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EFFECTIVENESS OF ISSRS TO DETERMINE THE GENETIC RELATIONSHIPS BETWEEN THIRTY SPECIES FROM ELEVEN GENERA OF BAMBOO

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Abstract– The identification of bamboo is traditionally dependent on vegetative characters due to its long and unpredictable flowering cycle. Molecular techniques are suited and consistent for resolving the genetic relationship between bamboo species and that was studied among 30 species from 11 genera using ISSRs markers. Thirteen ISSR primers revealed 305 polymorphic loci among the accessions Polymorphic information content and ISSR primer index ranged from 0.26 to 0.40 and 4.77 to 9.91 respectively. The Neighbour-Joining dendrogram of 30 species resulted in two major groups. all species of *Bambusa* were grouped in cluster I along with three species of *Dendrocalamus*, the remaining species of *Dendrocalamus* were distributed in cluster II along with nine other bamboo genera. This study supports the monophyletic origin of *Bambusa* and the paraphyletic origin of the genus *Dendrocalamus*.

INTRODUCTION

The bamboos belong to the family Poaceae and sub-family Bambusoideae. Nearly, 1575 species from 111 genera have been identified worldwide (Sharma *et al.*, 2008; Basumatary *et al.*, 2017). Bamboo is one of the important natural resources, it has commercial importance and versatile use for industrial and domestic purposes e.g. construction, paper pulp, and food (Sharma *et al.,* 2014). The unusually long flowering cycles of bamboo that can extend up to 150 years (Sharma *et al.*, 2008; Das *et al.*, 2007) have compelled the use of vegetative characters such as rhizomes, culms, branching, and leaves for delimiting genera, while characters pertaining to culm sheath, ligule, and auricle have been used in species delimitation (Ohrnberger and Goerrings, 1986). However, the vegetative characters are often influenced by environmental factors such as temperature, precipitation, and local adaptation (Merilä and Crnokrak, 2001; Wu, 1962), which make the characters unreliable for resolving the genetic differences among bamboo species especially at field conditions in the absence of flowering (Bhattacharya *et al*., 2006).

Molecular markers have been successfully employed to study genetic diversity in bamboo and to resolve taxonomic ambiguities by providing an unbiased approach in comparison to the use of vegetative characters (Das *et al.,* 2007). Though DNA based marker systems such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLP), and Diversity Arrays Technology (DArT) provide useful information on the various aspects of taxonomy and have emerged as popular tools to evaluate genetic diversity among taxa (Nybom *et al*., 2014). The sequence and structure of ITS1 and ITS2 have been used to reconstruct the phylogeny of 21 tropical bamboo species, suggesting the reliability of molecular techniques for species identification (Ghosh *et al.,* 2017). The less availability of SSR markers in bamboo, transferability of marker information among closely related taxa has enabled the use of SSR markers of rice to resolve 21 different bamboo species with 68% transferability (Chen *et al*., 2010).

Among the molecular markers, ISSRs are

preferred due to the high polymorphism, reproducibility, and cost-effectiveness (Reddy *et al.,* 2002). ISSRs have been successfully employed to study genetic relationships among 22 bamboo taxa (Mukherjee *et al*., 2010) as well as to distinguish the genera *Bambusa*, *Phyllostachys,* and *Dendrocalamus* (Desai *et al.,* 2015). ISSRs showed higher efficiency in resolving taxa than the sequences of plastid *psbA-trnH* regions, which has been attributed to the genome-wide occurrence of ISSRs (Kumar *et al*., 2016).

In the present study, thirteen ISSR markers were used to study genetic relationships within 30 species and 11 genera of bamboo.

MATERIALS AND METHODS

Plant material

Fresh leaves of 84 clums of bamboo from 11 genera and 30 species were sampled (Table 1) represents collections from various places e.g. Bamboosetum at BAIF, KFRI, Kerala, Dapoli Krishi Vidyapeeth, Maharashtra, and wild collections (Kurg, Belgao - Karnataka; Vidarbha, Bhor – Maharashtra, Agartala, Gujrat, etc.), Seeds received from Forest Department Assam, etc. In the laboratory, leaves were flash-frozen in liquid nitrogen and stored at -70^{0}C until further use.

DNA extraction and PCR amplification

The leaf tissue (100 mg) was ground to a fine powder in liquid nitrogen. DNA was extracted using a modified CTAB method (Murray and Thompson 1980) with 1% (w/v) of polyvinyl pyrrolidone (PVP). After two chloroform: isoamyl alcohol (24:1) washes, DNA was precipitated with 2-propanol and the resultant pellet was dissolved in TE buffer. DNA concentration of each sample was determined using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, India). DNA fingerprinting was carried out using thirteen ISSR primers (Table 2). PCR reaction of 20 µl was performed using 20 ng DNA in the 1x GoTaq Green master mix (Promega, India) and 0.3 µM of primer. PCR cycling was used as detailed before (Bahulikar *et al*., 2004; Nimbalkar *et al.,* 2018). The amplified products were separated on 2.0% agarose gel in 1.0X TAE buffer and the resolved bands were detected by ethidium bromide staining. Gels were photographed using a gel documentation system (Bio-Rad, India). The reproducibility of the amplification was confirmed by repeating the experiment.

Data analysis

All polymorphic and reproducible bands were scored manually for the presence (1) or absence (0) of alleles. Genetic variability was interpreted as the rate of polymorphism (%) and Polymorphism Information Content (PIC) (Nagy *et al.,* 2012). The ISSR Primer Index (SPI) was calculated by summing up the PIC values of all the loci amplified by the given primer. The data for each species were combined using the Additive Band Profile option of POPGENE var 1.32 (Yeh *et al.,*1999) and the dendrogram was constructed using the neighbor-joining method by PAST software (ver. 3.14) (Hammer *et al*., 2001).

RESULTS AND DISCUSSION

Morphological characters being used for the identification of the bamboo species due to its unusually long flowering cycles (Sharma *et al.,* 2008; Das *et al*., 2007). The vegetative characters may show environmental influence and make identification difficult (Merilä and Crnokrak, 2001; Wu, 1962). Molecular tools have been helping to differentiate taxa at a great level. In this study, ISSRs were used to generate fingerprinting patterns as a step towards a molecular identification of bamboo species.

Initially, 25 primers were screened for the PCR amplification, out of which 13 dinucleotide primers showing clear and reproducible bands were selected for final analysis. It gave rise to 305 bands with absolute polymorphism and at an average of 23 bands per primer. Primer (CA) $_8$RG gave the maximum number of bands (33 bands) and a minimum number of bands (19 bands) were observed for primers (CT)$_8$ RG and (AG)$_8$T. PIC value was ranged from 0.26 (CA) $_8$RC to 0.40 (GA) $_8$C. The ISSR primer index (SPI) which is indicative of the efficiency of the maker system, ranged from a low value of 4.77 (CA) $_8$RC to a high value of 9.91 (AG) $_8$YT (Table 2).

All 30 species of bamboo from 11 genera showed a high percentage of polymorphism when analyzed by using ISSR markers. The ISSRs are considered as the neutral type of markers and based on the previous reports (Nimbalkar *et al*., 2018; Takawale *et al*., 2019), the di nucleotide-based primers were applied. Used ISSR primers yielded absolute polymorphism and such higher polymorphism was also reported previously for bamboo species (Desai

Table 1. Details of bamboo species collection*. The species have been either wild collections, grown from seeds or clone of CPC's from various sources. Table include no of species of a taxa, sample size of species and private alleles obtained for the genus by ISSRs

Sr. No.	Plant Genera	No of Species	No. of private alleles	Plant Species used in present	Sample Size	Area of Collection
1	*Bambusa*	12	6	*B. arundinacea* (Retz.) Willd.	1	Pune, Maharashtra
				B. balcooa Roxb.	3	Pune, Maharashtra and Gujarat
				B. bambos (L.) Voss	3	Pune, Maharashtra
				B. cacharensis R. B. Majumdar	2	Assam and Gujarat
				B. jaintiana R. B. Majumdar	1	Gujarat
				B. multiplex (Lour.) Raeusch. ex Schult. & Schult. F	7	Pune, Maharashtra
				B. nutans Wall. ex Munro	1	Gujarat
				B. paraguayana (Döll) Bertoni	1	Gujarat
				B. tulda Roxb.	4	'Gujarat and Tripura
				B. ventricosa McClure	4	Pune, Maharashtra
				B. vulgaris Schrad. ex J.C. Wendl.	4	Pune, Maharashtra
				B. wamin Brandis	1	Pune, Maharashtra
2	*Dendrocalamus*	7	8	*D. asper* (Schult. & Schult. f.) Backer ex K. Heyne	2	Gujarat
				D. brandisii (Munro) Kurz	5	Gujarat, Pune Maharashtra and Banglore, Karnataka
				D. giganteus Wall. ex Munro	3	Gujarat, Kerala and Jawhar Maharashtra
				D. hamiltonii Nees & Arn. ex Munro	1	Pune, Maharashtra
				D. longispathus Kurz	3	Tripura and Kerala
				D. stocksii Munro	14	Wild collection Bhor, Maharashtra
				D. strictus (Roxb.) Nees	4	Maharashtra and Gujarat
3	*Dinocloa*	1	0	*D. andamanica* Kurz	1	Pune Maharashtra
4	*Gigantochloa*	1	0	*G. atroviolacea* Widjaja	2	Pune Maharashtra
5	*Guadua*	1	1	*G. angustifolia* Kunth	1	Pune Maharashtra
6	*Melocanna*	1	1	*M. baccifera* (Roxb.) Kurz	4	Dapoli Maharashtra and Tripura
7	*Ochlandra*	1	2	*O. travancorica* (Bedd.) Benth. ex Gamble	3	Kerala and Kurg Karnataka
8	*Pseudoxytenanthera*	1	0	*P. ritchii* (Munro) H. B. Naithani	1	Pune Maharashtra wild collection
9	*Sasa*	1	0	*S. fortune* (Van Houtte) Fiori	1	Gujarat
10	*Schizostachyum*	2	0	*S. brachycladum* Kurz	1	Pune Maharashtra
				S. dullooa (Gamble) R.B. Majumdar	1	Gujarat
11	*Thyrsostachys*	2	0	*T. siamensis* Gamble	1	Pune Maharashtra
				T. oliveri Gamble	4	Pune, Maharashtra and Kerala
	Total	30	18		84	

* Except few wild collections, clones or seedlings have been collected and being maintained on campus at authors institute

Table 2. Comparison of primers, number of fragments scored, number of polymorphic bands, percentage polymorphism and number of unique markers for amplification profiles of 84 individuals of bamboo from 11 genera and 30 species generated using 13 ISSR markers

Sr. No.	Sequence	Total bands scored	% polymorphism	PIC	ISSR Primer Index (SPI)
1	(AG)8YC	20	100	0.40	6.34
2	(CT)8G	24	100	0.36	8.05
3	(AC)8G	32	100	0.33	9.41
4	(AG)8YA	25	100	0.36	8.16
5	(CA)8RG	33	100	0.32	9.07
6	(GA)8A	22	100	0.35	6.94
7	(GA)8C	23	100	0.40	8.21
8	(AG)8YT	28	100	0.39	9.91
9	(CA)8RC	21	100	0.26	4.77
10	(CT)8RG	19	100	0.33	5.85
11	(AG)8T	19	100	0.29	4.88
12	(CA)8A	22	100	0.38	6.21
13	(AC)8YT	21	100	0.33	6.47
	Total bands	305	100		

et al., 2015; Mukherjee *et al*., 2010; Amom *et al*., 2018), and tree species such as *Eurya nitida, Symplocos racemosa, Gaultheria fragrantissima* (Deshpande *et al*., 2001; Bahulikar *et al*., 2004) and *Madhuca indica* (Nimbalkar *et al*., 2018). PIC and SPI are used to detect polymorphism within a population by considering both the number of alleles that are expressed and also the relative frequencies of those alleles (Nagy *et al*., 2012). The informative range of molecular markers based on PIC is 0.3- 0.59 (Hafzari *et al*., 2019) and the PIC value of ISSR in the present study falls in the informative range.

Genetic relationship among species

A neighbor-joining dendrogram of 30 species from 11 genera of bamboo was constructed using Jaccard's Coefficient resulted in two major clusters (Fig. 1). Cluster I have represented 12 species of *Bambusa*, along with four species of *Dendrocalamus* (*D. asper, D. brandisii, D. giganteus* and *D. hamiltonii*) among them *D. hamiltonii* outgrouped cluster I.

The genetic relatedness among the taxa detected by cluster analysis. In the present work, cluster analysis based on ISSRs differentiated bamboo species into two main clusters. Twelve species of *Bambusa* clustered together showing a close relationship of *B. vulgaris* with *B. ventricosa*, which was corroborated with the findings of Nayak *et al.* (2003) demonstrating a close relationship between same species using RAPD markers. However, another study indicated the close genetic relatedness between *B. vulgaris* and *B. nutans* using RAPD markers. *B. ventricosa* is reported to be a cultivated variety of *B. vulgaris* (Chua *et al*., 1996), which underscores the close molecular phylogeny of these species revealed by several molecular analyses.

In the present study, all *Bambusa* species were clustered together with three *Dendrocalamus* species, which was contrary to the RAPD marker-based phylogeny where the thorny *Bambusa* group was distinctly separated from the *Dendrocalamus* group (Sun *et al*., 2006). Moreover, all *Bambusa* species were grouped, which was inconsistent with the paraphyletic origin of *Bambusa* suggested by RAPD (Das *et al*., 2007) and AFLP phylogenetic analyses (Loh *et al*., 2000). Overall this study supports the monophyletic origin of *Bambusa.*

Cluster II contained 14 species belonging to ten genera, with three species of *Dendrocalamus* (*D. stocksii, D. strictus, and D. longisphathus*), and one species each of *Melocanna baccifera*, *Ochlandra travancorica* were grouped. Two species of *Thyrostachys* (*T. siamensis* and *T. oliveri)* separated with 100 bootstrap values. *Whereas other species form a small subgroup.*

The seven species of *Dendrocalamus* were distributed in both clusters, the first group consisting of *D. asper, D. gigantieus, D. brandisii* and *D. hamiltoni* was clustered with *Bambusa*, and the second group consisting of *D. longisphathus, D. strictus, D. strocksii*, and grouped with the other bamboo species. This supports a paraphyletic origin of the genus *Dendrocalamus* as was also reported using RAPD markers (Nayak *et al*., 2003), ITS, and AFLP markers (Pattanaik and Hall 2011) and nuclear and plastid gene sequences (Yang *et al*., 2010). Nuclear and plastid gene sequences demonstrated a close relation between *D. brandissii,*

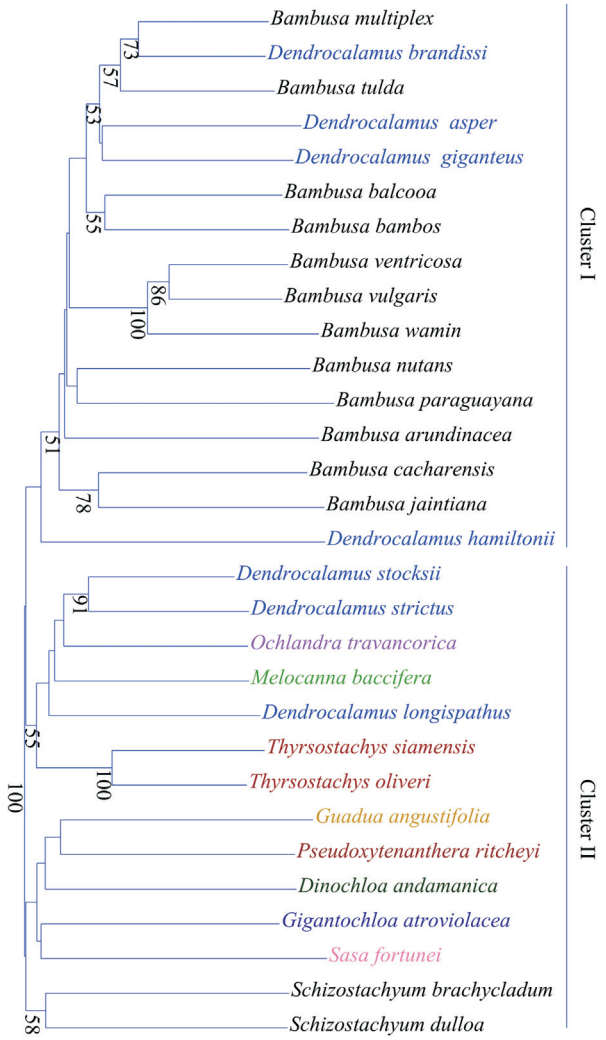

Fig. 1. The Neighbor-joining dendrogram of 30 bamboo species from 11 genera was constructed using 13 ISSR markers and 305 loci combined using the additive Band Profile option of POPGENE software for a species.

D. hamiltonii, and *D. asper* (Yang *et al.*, 2010), which was in accordance with the present study.

This study revealed a close association between *G. atroviolacea* and *Sasa fortunei*, however, it was reported that *G. atroviolacea* genetically similar to *Bambusa* by AFLP (Loh *et al.*, 2000) and to *D. giganteus* by RAPD (Das *et al.*, 2007) and ITS 1 markers (Ghosh *et al.*, 2017).

M. baccifera was distinctly separated from the *Bambusa* cluster, similar to the findings with combined phylogenetic analysis using nuclear GBSSI gene and plastid psbA-trnH, rpl32-trnL, and rps16 intron DNA sequences, where *M. baccifera* was suggested as an outgroup to the genus *Bambusa* (Yang *et al.*, 2010). *M. baccifera* was reported to be genetically related to two species of *Thyrsostachys* species (Yang *et al.*, 2010), which was in accordance with this study.

Another ISSR study demonstrated that *Schizotachyum dullooa* was grouped into a separate cluster than *Bambusa* and *Dendrocalamus* species (Amom *et al.*, 2018). Also three *Schizotachyum* species formed a separate cluster showing genus-specific clustering (Amom *et al.*, 2018). The monophyletic origin of *Schizotachyum* has been supported by *the* GBSSI gene - TrnL-F (Yang *et al.*, 2007) which was also observed in present study.

Similarly, based on multigene phylogeny, *G. angustifolia* was shown to be well separated from *Bambusa* and *Dendrocalamus* species (Sungkaew *et al.*, 2009) and *Sasa* species were reported to be well separated from *Bambusa* sp. using EST-SSR markers (Barkley *et al.*, 2005), which is in accordance with the present study.

CONCLUSION

The present study could differentiate 30 species from 11 bamboo genera using ISSR. It supports the monophyletic origin of Bambusa and the paraphyletic origin of the Dendrocalamus. This study also supports the paraphyletic origin of the *Dendrocalamus* and support was observed to the phylogeny of the other bamboo species. Further studies using more accessions for intra- and inter-species variation would be required to get a more comprehensive molecular phylogenetic relationship among the bamboo species, which will complement and enrich the traditional taxonomic classifications and hierarchy of bamboos.

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