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Research Article

Morphological and Molecular Phylogenetic Analysis of the Genus *Piper* (Piperaceae) in Assam, India

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Abstract

Morphological studies along with the molecular characterization of 18 Piper species were resorted to in the present investigation. Proper characterization of genetic resources such as that of *Piper* is expected to enhance understanding of the relationships of *Piper* species, providing valuable insights into its diversity and evolution. DNA sequence polymorphism of the chloroplast gene, *mat*K resolved the genetic differences among the experimental *Piper* species.

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

Introduction

Assam is considered a biodiversity hotspot in Northeast India and is renowned for its rich flora and fauna. Among its diverse plant species, the genus *Piper* holds significant importance due to its enormous economic and medicinal value. This genus also happens to be the largest among the basal angiosperms (Kubitzki *et al.*,1993; Soltis *et al.*,1999). The Northeastern region of India, encompassing Sikkim, has been documented to host approximately 65 species of *Piper* (Hooker, 1886; de Candolle, 1869, 1912a, 1914, 1923; Gajurel *et al.*, 2008), of which some are restricted in their distribution to this part of the country. *Piper* species are known for their morphological diversity and intricate genetic makeup, making them intriguing subjects for research. The plants within this genus are characterized by the presence of swollen nodes, heart-shaped leaves, densely clustered spike inflorescences, and diminutive flowers.

Previously morphological markers were primarily adopted for the characterization of plants. However, due to limitations associated with morphological markers, molecular markers have gained prominence over morphological markers. Molecular markers are considered advantageous as they remain unaffected by the age of the organism, environmental conditions, and its physiological state (Nadler, 1995; Poczai, *et al.*, 2013). Such markers may be extracted from any type of tissue and at any developmental stage enhancing their versatility in application. Molecular markers are now regularly employed for the study of biodiversity (Kumari & Rai, 2020), proper identification and characterization (Egydio *et al.*, 2020), exploring relationships (Chowdhury & Baruah, 2021), and determination of evolutionary patterns. Molecular marker-assisted analysis is recognized as a crucial method for the in-situ conservation of plant genetic resources (Ketema, 2020). The present study focuses on the comparative assessment of morphological and molecular phylogenetic analysis of *Piper* species in Assam, India.

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Materials and Methods

Collection and identification of plant sample

A total of 18 *Piper* species were gathered from the geographical confines of the state of Assam, India. The *Piper* species considered for the study are, *P. sarmentosum, P. thomsonii, P. attenuatum, P. hymenophyllum, P. mullesua, P. rhytidocarpum, P. griffithii, P. peepuloides, P. nepalense, P. betleoides, P. lonchites, P. brachystachyum, P. pedicellosum, P. sylvaticum, P. diffusum* and three cultivated forms viz. *P. nigrum, P. betle* and *P. longum*. The identification of collected plant materials was carried out through consultation with diverse literature and monographs (Hooker, 1882; Bentham & Hooker, 1883; Deb, 1983; Kanjilal *et al.*,1939; Kanjilal *et al.*,1940). Additionally, for verification purposes, voucher specimens were cross-referenced with preserved samples available in Herbaria such as GUBH, CAL, and ASSAM.

Morphological study of the experimental plants

Morphological analysis was conducted on the experimental plants, encompassing a comprehensive study of their habit and morphological features, including characteristics of the stem, leaves, inflorescence, flowers, fruits, and nodes. The % similarity matrix was calculated using the formula:

$$S = \frac{NS}{NS + ND} \text{ X } 100$$

Where,

S = Similarity value expressed in percentage.

NS = Number of similarities shared by any two species.

ND = Number of dissimilarities shared by any two species.

Extraction of genomic DNA and PCR amplification

Cryogenic grinding of leaf tissues employing liquid nitrogen was carried out. This was followed by DNA extraction by using the HiPurATM Plant Genomic DNA Miniprep Purification Kit (MB507-50PR, Himedia, India) following the recommended protocol of the manufacturer. Electrophoresis was performed on a 0.8% Agarose gel using 2 µL of extracted genomic DNA from each plant sample. The samples were run alongside uncut λ DNA in 1× TAE buffer containing 0.5 mg/mL of Ethidium Bromide (EtBr). The visual inspection of DNA bands was carried out under UV light. For the PCR amplification, SSR primers were sourced from Operon Technologies Inc., CA, USA (**Table 1**). The PCR reactions were conducted using a SimpliAmp™ Thermal cycler.

The amplification process commenced with DNA denaturation at 95°C for 5 minutes, succeeded by 35 cycles of exposure at 95°C for 1 minute, annealing at a temperature of 50°C for 1 minute, and elongation at 72°C for 1 minute. A final extension was carried out at 72°C for 5 minutes, and the reaction was concluded at 4°C. Subsequently, the amplified products were scrutinized through electrophoresis on a 2% agarose gel (Hi Media), run alongside a 100 bp molecular marker. The gel photograph was captured using a gel documentation system from Thermo Fisher Scientific, USA.

Table 1: List of primers employed for phylogenetic analysis.

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

Sequencing of the matK region and construction of the phylogenetic tree

Sequencing of the Amplified *mat*K region was resorted to by Automated DNA Sequencer (Applied Biosystems, ABI3730 xl). Bidirectional sequencing was carried out with respective forward and reverse primers. Following sequencing, chromates were manually inspected and visualized using ChromasPro software. Phylogenetic tree was constructed using the maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) methods with the help of MEGA 7.0 software.

Results and Discussion

Computation of % similarity matrix based on the morphological traits of the Piper species

% similarity among the experimental *Piper* species was determined using contrasting morphological characters. Roman numeric codes were assigned to the experimental plants (**Table 2)**. The characters considered for the study were given word codes (**Table 3**). A maximum of 8 states coding has been conducted. The coding assigned to each taxon based on their morphological variations is shown in **Table 4.** Morphological characters considered and the calculations of the % similarity matrix are tabulated in **Table 5**. **Table 6** displays the % similarity matrix among the experimental plants.

Table 2: List of code numbers assigned to the *Piper* spp.

Sl. No.	Names of the sample plant	Code numbers
1.	*Piper thomsonii*	i
2.	*Piper sarmentosum*	ii
3.	*Piper attenuatum*	iii
4.	*Piper hymenophyllum*	iv
5.	*Piper nigrum*	v
6.	*Piper rhytidocarpum*	vi
7.	*Piper mullesua*	vii
8.	*Piper peepuloides*	viii
9.	*Piper griffithii*	ix
10.	*Piper nepalense*	x
11.	*Piper lonchites*	xi
12.	*Piper diffusum*	xii
13.	*Piper betleoides*	xiii
14.	*Piper longum*	xiv
15.	*Piper betle*	xv
16.	*Piper pedicellosum*	xvi
17.	*Piper brachystachyum*	xvii
18.	*Piper sylvaticum*	xviii

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Table 3: Morphological characters used for numerical evaluation.

Sl. No.	Morphological characters	Word code
1.	Habit.	H
2.	Habit lateral branch.	HLB
3.	Holding capacity to support.	HCS
4.	Stem texture.	ST
5.	Nature of node in flowering shoot.	NFS
6.	Adventitious roots.	AR
7.	Leaf texture.	LT
8.	Leaf thickness	LTh
9.	Leaf shape (fertile branch).	LSF
10.	Leaf apex.	LA
11.	Leaf base.	LB
12.	Shape of male spike.	SMS
13.	Shape of female spike.	SFS
14.	Bract type.	BT
15.	Shape of fruiting spike.	SFrS
16.	Orientation of fruiting spike.	OFS
17.	Arrangement of fruit.	AF
18.	Colour of mature fruit.	CMF

Table 4: List of coding of characters.

Sl. No.	Characters	Grouping of characters into different character states							
		1	2	3	4	5	6	7	8
1.	Habit.	Trailing shrub ii, xiv	Erect shrub vii, xi, xvii	Climber i, iii, iv, v, vi, viii, ix, x, xii, xiii, xv, xvi	Trailing/ climbing shrub xviii	_	_	_	_
2.	Habit lateral branch.	Erect i, ii, iii, vii, xi, xii, xiv, xviii	Pendent iv, v, vi, viii ix, xiii, xv, xvi, xvii	Horizontal x	_	_	_	_	_
3.	Holding capacity to support.	Nil ii, iii, vii, xiv, xvii	Weak x, xi, xviii	Strong i, iv, v, vi, viii, ix, xii, xiii, xv, xvi,	_	_	_	_	_
4.	Stem texture.	Glabrous iii, v, vi, viii, ix, x, xi, xii, xvi, xvii, xviii	Pubescent i, ii, iv, vii, xiii, xv	Hispid xiv	_	_	_	_	_
5.	Nature of node of flowering shoot.	Slightly swollen iii, x, xi, xiv, xviii	Swollen i, ii, iv, viii, ix, xiii, xvi, xvii	Highly swollen v, vi, vii, xii, xv,	_	_	_	_	_
6.	Adventitious roots.	Nil vii, xi	Few ii, iii, iv, x, xii, xvii	Many i, v, vi, viii, ix, xiii, xiv, xv, xvi, xviii	_	_	_	_	_
7.	Leaf texture	Glabrous v, vi, vii, ix, xi, xii, xvi, xvii	Pubescent i, ii, iv, x, xiii, xiv, xv, xviii	Hispidulous iii	Stellate viii	_	_	_	_
8.	Leaf thickness.	Membranous i, ii, iii, iv, viii, xiii, xiv, xv, xvii,	Thinly coriaceous vii, ix, x, xi,	Thickly coriaceous v, vi,	_	_	_	_	_

		xviii	xii, xvi						
9.	Leaf shape (Fertile branch).	Ovate i, ii, iii, iv, v, vi, vii, xii, xiv, xvi	Elliptic i, v, xvi	Lanceolate vii, xiii, xiv	Ovate-elliptic iv	Ovate-lanceolate i, ii, ix, x, xi, xv	Elliptic-lanceolate iii, ix, xii, xviii	Linear viii	Oblong-ovate viii
10.	Leaf apex	Acuminate i, iv, vii, viii, ix, x, xi, xii, xiii, xiv, xv, xvi, xvii, xviii	Acute ii, v, vi, xvi	Cuspidate iii,	Mucronate iii,	Caudate viii, xvii	_	_	_
11.	Leaf base.	Rounded i, ii, iii, iv, v, vi, vii, ix, x, xii, xiv, xv, xvi, xvii	Cordate i, ii, iii, vi, viii, xii, xiii, xv, xvi, xvii, xviii	Cuneate i, ii, vii, ix, x, xi, xii, xviii	Truncate iii, x, xvi	Acute iv, xi, xvii	Oblique i, iii, v, vi, vii, viii, ix, x, xiii, xiv, xvi, xviii	_	_
12.	Shape of male spike.	Cylindrical i, ii, xi, xiii, xiv, xvi, xvii	Filiform iii, iv, vi, vii, viii, ix, x, xv, xviii	_	_	_	_	_	_
13.	Shape of female spike.	Cylindrical ii, iii, v, viii, x, xi, xiii, xiv, xv, xvi, xviii	Filiform iv, vi, ix	Globose i, vii, xii, xvii	_	_	_	_	_
14.	Nature of bract.	Adnate iii, iv, v, vi, vii, ix, x, xi, xiv	Peltate i, ii, viii, xii, xiii, xv, xvi, xvii, xviii	_	_	_	_	_	_
15.	Shape of fruiting spike.	Straight iii, iv, vi, ix, x, xi	Cylindrical ii, viii, xiii, xiv, xv, xvi, xviii	Twisted v	Globose i, vii, xii, xvii	_	_	_	_
16.	Orientation of fruiting spike.	Erect i, ii, vii, viii, xi,	Pendent iii, iv, v, vi,	_	_	_	_	_	_

		xii, xiv, xvii, xviii	ix, x, xiii, xv, xvi						
17.	Arrangement of fruit.	Loose iii, iv, v, vi, ix, x, xi, xiv	Compact i, ii, vii, viii, xii, xiii, xv, xvi, xvii, xviii	–	–	–	–	–	–
18.	Colour of mature fruit.	Black i, ii, iii, iv, vi, vii, viii, ix, x, xii, xiii, xiv, xv, xvi, xvii, xviii	Orange xi	Yellow v	–	–	–	–	–

Table 5: Tabulation of character states against the experimental *Piper* spp.

Character	*Piper* species																	
	P. thomsonii	*P. sarmentosum*	*P. attenuatum*	*P. hymenophyllum*	*P. nigrum*	*P. rhytidocarpum*	*P. mullesua*	*P. peepuloides*	*P. griffithii*	*P. nepalense*	*P. lonchites*	*P. diffusum*	*P. betleoides*	*P. longum*	*P. betle*	*P. pedicellosum*	*P. brachystachum*	*P. sylvaticum*
H	3	1	3	3	3	3	2	3	3	3	2	3	3	1	3	3	2	4
HLB	1	1	1	2	2	2	1	2	2	3	1	1	2	1	2	2	2	1
HCS	3	1	1	3	3	3	1	3	3	2	2	3	3	1	3	3	1	2
ST	2	2	1	2	1	1	2	1	1	1	1	1	2	3	2	1	1	1
NFS	2	2	1	2	3	3	3	2	2	1	1	3	2	1	3	2	2	1
AR	3	1	3	2	3	3	1	3	3	2	1	2	3	3	3	3	2	3
LT	2	2	3	2	1	1	1	4	1	2	1	1	2	2	2	1	1	2
LTh	1	1	1	1	3	3	2	1	2	2	2	2	1	1	1	2	1	1
LSF	1,2,5	1,5	1,6	1,4	1,2	6	1,3	7,8	5,6	5	5	1,6	3	1,3	5	1,2	4,6	6
LA	1	2	3,4	1	2	2	1	1,5	1	1	1	1	1	1	1	1,2	1,5	1
LB	1,2,3,6	1,2,3	1,2,4,6	1	1,6	1,3,6	1,3,6	2,6	1,3,6	1,4,6	3,5	1,2,3	2,6	1,6	1,2	1,2,4,6	1,2	2,3,6
SMS	1	1	2	2	_	2	2	2	2	2	1	_	1	1	2	1	1	2
SFS	3	1	1	2	1	2	3	1	2	1	1	3	1	1	1	1	3	1
BT	2	2	1	1	1	1	1	2	1	1	1	2	2	1	2	2	2	2
SFrS	1	2	1	1	3	1	4	2	1	1	1	4	2	2	2	2	4	2
OFS	1	1	3	2	2	2	1	1	2	2	1	1	2	1	2	2	1	1
AF	2	2	1	1	1	1	2	2	1	1	1	2	2	1	2	2	2	2
CMF	1	1	1	1	3	1	1	1	1	1	2	1	1	1	1	1	1	1

Table 6: Morphological % Similarity matrix.

	P. thomsonii	*P. sarmentosum*	*P. attenuatum*	*P. hymenophyllum*	*P. nigrum*	*P. rhytidocarpum*	*P. mullesua*	*P. peepuloides*	*P. griffithii*	*P. nepalense*	*P. lonchites*	*P. diffusum*	*P. betleoides*	*P. longum*	*P. betle*	*P. pedicellosum*	*P. brachystachum*	*P. sylvaticum*
P. thomsonii	100	66.7	44.4	61.1	29.4	33.3	50.0	61.1	44.4	38.9	38.9	64.7	72.2	55.6	66.7	61.1	55.6	50,0
P. sarmentosum		100	38.9	38.9	23.5	16.7	44.4	50.0	16.7	27.8	38.9	41.2	61.1	66.7	55.6	55.6	38.9	55.6
P. attenuatum			100	50.0	47.1	50.0	38.9	44.4	55.6	44.4	38.9	35.3	33.3	61.1	38.9	38.9	33.3	55.6
P. hymenophyllum				100	47.1	61.1	38.9	44.4	72.2	61.1	22.2	35.3	55.6	44.4	61.1	50.0	44.4	27.8
P. nigrum					100	76.5	29.4	41.2	58.9	41.2	29.4	41.2	41.2	35.3	47.1	64.8	23.5	23.5
P. rhytidocarpum						100	33.3	44.4	**83.3**	50.0	33.3	47.1	33.3	27.8	50.0	55.6	33.3	33.3
P. mullesua							100	33.3	38.9	33.3	50.0	70.1	33.3	44.4	38.9	38.9	55.6	38.9
P. peepuloides								100	55.6	38.9	22.2	52.9	72.2	44.4	72.2	72.2	50.0	66.7
P. griffithii									100	66.7	50.0	52.9	44.4	33.3	55.6	66.7	44.4	38.9
P. nepalense										100	55.6	41.2	38.9	44.4	50.0	44.4	22.2	50.0
P. lonchites											100	41.2	16.7	44.4	16.7	33.3	27.8	44.4
P. diffusum												100	41.2	35.3	47.1	64.7	70.1	52.9
P. betleoides													100	55.6	**83.3**	77.8	50.0	55.6
P. longum														100	44.4	44.4	38.9	61.1
P. betle															100	66.7	38.9	61.1
P. pedicellosum																100	55.6	50.0
P. brachystachyum																	100	50.0
P. sylvaticum																		100

Computation of the % similarity matrix unrevealed a range of moderate to high morphological resemblances revealing clear morphological distinctiveness within the studied *Piper* plants. The highest morphological similarity of 83.3 % was observed between *Piper griffithii* and *P. rhytidocarpum* and between *P. betleoides* and *P. betle*. *P. sarmentosum* is most distant from both *P. griffithii* and *P. rhytidocarpum*, displaying a mere 16.7 % similarity. Similarly, *P. lonchites* stands out as the most morphologically distant from both *P. betleoides* and *P. betle* with a low % similarity matrix value of 16.7 %.

PCR amplification

The extracted genomic DNA from the experimental plants displayed nearly identical bands of high molecular weight. PCR Amplification of the targeted *mat*K gene produced bands of approximately ~950 bp, as observed on a 1.5% (w/v) agarose gel in comparison to a 100 bp DNA ladder (**Plate 1**).

matK gene sequencing

Automated DNA sequencer yielded ~ 950 bp sized amplicons for the PCR products of the *mat*K gene. The *mat*K sequences of the experimental plants have been submitted to NCBI. The relevant accession numbers are shown in **Table 7.**

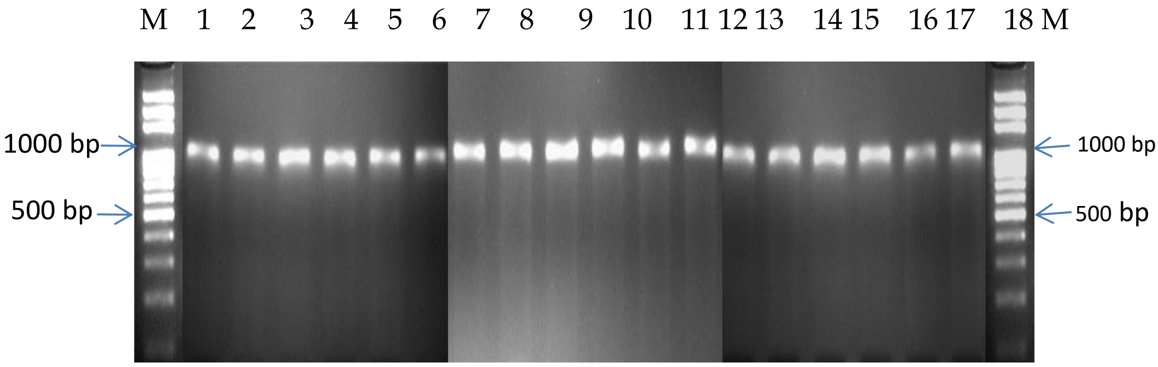

Plate 1: PCR amplified *mat*K gene of the 18 Piper spp. at 1.5% (w/v) agarose gel. Lane M: Molar mass marker (100 bp DNA ladder); Lane 1: *P thomsonii*; Lane 2: *P sarmentosum*; Lane 3: *P attenuatum*; Lane 4: *P hymenophyllum*; Lane 5: *P nigrum*; Lane 6: *P rhytidocarpum*; Lane 7: *P mullesua*; Lane 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 7: Accession numbers obtained from NCBI shown against the 18 *Piper* spp.

Sl No	Name of the *Piper* species	Accession No.
1.	*Piper thomsoni*	MW617342
2.	*Piper sarmentosum*	MW617343
3.	*Piper attenuatum*	MW617344
4.	*Piper hymenophyllum*	MW617345
5.	*Piper nigrum*	MW617346
6.	*Piper rhytidocarpum*	MW690589
7.	*Piper mullesua*	MW617347
8.	*Piper peepuloides*	MW617348
9.	*Piper griffithii*	MW617348
10.	*Piper nepalense*	MW810865
11.	*Piper lonchites*	MW810866
12.	*Piper diffusum*	MW810867
13.	*Piper betleoides*	MW617350
14.	*Piper longum*	MW617351
15.	*Piper betle*	MW617352
16.	*Piper pedicellosum*	MW617353
17.	*Piper brachystachyum*	MW810868
18.	*Piper sylvaticum*	MW690590

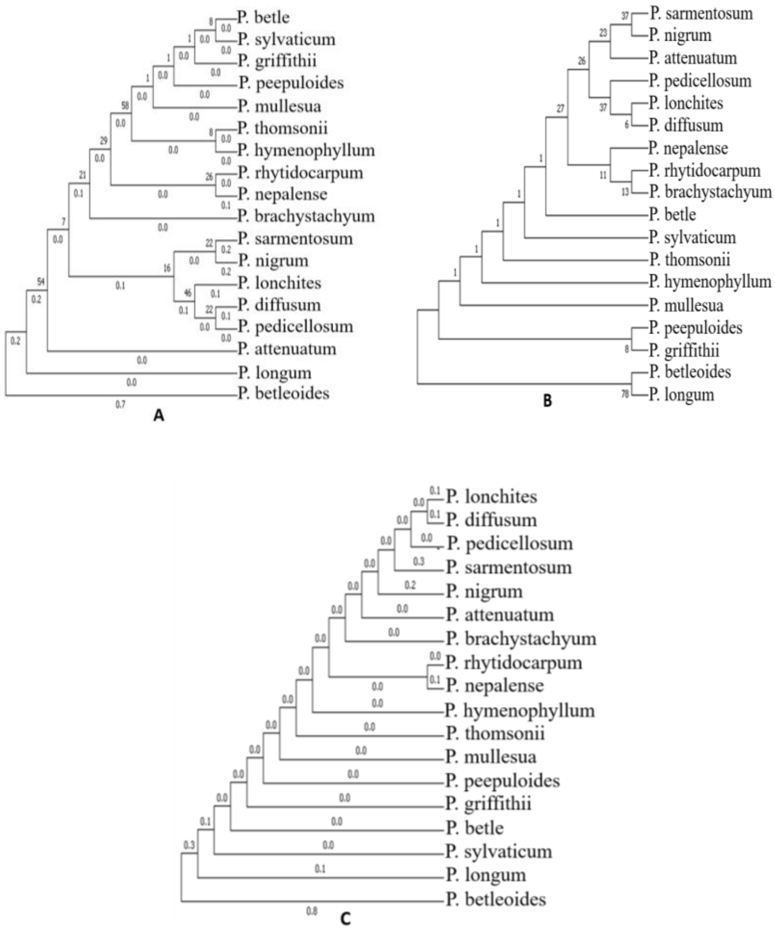

Fig. 1: Phylogenetic tree constructed based on matK sequences of *Piper* spp. using MEGA 7 package. A-ML, B-MP & C-NJ.

Phylogenetic relationships of the experimental plants based on the matK sequences

The phylogenetic relationships among the 18 experimental *Piper* species, adopting maximum parsimony (MP), maximum likelihood (ML), and neighbor joining (NJ) methods are illustrated in **Figure 1**. As per the ML and MP methods, *Piper sarmentosum* exhibited maximum proximity towards *P. nigrum* with comparatively high bootstrap values. Despite not sharing the same most recent common ancestors (MRCAs), the NJ analysis also revealed comparable proximity between the two species. Similarly, the MP and NJ analyses indicate that *P. lonchites* is closest to *P. diffusum*. The ML analysis also demonstrated proximity between *Piper diffusum* and *Piper lonchites* although they shared distinct most recent common ancestors (MRCAs). In the ML and NJ phylogenetic tree, *P. rhytidocarpum* was identified as being closest to *P. nepalense*. The MP phylogenetic tree also indicated a close relationship between the two species. The remaining experimental species were distributed across distinct clades as per the three *mat*K phylogenetic trees, indicating their distant relationships. *P. betleoides* and *P. longum* are grouped as a monophyletic clad as per the ML analysis and can be considered as sister species. Nevertheless, these two species are positioned as outgroup species in all three phylogenetic trees, indicating their considerable distance from the remaining *Piper* species. The genetic variations identified through the utilization of SSR markers closely align with the findings reported by previous researchers (Jiang and Liu, 2011; Chowdhury *et al.,*2014; Singh *et al.,*2016; Chaveerach *et al.,* 2016; Naim and Mahboob, 2020).

Comparative study of morphological variations and molecular phylogenetic analysis

According to the molecular study using *mat*K sequences, *P. sarmentosum* exhibited the closest relation to *P. nigrum*. However, morphological analysis presented a significantly low similarity matrix of 23.5% between these two species. Furthermore, *P. lonchites* was identified as the closest relative to *P. diffusum* through multiple molecular phylogenetic analyses (MP, ML, and NJ). Nevertheless, these two species displayed only a moderate morphological similarity of 41.2%. The most noteworthy morphological similarity was observed between *P. rhytidocarpum* and *P. griffithii* (83.3%), as well as between *P. betleoides* and *P. betle* (83.3%). Surprisingly, despite the high morphological resemblance, these species exhibited considerable genetic distance in the molecular phylogenetic analysis. *P. sarmentosum* exhibited a considerable morphological distance from *P. rhytidocarpum* and *P. griffithii* (16.7% similarity) based on morphological traits. Similar distances were also affirmed through molecular phylogenetic analysis. Notably, the morphological distances between *P. lonchites* and *P. betleoides* (16.7% similarity) and between *P. lonchites* and *P. betle* (16.7% similarity) were congruently supported by molecular phylogenetic analysis. The results of % similarity matrices derived from morphological characteristics of the 18 *Piper* species are partially aligned with the findings from molecular phylogenetic analysis based on *mat*K sequences.

Conclusion

A comprehensive investigation into the *Piper* is imperative owing to a great deal of variations both within and between species. The results of the morphological analysis are partly congruent with the results of the molecular phylogenetic study based on the *mat*K sequences. Partial congruence suggests a complex interplay between observable traits and genetic evolution. Certain morphological traits might be influenced by genetic factors, while others could be shaped by environmental conditions. Intra-specific variations found in the genus *Piper* may be due to phenotypic plasticity, where a single genotype can give rise to different phenotypes based on environmental cues. While molecular analysis is powerful, morphological traits remain essential for a holistic understanding of the genus.

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Author contributions
UC conceptualized the study. UC and PKB designed the experimental protocols. UC conducted the experiments and wrote the manuscript with inputs from the co-author.

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