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RESEARCH ARTICLE

Reconstruction of molecular phylogeny of closely related *Amorphophallus* species of India using plastid DNA marker and fingerprinting approaches

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Abstract Plastid DNA markers sequencing and DNA fingerprinting approaches were used and compared for resolving molecular phylogeny of closely related, previously unexplored *Amorphophallus* species of India. The utility of individual plastid markers namely *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD*, their combined dataset and two fingerprinting techniques viz. RAPD and ISSR were tested for their efficacy to resolves *Amorphophallus* species into three sections specific clades namely *Rhaphiophallus*, *Conophallus* and *Amorphophallus*. In the present study, sequences of these four plastid DNA regions as well as RAPD and ISSR profiles of 16 *Amorphophallus* species together with six varieties of two species were generated and analyzed. Maximum likelihood and Bayesian Inference based construction of phylogenetic trees indicated that among the four plastid DNA regions tested individually and their combined dataset, *rbcL* was found best suited for resolving closely related *Amorphophallus* species into section specific clades. When analyzed individually, *rbcL* exhibited better discrimination ability than *matK*, *trnH–psbA*, *trnLC–trnLD* and combination of all four tested plastid markers. Among two fingerprinting techniques used, the resolution of *Amorphophallus* species using RAPD was better than ISSR and combination of RAPD +ISSR and in congruence with resolution based on *rbcL*.

Keywords *Amorphophallus* · Molecular phylogeny · DNA finger printing · *rbcL* · *matK* · Maximum likelihood · Bayesian inference

Introduction

Genus *Amorphophallus* Blume ex Decne belongs to the family *Araceae* and comprises of perennial herbaceous plants that are mainly adapted to grow in the shady and mountainous areas. This genus is represented by 200 species distributed in the tropical Africa, Madagascar, tropical and subtropical Asia, the Malay Archipelago, Melanesia and Australia (Mayo et al. 1997). The genus has economic potential as starch and other carbohydrate yielding plants. Some of the species are used as food and vegetables while others have ornamental potentials. With the merger of *Amorphophallus* sect. *synantherias* into section *Rhaphiophallus* (Sivadasan 1989), the genus at present has 10 sections, of which 3 sections viz. *Candarum* Engl., *Conophallus* (Schott) Engl. and *Rhaphiophallus* (Schott) Engl. are represented in India. Recently section *Candarum* has been revised and renamed as section *Amorphophallus* (Jaleel et al. 2014). In India, these sections are represented by 18 species and 7 varieties of which 12 species and 3 varieties are endemic to the country. Identification and discrimination of *Amorphophallus* species is difficult due to closely related morphological characters. Because of the timing of emergence of inflorescences and their relatively short active period of existence, the taxonomic

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identification of *Amorphophallus* species becomes difficult. Moreover, close similarity of morphological characters of the leaves of many *Amorphophallus* species makes their identification with vegetative specimens difficult or impossible (Jaleel et al. 2011).

Previously, several studies investigated the phylogenetic relationships within the genus *Amorphophallus.* Recently, Grob et al. (2002, 2004) used molecular data for phylogenetic reconstruction and attempted to interpret morphological character evolution in *Amorphophallus* based on a combined nuclear and plastid phylogeny. This study based on *matK* and *trnL* intron sequences indicated that *Amorphophallus* can be divided into five well supported clades (Grob et al. 2002). Also, Sedayu et al. (2010) studied the morphological character evolution of *Amorphophallus* based on a combined phylogenetic analysis of *trn*L, *rbc*L and *LEAFY* second intron sequences. Three major clades were identified which reflected the biogeographical distribution of *Amorphophallus* and some of the clades were supported by morphological characters. The study conducted by Grob et al. (2002) included only three *Amorphophallus* species from India namely *A. commutatus*, *A. margaritifer* and *A. smithsonianus* whereas the study by Sedayu et al. (2010) included six species from India namely *A. commutatus*, *A. hirsutus*, *A. hohenackeri*, *A. konkanensis*, *A. longiconnectivus* and *A. margaritifer*. The molecular phylogeny of remaining *Amorphophallus* species from India is still not known.

It is generally agreed that a multilocus approach based on plastid ('chloroplast') data is currently the most effective strategy for species identification and species recognition in plants (Chase et al. 2005, 2007a, b; Kress et al. 2005, Kress and Erickson 2007; Newmaster et al. 2006, 2008; Cowan et al. 2006). Beside multilocus DNA barcoding approach, molecular marker based fingerprinting techniques can also be used to study the similarity of genome of the different species distributed in same geological area (Kumar et al. 2008). Molecular markers such as randomly amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) have been successfully utilized for phylogenetic studies of many plant species (Fang et al. 1997; Joshi et al. 2000; Rajesh et al. 2003; Arnau et al. 2003).

In this report, we reconstructed the molecular phylogeny of *Amorphophallus* species from India using four different plastid gene regions sequences viz. *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD* and two fingerprinting markers namely RAPD and ISSR. Molecular phylogeny was reconstructed to infer species relationship and to test if molecular phylogeny resolves *Amorphophallus* species into three section specific clades namely *Rhaphiophallus*, *Conophallus* and *Amorphophallus*. We evaluated the sequences of these plastid gene regions individually as well as their combined data for reconstruction of phylogenetic trees using for maximum likelihood (ML) and Bayesian Inference (BI) analyses and compared their efficacies with RAPD and ISSR. The objective of the present study was to assess if the resolution of closely related *Amorphophallus* species of India into three sections is also supported by molecular phylogeny. This was achieved by using (1) multi-locus plastid gene regions sequencing approach (2) DNA fingerprinting method, and (3) and comparing the performance of both approaches.

Materials and methods

Sampling

Tubers or plantlets of 16 *Amorphophallus* species together with six varieties of two species were initially collected from different locations in India and maintained in Lead Botanical Garden of Department of Botany, Shivaji University, Kolhapur, India. Our specimen included ten species from section *Rhaphiophallus*, four species together with four varieties of *Amorphophallus commutatus* from section *Conophallus*, two species together with two varieties of *Amorphophallus paeoniifolius* from section *Amorphophallus*, and *Sauromatum venosum* as an out-group (Table 1). Specimens were taxonomically identified by studying floral characters (Fig. 1), using basic floras, monographs; papers and herbarium were submitted at Dept. of Botany, Shivaji University, Kolhapur (SUK), India.

DNA extraction, PCR amplification and sequencing

Total genomic DNA from each species under study was isolated from approximately 0.5 g of fresh leaf material using CTAB method (Doyle and Doyle 1987). The extracted DNAs were checked for their purity on agarose gel (1%) and quantified spectrophotometrically. Prior to PCR amplification of barcoding, RAPD and ISSR markers, working stock (20 ng/µl) of each of genomic DNA was prepared and used. The PCR amplifications of various barcode regions were performed with optimized annealing temperatures (Ta) depending upon the primers used (Table 2). DNA was amplified in 50 µl reaction mixtures containing 2U of high fidelity *Taq* DNA polymerase (Merck Bioscience Pvt. Ltd, Bangalore, India) with 1 X Buffer A, 2.5 mM $MgCl_2$ (Merck Bioscience Pvt. Ltd, Bangalore, India), 400 µM dNTPs, 400 µM of each primer and, 40 ng of template DNA. PCR conditions were as follows: initial denaturation at 96 °C for 5 min., followed by 35 cycles of denaturation at 96 °C for 1 min., annealing at optimized temperature for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. Successful

Table 1 Collection localities and sections of *Amorphophallus* species and varieties

Sr. no.	Name of species	Vouchers specimens	Sections	Locality
1	*A. commutatus* var. *commutatus*	ARG-19	*Conophallus*	Amba, Maharashtra, India.
2	*A. commutatus* var. *anmodensis*	ARG-21	*Conophallus*	Anmod Ghat, Goa, India
3	*A. commutatus* var. *wayanadensis*	ARG-20	*Conophallus*	Mulshi, Maharashtra, India
4	*A. commutatus* var. *anshiensis*	ARG-22	*Conophallus*	Anshi National Park, Karnataka, India
5	*A. bulbifer*	ARG-18	*Conophallus*	Jog fall, Karnataka, India
6	*A. hirsutus*	ARG-35	*Amorphophallus*	Andaman Island, India
7	*A. oncophyllus*	ARG-36	*Conophallus*	Andaman Island, India
8	*A. konkanensis*	ARG-12	*Rhaphiophallus*	Belgaum, Karnataka, India
9	*A. hohenackeri*	ARG-16	*Rhaphiophallus*	Kaddan pedika, Kerala, India
10	*A. margaritifer*	ARG-8	*Rhaphiophallus*	Parasnath, Jharkhand, India
11	*Amorphophallus species*	ARG-24	*Rhaphiophallus*	Gondia, Maharashtra, India
12	*A. paeoniifolius* var. *campanulatus*	ARG-34	*Amorphophallus*	Botanical Garden. Shivaji Uinversity, India
13	*A. paeoniifolius* var. *paeoniifolius*	ARG-37	*Amorphophallus*	Botanical Garden. Shivaji Uinversity, India
14	*A. bhandarensis*	ARG-38	*Rhaphiophallus*	Bhandara, Tumsar, Maharashtra, India
15	*A. bonaccordensis*	ARG-13	*Rhaphiophallus*	Bonaccord, Kerala, India
16	*A. longiconnectivus*	ARG-3	*Rhaphiophallus*	Khandva, Madhya Pradesh, India
17	*A. mysorensis*	ARG-33	*Rhaphiophallus*	Billigirirangan Hills., Karnataka, India
18	*A. nicolsonianus*	ARG-15	*Conophallus*	Bonaccord, Kerala, India
19	*A. smithsonianus*	ARG-14	*Rhaphiophallus*	Bonaccord, Kerala, India
20	*A. sylvaticus*	ARG-40	*Rhaphiophallus*	Pillathikkuppam, Tamil Nadu, India.
21	*Sauromatum venosum*	ARG-39	Outgroup	Botanical Garden. Shivaji Uinversity, India

amplification was checked on 1% agarose gel, amplified products were purified using PCR purification Kit (Sigma Aldrich, India). Purified PCR products were sequenced bidirectionally on ABI's 3500×L genetic analyzer using a BigDye Terminator v3.1 cycle sequencing kit (Invitrogen, Carlsbad, USA) using standard conditions.

Phylogenetic reconstructions and analyses

Sequences were analyzed, edited and assembled using ChromasPro software (http://www.technelysium.com.au/ChromasPro.html). For phylogenetic analysis, multiple sequence alignments of sequences were performed with ClustalW (Thompson et al. 1994) and edited manually using DAMBE (Xia and Xie, 2001) to obtain an unambiguous sequence alignment. For the analyses of combined datasets, sequences were concatenated using DAMBE. Interspecific genetic divergences was calculated using Kimura 2-Parameter (K2P) distances in MEGA 5.0 following the instruction of the CBOL for distance calculations (Tamura et al. 2011).

Maximum likelihood (ML) analyses for individual and combined datasets of *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD* were carried out in RAxML (Stamatakis, 2006, 2014) using the application raxmlGUI 1.5b1(Silvestro and Michalak, 2011). The clade support was estimated using ML + rapid bootstrap with 1000 replicates based on a maximum likelihood analysis with the model GTRGAMMA using RAxML version 8.0.0 (Stamatakis 2014).

Bayesian inference (BI) of phylogeny using Monte Carlo Markov chains (Yang and Rannala, 1997) was performed with MrBayes v. 3.2 (Ronquist et al. 2012). Individual and combined datasets of *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD* were tested for the appropriate model of nucleotide evolution with MrModelTest 2.3 (Nylander, 2004). The optimal model was implemented according to the Akaike information criterion and then implemented for the analyses (Posada and Buckley 2004). In BI analyses, 1,000,000 generations of two independent Monte Carlo Markov chains with equal rates until convergence was achieved, implemented as 'stoprule' when average deviation of split frequencies was below 0.01, were carried out. Trees were sampled every 100 generations and initial 10% trees before stationarity were discarded as burn-in (set to 0.1). Plot of the generation versus log probability was generated by giving the "sump" command and used for estimation of stationarity of the analyses. Convergence between runs was reported by MrBayes using ''mcmcdiagn'' command. The majority-rule consensus trees with posterior probabilities (PP) values were constructed using remaining trees. PP values between 0.8 and 0.89 were considered as moderate support whereas PP values over 0.9 were considered as high support.


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◀**Fig. 1** Kinds of inflorescence/flower in 16 *Amorphophallus* species and six varieties of India included in this study: *A. commutatus* var. *commutatus* (**a**) *A. commutatus* var. *anmodensis* (**b**), *A. commutatus* var. *wayanadensis* (**c**), *A. commutatus* var. *anshiensis* (**d**), *A. bulbifer* (**e**), *A. hirsutus* (**f**), *A. oncophyllus* (**g**), *A. konkanensis* (**h**), *A. hohenackeri* (**i**), *A. margaritifer* (**j**), *Amorphophallus species* (**k**), *A. paeoniifolius* var. *campanulatus* (**l**), *A. paeoniifolius* var. *paeoniifolius* (**m**), *A. bhandarensis* (**n**), *A. bonaccordensis* (**o**), *A. longiconnectivus* (**p**), *A. mysorensis* (**q**), *A. nicolsonianus* (**r**), *A. smithsonianus* (**s**), *A. sylvaticus* (**t**), *Sauromatum venosum*-outgroup (**u**)

Nucleotide accession numbers

Sequences generated in this study have been submitted to NCBI's GeneBank under following accession numbers. *rbcL:* KM093835–KM093855, *matK:* KM113324–KM113344, *psbA–trnH:* KM113366–KM113372, *trnLC–D:* KM113345–KM113365.

RAPD and ISSR analyses

Initially, a total of 25 decamer RAPD primers from Bangalore Genei Kit (Merck Bioscience Pvt. Ltd, Bangalore, India) and 50 ISSR primers (UBC primer set No. 9, University of British Columbia, Canada) were screened for RAPD and ISSR analyses respectively. In order to select ISSR primers that could give good amplification and suitable annealing temperatures, a preliminary screening was carried out using gradient annealing temperatures (Ta). The PCR amplification reaction (25 µl) consisted of 40 ng of DNA, 1X PCR buffer A (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 100 µM of each of the four dNTPs, 0.4 µM of RAPD or ISSR primer and 1U of *Taq* DNA polymerase (Merck Bioscience Pvt. Ltd, Bangalore, India). PCR amplifications were performed in C1000 Touch Thermal Cycler (Biorad, India) with an initial denaturation at 94 °C for 3 min followed by 45 cycles at 94 °C for 45 s, 36 °C for 30 s and 72 °C for 2 min with a final extension at 72 °C for 7 min. Regardless of the marker system used, all the PCR amplifications included a negative control (no DNA) to avoid erroneous interpretations. The amplified PCR products were separated on 1.8% agarose gel in 1X TAE buffer by electrophoresis at 100 V for 3 h., stained with ethidium bromide and documented on G:Box gel imaging system (Syngene Bioimaging Pvt. Ltd, India). To ensure reproducibility, DNA amplified profiles with ISSR and RAPD markers were tested for repeatability at least thrice. This was done by repeating the PCR reactions on two different thermal cyclers. The generated band profiles were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer. Only clear and intense bands were considered and scored while faint bands were not scored for the analysis.

Statistical analysis

For each primer, the presence (1) or absence (0) of polymorphic bands in each species profile was scored using GeneTool image analysis software (Syngene Bioimaging Pvt. Ltd, India) and set in a binary matrix. Genetic similarities were calculated using Jaccard similarity coefficient for RAPD and ISSR polymorphisms individually as well as together. The Mantel test of significance was determined to measure the goodness of fit between the similarity matrices

Table 2 List of primer pairs and polymerase chain reaction (PCR) annealing temperatures of candidate barcodes markers used

Locus	Primer name	Sequence	Annealing temperature	References
rbcL	*rbcL 1F*	ATGTCACCACAAACAGAAAC	46	Fay et al. (1998)
	rbcL 724R	TCGCATGTACCTGCAGTAGC		
	rbcL aF	ATGTCACCACAAACAGAGACTAAAGC	48	Levin et al. (2003), Kress et al. (2009)
	rbcL aR	GTAAAATCAAGTCCACCRCG		
matK	*mat K 390*	CGATCTATTCATTCAATATTC	48	Cuénoud et al. (2002)
	matK 1326	TCTAGCACACGAAAGTCGAAGT		
	matK F	ACCCAGTCCATCTGGAAATCTTGGTTC	50	Fofana et al. (1997)
	matK R	CGTACAGTACTTTTGTGTTTACGAG		
ITS	*ITS 8F*	AACAAGGTTTCCGTAGGTGA	58	Wen and Zimmer (1996)
	ITS 9R	TATGCTTAAAYTCAGCGGGT		
	ITS 10F	CGAACACGTTACAATACCG	55	
	ITS 11R	ACCACTTGTCGTGACGTCC		
trnLC–trnLD	*trnL C*	CGAAATCGGTAGACGCTACG	48.5	Taberlet et al. (1991)
	trnL D	GGGGATAGAGGGACTTGAAC		
trnH–psbA	*psbA*	GTTATGCATGAACGTAATGCTC	55	Sang et al. (1997)
	trnH	CGCGCATGGTGGATTCACAATCC		Tate and Simpson (2003)

produced with the two marker systems. Dendrograms were constructed using the un-weighted pair-group method with an arithmetic average (UPGMA) using the NTSYS PC version 2.11 (Applied Biostatistics Inc, Setauket, USA).

Results

PCR amplification and sequence analyses

To evaluate the utility of different plastid DNA markers for assessing the phylogenetic relationships among the *Amorphophallus* species of India, we initially tested different plastid regions such as *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD*, *rpoB and rpoC* and nuclear locus *ITS*. The efficiency of PCR amplification of *ITS*, *rpoB* and *rpoC* were less than 40% (Data not shown), therefore were not used further. The most successful PCR rate (100%) was observed for *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD*. Except *trnH-psbA*, substantial sequence length differences were not detected in these three markers and PCR products were sequenced without complications.

For individual marker regions, aligned sequence lengths was 524 for *rbcL*, 724 for *matK*, 144 for *trnH–psbA*, and 529 for *trnLC–trnLD* (Table 3). Among the four loci analyzed, *rbcL* was found most conserved (93.12%) in *Amorphophallus* species and *trnH-psbA* was least conserved (22.22%). Locus *trnH-psbA* was also noted with highest percentage of Parsimony Informative (PI, 66.66%) characters and maximum mean interspecific K2P distance (0.58). When sequence of all four studied loci were concatenated, it resulted aligned length of 2014 bp with 65.59% conserved sites, 11.71% PI characters and interspecific K2P distance of 0.05.

Maximum likelihood analyses

The majority rule consensus trees with bootstrap support values resulting from the ML analyses based on *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD* and combined dataset are shown in Fig. 2 and Supplementary Figure S1. The ML analysis based on *rbcL* marker resolved tested taxa in three strongly supported section specific clades I (BS 100%), clade II (BS 99%) and clade III (BS 93%) (Fig. 2a). In clade I, resolved topology strongly (BS 100%) supported the monophyly of all three tested members of section *Amorphophallus*. Clade II comprised of monophyletic group of four tested varieties of *A. commutatus* of section *Conophallus* (BS 94%) whereas remaining two members namely *A. bulbifer* and *A. oncophyllus* were sister (BS 100%) to clade III. In Clade III, seven of the eleven species of section *Rhaphiophallus* were resolved as monophyletic with 99% BS and remaining four were resolved either as paraphyletic or polyphyletic (Fig. 2a).

Section specific clades were not resolved in ML tree based on *matK* markers and tree was highly polytomous with low bootstrap supports (Fig. S1a). ML tree based on *trnH–psbA* resolved the taxa in two clades and the topologies in both of these clades were polytomic and none resolved tested taxa into the section specific clades (Fig. S1b). Clade I was strongly supported with BS 100% and comprised of all of the tested species of sections *Conophallus* and *Amorphophallus*, whereas poorly supported (BS 41%) clade II comprised of species of section *Rhaphiophallus*. The members of section *Amorphophallus* were found embedded in the clade I that was composed of most of the members of section *Conophallus*. The monophyly of tested section was not supported in both these clades. The topology of ML tree based on *trnLC–trnLD* was similar to that obtained in ML tree based on *matK*. Although species were resolved in two clades, they were polytomy and none of these clades was section specific (Fig. S1c).

Similar to resolutions and tree topologies resulted from ML analysis of individual markers, resolutions and tree topology resulted from combined data was not in congruence with the *rbcL*. Unlike on the *rbcL* phylogeny, the resolution of relationships among *Amorphophallus* species

Table 3 Properties of the four plastid DNA loci and their combinations (the outgroup sequence is not included in the calculation)

Locci and their combinations	Align length (bp)	No. of variable sites	No. of conserved sites	No. of par-informative sites	No. of singleton sites	Mean interspecific K2P distance
rbcL	524	27 (5.15)	488 (93.12)	13 (2.48)	14 (2.67)	0.011 (0.002–0.034)
matK	724	201 (27.76)	455 (62.84)	68 (9.39)	122 (16.85)	0.046 (0.018–0.097)
trnH–psbA	144	110 (76.38)	32 (22.22)	96 (66.66)	14 (9.72)	0.585 (0.069–1.153)
trnLC–trnLD	529	101 (19.09)	378 (71.45)	59 (11.15)	42 (7.93)	0.069 (0.003–0.126)
rbcL + *matK* + *trnH–psbA* + *trnLC–trnLD*	2014	437 (21.69)	1321 (65.59)	236 (11.71)	190 (9.43)	0.051 (0.009–0.067)

Figures in the parenthesis for no. of variable sites, no. of conserved sites and no. of Parsimony informative sites indicate the percentage of aligned length; Figures in the paranthesis for Mean K2P interspecific distance indicate range of K2P interspecific distance in 16 *Amorphophallus* species and six varieties

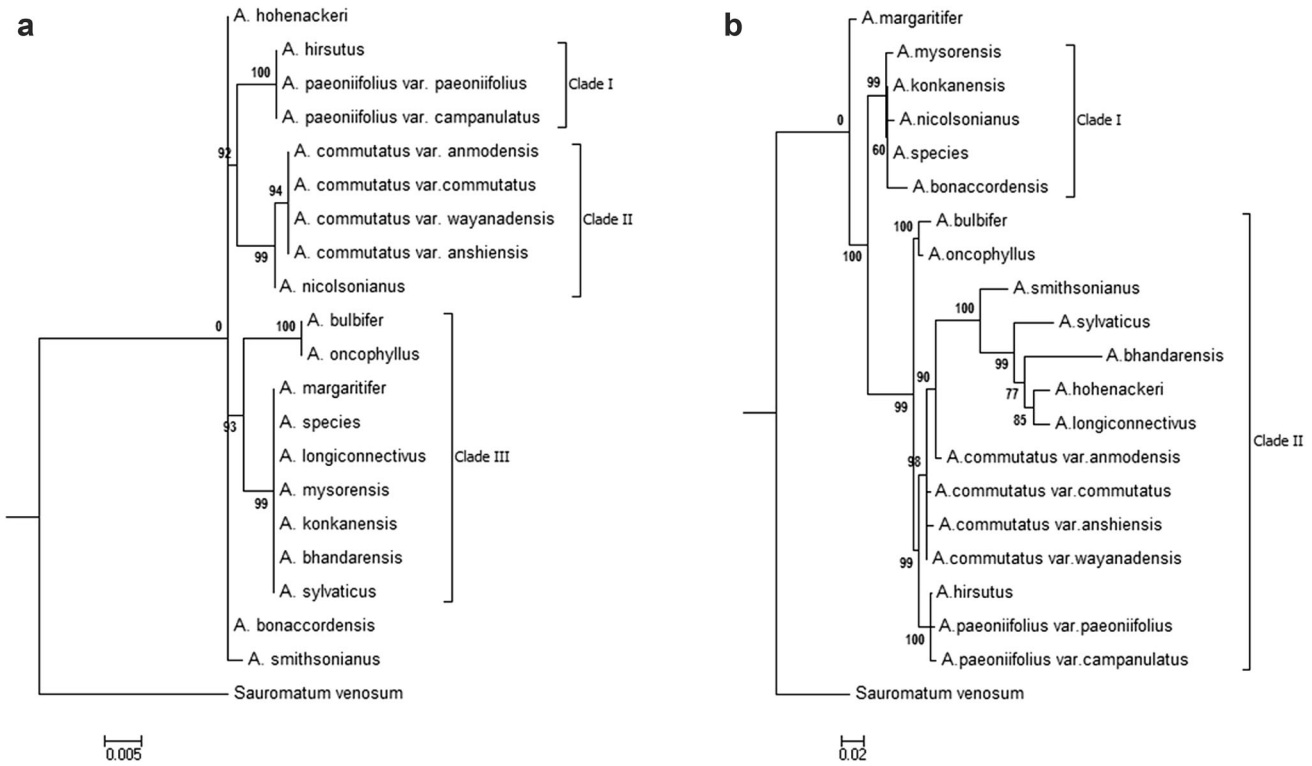

Fig. 2 Maximum likelihood majority rule consensus trees (RAxML) showing the relationships among *Amorphophallus* species of India based on **a** *rbcL* sequences and **b** combined dataset of *rbcL*, *matK*, *TrnH–psbA* and *TrnL C–D* sequences. ML bootstrap values are shown next to the node

based on combined data were poor. None of the two clades supported monophyly of tested sections and none resolved species into section specific clades. In clade II (BS 90%), five of the ten members of section *Rhaphiophallus* formed an unresolved polytomy together with members of section *Conophallus* whereas remaining members formed clade I (Fig. 2b).

BI analysis

The Bayesian 50% majority rule consensus trees with posterior probability values resulting from the BI analyses based on individual marker and their combined dataset are shown in Fig. 3 and Supplementary Figure S3.

When we carried out BI analysis, resolution of the species relationship and tree topology based on *rbcL* were similar to that obtained in ML analysis (Figs. 2a, 3a). Like ML analysis, BI analysis also resolved tested taxa into in three section specific clades viz. clade I, II and III that were strongly supported with higher posterior probabilities (PP 1, 1, and 0.9 respectively) (Fig. 3a). The clade I comprised of all three tested members of section *Amorphophallus* and their monophyly was strongly supported (PP 1). Clade II comprised of four tested varieties of *A. commutatus* of section *Conophallus* as monophyletic group with strong support (PP 1). Remaining two members of section *Conophallus* namely *A. bulbifer* and *A. oncophyllus* were embedded in clade III as sister to monophyletic group of seven of the ten species of section *Rhaphiophallus* (PP 1) (Fig. 3a).

The species resolutions and tree topologies in BI trees based on *matK* was better than ML tree and resolved tested species into three clades that were in congruence with ML and BI trees based on *rbcL* (Figs. S2a, 2a, 3a). The BI analysis of *trnH–psbA* and *trnLC–trnLD* were also similar to those obtained in ML trees (Fig. S2 b, c, Fig. S1 b, c). In BI trees of these three markers, section specific, monophyletic clades were not resolved and showed high polytomy with low supports of posterior probabilities.

When BI analysis was performed for the combined dataset of all four tested markers, species resolution and tree topology were comparable and in congruence with ML tree of combined dataset. Similar to ML tree, BI tree also resolved tested taxa in two clades (Fig. 3b). Major Clade I (PP 1) comprised of most of the species of all three section while minor clade II (PP 1) comprised of five species of section *Rhaphiophallus*. Similar to ML tree, section specific monophyly of tested species was not supported in either of these two clades and none resolved species into section specific clades (Fig. 3b).

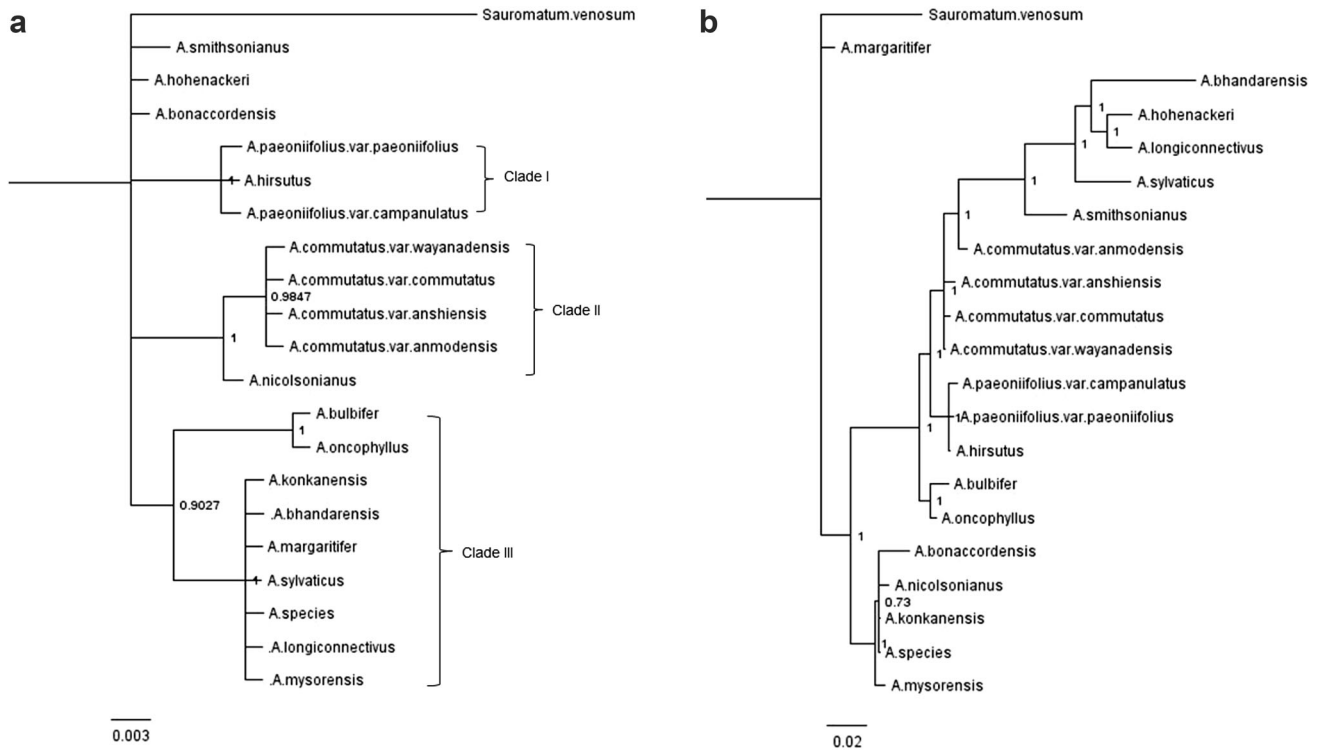

Fig. 3 The Bayesian 50% majority rule consensus trees showing the relationships among *Amorphophallus* species of India based on **a** *rbcL* sequences and **b** combined dataset of *rbcL*, *matK*, *TrnH–psbA* and *TrnL C–D* sequences. The Bayesian posterior probability values are shown next to the node

Resolution based on DNA fingerprinting approaches

Resolution based on RAPD

To study the suitability of fingerprinting techniques for resolving molecular phylogeny of *Amorphophallus* species, we initially screened 25 decamer RAPD primers and 50 ISSR primers. Out of 25 RAPD primers tested, nine primers produced amplification products all of which revealed polymorphic fingerprint patterns in tested *Amorphophallus* species. These nine RAPD primers produced a total of 162 reproducible and scorable bands, 126 (77%) were polymorphic with an average of 14 polymorphic bands per primer. The total number of bands per amplification varied from 13 to 23 in the molecular size range of 77–1507 bp. Maximum 23 bands were produced by primer RPi 4 whereas maximum 18 polymorphic bands were produced by RPi 1 and RPi 4. Using RAPD, the percentage of polymorphism ranged from 64.79% (RPi 6) to 85% (RPi 2) with an average polymorphism of 77.30% per primer (Table 4).

The genetic similarity based on Jaccard coefficients among 16 *Amorphophallus* species and six varieties was used to construct a dendrogram (Fig. 4) by UPGMA method. The genetic distance among the *Amorphophallus* species ranged from 0.11 to 0.84. The dendrogram clustered 16 *Amorphophallus* species and six varieties into clusters that were in congruent with the morphological characters. All of the three species and four varieties *A. commutatus* of section *Conophallus* were clustered together. However, this resolved group of *Conophallus* also contained *A. sylvaticus*, a member of *Rhaphiophallus*, whereas eight of the remaining nine species of *Rhaphiophallus* were clustered together in two groups. With the exception of *A. hirsutus* that was clustered with species of section *Rhaphiophallus*, remaining two varieties of *A. paeoniifolius* of section *Amorphophallus* were also found clustered together (Fig. 4).

Resolution based on ISSR

Of the 50 ISSR primers screened, 16 primers resulted amplification of which ten primers generated polymorphic banding patterns. Out of 101 bands (10.1 per primer), 86 were polymorphic indicating an average of 8.6 polymorphic bands per primer. With tested ISSR primers, the number of bands amplified per primer ranged from eight (UBC 4) to 14 (UBC 3 and 36) with band size between 170 bp and 3.2 kb. The percentage of polymorphism ranged from 57.14% (UBC 3) to 87.50% (UBC 4) with an average polymorphism of 76.59% across the *Amorphophallus* species studied. The genetic distance based on Jaccard coefficients ranged from 0.15 to 0.71. UPGMA dendrogram based on ISSR profile

Table 4 RAPD and ISSR primers and their characteristics

No	Name	Annealing temperature used (Ta)	No. of Bands	Band size (bp)	No. of polymorphic bands	% polymorphism
RAPD						
1	RPi 1	38.0	22	119–1507	18	81.18
2	RPi 2	39.0	20	85–660	17	85.00
3	RPi 3	38.5	21	85–117	17	80.95
4	RPi 4	38.0	23	313–1082	18	78.36
5	RPi 5	40.0	16	530–1340	13	81.25
6	RPi 6	39.0	17	450–920	11	64.79
7	RPi 7	40.0	13	480–1340	10	76.92
8	RPi 9	38.5	14	77–682	11	78.57
9	RPi 10	38.0	16	80–387	11	68.75
ISSR						
1	UBC 2	44.6	11	170–2900	9	81.81
2	UBC 3	44.6	14	340–2050	8	57.14
3	UBC 4	44.6	8	300–1950	7	87.50
4	UBC 5	49.2	10	450–2370	8	80.00
5	UBC 6	49.2	9	250–3010	7	77.77
6	UBC 18	59.8	12	300–1810	9	75.00
7	UBC 22	55.0	13	1060–2240	10	76.92
8	UBC 26	60.0	13	200–3200	11	84.61
9	UBC 27	60.0	9	600–1790	6	66.66
10	UBC 36	59.8	14	470–2740	11	78.57

did not resolve *Amorphophallus* species into section specific clusters congruent with the sections proposed by Sivadasan (1989) (Fig. S3). This clearly indicated that the DNA based marker ISSR alone is not suitable for resolution of *Amorphophallus* species.

Resolution based on combined data of RAPD and ISSR

The NJ analysis and UPGMA dendrogram of the combined 126 RAPD and 86 ISSR bands for 16 species six varieties clustered together all of the three species of section of *Amorphophallus* while 9 of the ten species of section *Rhaphiophallus* were also clustered together. The species of section *Conophallus* did not clustered together (Fig. S4). The resolution based on combined data of RAPD and ISSR also could not resolve *Amorphophallus* species completely into three proposed section. In comparison, the resolution based on combined data of RAPD and ISSR was better than the ISSR alone but not better that RAPD alone.

Discussion

In the present study, the molecular phylogeny of *Amorphophallus* species of India was reconstructed using plastid gene regions' sequences and two DNA fingerprinting markers namely RAPD and ISSR. We tested the performances of sequences of four plant loci viz. *rbcL*, *matK*, *trnH–psbA*, *trnLC–trnLD* for the resolution of *Amorphophallus* species into three Indian sections namely *Rhaphiophallus*, *Conophallus*, and *Amorphophallus*. In addition, the utility of two DNA finger printing techniques namely RAPD and ISSR for species resolution was also tested. Our study included most of the previously unexplored species of *Amorphophallus* occurring in India. Because of rare and endangered status of some of *Amorphophallus* species, we could not include multiple specimen and accessions. For the selection of suitable locus regions, criteria of efficiency of reproducible amplification and ease of sequencing were applied. The efficiency of PCR amplification, which is an important index to evaluate the candidate barcode, was determined to select the suitable candidate barcoding marker. In comparison to *rbcL*, *matK*, *trnH–psbA* and *trnLC–trnLD*, the efficiency of PCR amplification of *ITS*, *rpoB* and *rpoC* was low (<40%), therefore were not used further. Unlike two barcode markers namely *matK* and *rbcL* that have already been established and recommended for plant barcoding (CBOL 2009), the use of *ITS2* is still debated for its potential use as a DNA barcode in different plant groups. In our study, the use of *ITS* specific primers was consistently resulting lower amplification efficiency and amplification of multiple bands.

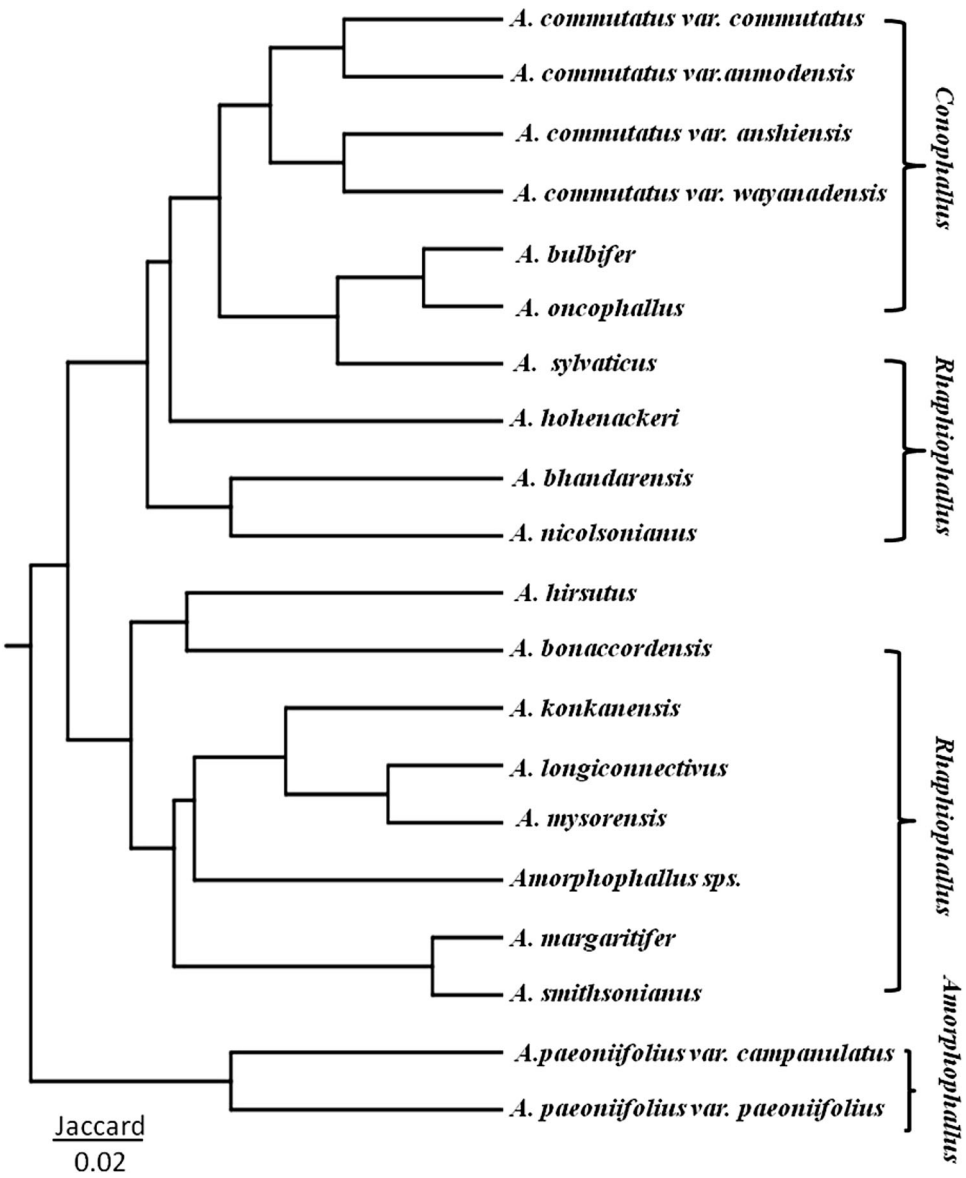

Fig. 4 Species dendrogram of 16 *Amorphophallus* species and six varieties based on 126 polymorphic bands from RAPD profile

Despite of our attempts to amplify other barcode regions, successful amplifications were achieved only for *rbcL*, *matK*, *trnH–psbA* and *trnLC–trnLD*. For these loci, amplification and sequencing success was 100%. Among the tested markers, substantial difference in the length of amplified product was not observed for *rbcL*, *matK and trnLC–trnLD*, however, despite of 100% amplification efficiency of *trnH–psbA*, the length of amplified product varied in the range of 140–200 bp. Similar variation in amplified length of *trnH–psbA* was demonstrated in *Umbelliferae* and in *Araliaceae* (Degtjareva et al. 2012). The ease of amplification of *rbcL* and *matK* in *Amorphophallus* species was congruent with those reported by Grob et al. (2002, 2004) and Sedayu et al. (2010). The performance of these four loci was analyzed individually as well as in combinations of four loci.

Locus *rbcL* as one of the core plant plastid barcode regions has been recommended as universal and was found easy to be sequenced and aligned in group of plants such as ferns, mosses and angiosperms (Hasebe et al. 1995; Hollingsworth et al. 2009; Liu et al. 2011). Despites of some doubts in few plant groups about the universality and applicability, another plastid coding region *matK* has also been recommended as one of the core plant barcode by CBOL (Cuénoud et al. 2002; Chase et al. 2007a, b). In the view of universality and applicability of *rbcL* and *matK*, we anticipated good resolution of phylogeny of *Amorphophallus* species using these two barcode markers. However, in our study, *rbcL* as a single locus was found most conserved than other three loci analyzed. It also contained low percentages of PI sites, singleton sites and lowest interspecific K2P distance. Similar observation

wherein *rbcL* possessed very low inter-specific variation in closely related species was made by few workers (Newmaster et al. 2008; Kress et al. 2005). Among four loci tested, *rbcL* was found most suitable for resolving *Amorphophallus* species than *matK*, *trnH–psbA* and *trnLC–trnLD*. Surprisingly, in the order of their efficacy for resolution of tested species, the performance of small intergenic spacer region *trnH–psbA* was found better suited than widely used plastid barcode marker *matK*. *trnH–psbA* was also noted with the highest percentage of variable sites, PI sites, interspecific K2P distance. Using *matK* and *trnLC–trnLD*, tree topology and relationships among the *Amorphophallus* species in ML and BI trees were not fully resolved. In comparison to ML and BI trees based on *matK*, *trnH–psbA* and *trnLC–trnLD*, the ML and BI trees based on *rbcL* clearly resolved *Amorphophallus* species into three clades which were strongly supported with boorstrap, posterior probabilities values and morphological characters. As expected, four tested varieties of *A. commutatus* of section *Conophallus* were resolved as monophyletic in ML and BI tress based on *rbcL*. Two members of *Conophallus* namely *A. oncophyllus* and *A. bulbifer* always grouped together as monophyletic with strong support and morphological characters. Also the species of section *Amorphophallus* namely *A. hirsutus* and two varieties of *A. paeoniifolius* namely var. *paeoniifolius* and *campanulatus* were recovered as monophyletic with strong support and morphological characters.

The monophyly of species of sections *Rhaphiophallus*, *Conophallus* and *Amorphophallus* was not fully supported and section specific clades were not recovered when ML and BI analyses were performed on combined datasets as well. In comparison, the efficacy and performance of *rbcL* alone was found better than *matK*, *trnH–psbA* and *trnLC–trnLD* and combined dataset for species resolution and recovery of section specific clades. The lower efficacy and poor performances of *matK*, *trnH–psbA* and *trnLC–trnLD* was also reflected in efficacy and performances of combined dataset.

In the view of our observation that individual locci *matK*, *trnH–psbA* and *trnLC–trnLD* were not able to resolve *Amorphophallus* species into clades supported by morphological characters, we further carried out ML and BI analyses on concatenated sequences of various combinations of two and three loci and found that their performances and efficiencies were also poor in terms of complete resolution and recovery of tested taxa into section specific clades (Data not shown). The Indian section *Rhaphiophallus* was founded by Engler and it is still in use (Grob et al. 2002). In our analysis, the species forming the clade of *Rhaphiophallus* were supported by morphological characters such the presence of stipitate spadix that contains staminodes between the female and the male zone, large stigma and undifferentiated spathe, whereas the species forming clade of *Conophallus* were supported by characters like globose or disciform tuber, with globose or rhizomatus offsets, spadix longer than spathe. Species forming clade of *Amorphophallus* had globose or depressed tuber with distinct annular root-scar, highly variable appendix and psilate pollen.

In previous analysis by Grob et al. (2002), two species namely *A. smithsonianus* and *A. margaritifer* of *Rhaphiophallus* section were not placed together. In congruence with this observation, we also observed that these two species were also not placed together, instead they were found polyphyletic in most of our ML and BI analyses. Based on these results, our study demonstrates and recommends that *rbcL* should also be considered better suited locus for resolving molecular phylogeny of closely related species of genus *Amorphophallus* of India. In terms of the resolving power, *trnH-psbA* had ranked second to *rbcL* and was found polymorphic, consequently suitable for resolving closely related *Amorphophallus* species. However, none of these tested loci completely resolved *Amorphophallus* species into *Rhaphiophallus*, *Conophallus*, and *Amorphophallus* section specific clades. The use of *matK*, *trnH–psbA* and *trnLC–D* as a single locus and multi-locus marker approach based on combination of two and three loci were not much suitable for resolution.

Recently, several workers have used RAPD and ISSR markers for the study of phylogeny and species discrimination. RAPD was successfully used for the study of relationships among *Verbascum* species (Muazaz et al. 2014) and combination of ISSR and RAPD was used to study genetic relationships in *Coffea* and Indian *Psilanthus* species (Kumar et al. 2008). In the view of these reports, we have also accessed the utility of RAPD and ISSR for resolving the phylogeny of *Amorphophallus* species. Since RAPD markers are known to suffer from a lack of reproducibility, we checked the consistency of the electrophoretic patterns and the polymorphism detected by repeating every PCR reaction twice on two different thermal cyclers. Phylogenetic dendrogarm based on RAPD profile clustered together all of the species of section *Conophallus* and species of section *Rhaphiophallus* were grouped in two clusters. In our study, to resolve the phylogeny of *Amorphophallus* species using DNA fingerprinting technique, RAPD alone performed better than ISSR and combination of RAPD and ISSR, but was not better than plastid gene region sequencing and analysis approach. In comparison, plastid gene region sequencing approach using *rbcL* was found most suitable for resolving molecular phylogeny and relationship among the *Amorphophallus* species of India.

Earlier studies of molecular phylogeny of *Amorphophallus* species by Grob et al. (2002, 2004) and Sedayu

et al. (2010) included only five *Amorphophallus* species each from India. Our study differs from these two reports in having studied most of the previously unexplored Indian species. Moreover, as plastid gene region sequencing approach, we have compared the performance of single and combinations of four barcode loci for resolution of phylogeny. In addition, the utility and performance of ISSR and RAPD fingerprinting technique has also been compared with plastid DNA region sequencing approach.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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