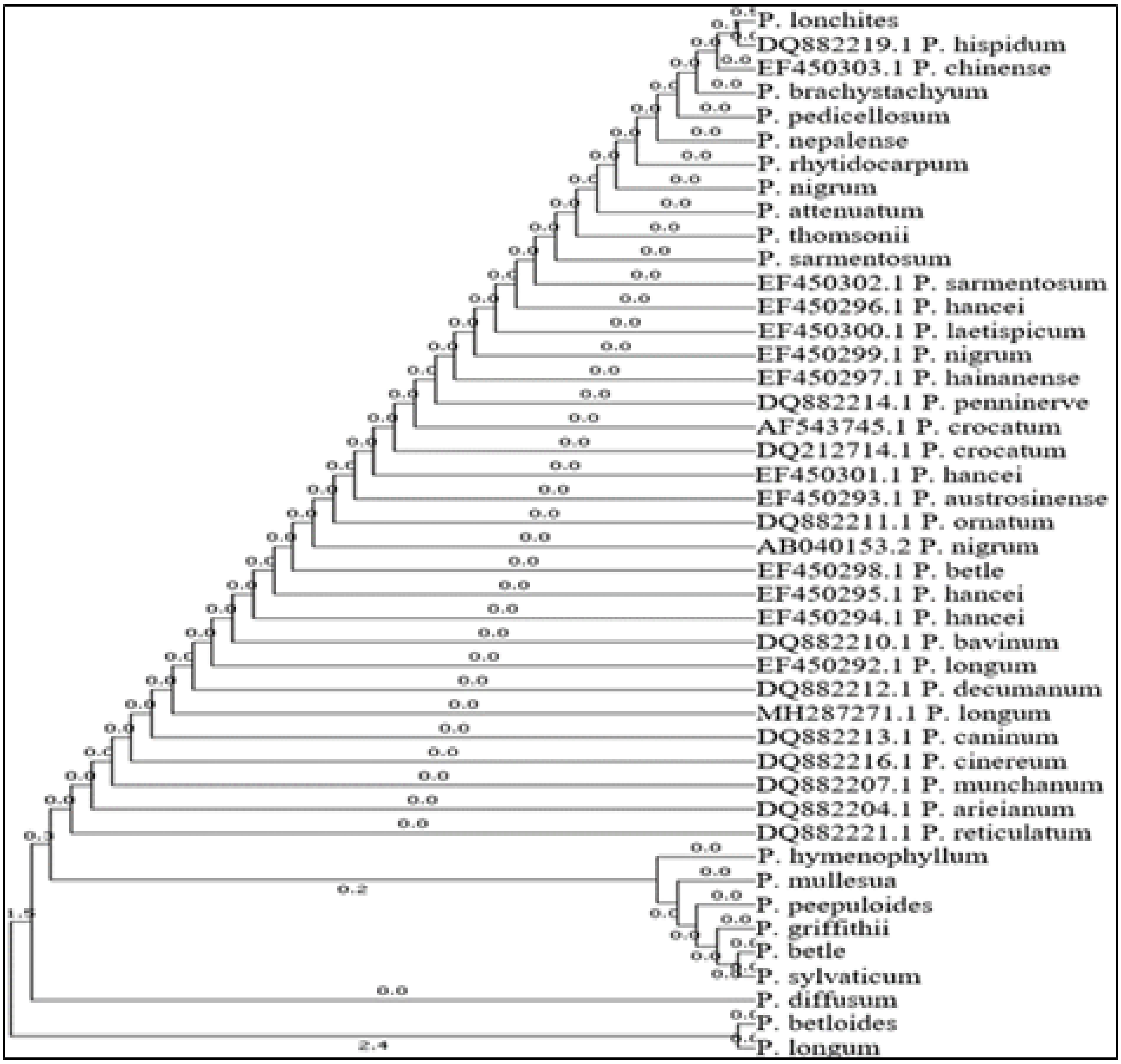

Fig 4: Phylogenetic tree based on the *mat*K sequences of experimental *Piper* spp. and homologous downloaded sequences of related spp. andusing MEGA 7 package adopting NJ (Neighbour Joining) method.

Conclusion

The *mat*K region has variable sites for the purpose of revealing genetic diversity among *Piper* species and thus offer good discriminating performance. The experimental *Piper* species exhibited considerable inter-specific variations. *mat*k datasets were helpful in resolving the phylogenetic relationships of the experimental *Piper* species. Information gathered from the molecular characterization of the experimental plants would assist in determination of strategies for their optimal conservation. Several wild *Piper* species bear characteristics better than the cultivated forms in terms of phytochemical constituents, disease resistance etc. Molecular level information is expected to help in the evaluation of the desired genotypes and would also help in breeding of the cultivated forms with the related wild species. The present global scale phylogenetic study would offer proper phylogenetic placements of the experimental plants.

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