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A GLANCE AT MOLECULAR IDENTIFICATION OF BAMBOO (*Poaceae: Bambusoideae*)

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ABSTRACT

Conservation of plant species plays a vital role in preventing the loss of valuable plant resources. The success of conservation depends on the correct identification and characterization of plant species. Bamboo is one of the most important plants with multiple uses that have contributed to the economy and socio-economy of many people in rural areas. It is under the subfamily of Bambusoideae that includes both woody and herbaceous bamboo. Conventionally, like other plants, bamboo has been classified dependently based on morphological characteristics. However, morphological identification leads to difficulties and misclassification of bamboo species due to their infrequent flowering behaviour and peculiar reproductive biology. Since then, molecular markers have been introduced to overcome the problems associated with bamboo taxonomy and phylogeny. This paper provides an overview of the diverse, predominantly molecular techniques used to assess and determine the genetic diversity of bamboo species.

Keywords: amplified fragment length polymorphism, DNA barcoding, random amplified polymorphic DNA, restriction fragment length polymorphism, sequence characterized amplified region, simple sequence repeat

INTRODUCTION

Bamboo is a plant member of the grass family known as Poaceae and constitutes a single subfamily Bambusoideae. The Bambusoideae subfamily consists of both herbaceous or Olyreae tribe and woody bamboos or the Bambuseae tribe (Ram, Thiruvengadam, & Vinod, 2007). Bamboo comprises approximately 1,290 species, which are naturally distributed worldwide (Hamzah, Hakeem, & Ibrahim, 2016). There is a rich abundance of bamboo species in the Asia-Pacific and South America (Bystriakova, Kapos, Lysenko, & Stapleton, 2003; Das, Bhattacharya, Singh, Filgueiras, & Pal, 2008). The species are mainly classified into three major categories: the pleiotropic woody bamboo, neotropical woody bamboo, and north temperate woody bamboo. The wide variety of species makes bamboo adaptable to many environments and is highly palatable to humans, domestic animals, and wildlife.

Bamboo has emerged as a prospective crop with over 1,500 diversified applications worldwide, ranging from medications to nutrient supplies and from producing toys to aircraft. It is considered the world's best material because of its high tensile strength compared to teak wood and mild steel. It may be a valuable resource for energy because of its fast growth rate (5 – 7 years to mature) and potential fuel characteristics (Bystriakova et al., 2003). The combination of fast-growing, natural vegetative propagation and their adequacy for making several products makes bamboo ideal for various industrial applications to replace other perennials and woody plants (Bonilla, Guarnetti, Almeida, & Giannetti, 2010; Hamzah et al., 2016). The traditional phenotypic approach for identifying bamboo species, however, is very complicated, leading to controversy over the classification based on their peculiar vegetative process and flowering characteristics (Isagi et al., 2004; Ramakrishnan et al., 2020; Sharma et al., 2008). These morphological features are often affected by the environment and are complicated, state-specific, and limited in number, contributing to the misclassification of bamboo at the genus and species level (Wu, 1962; Yeasmin, Ali, Gantait, & Chakraborty, 2015). It is, therefore, essential to have a reliable and precise taxonomic classification and nomenclature system for bamboo to be able to identify and select the best species for growing in the right environment for a purpose that is well suited to it.

TRADITIONAL IDENTIFICATION OF BAMBOO SPECIES

The identification of bamboo is traditionally based on its morphological characteristics, including rhizomes, buds, leaves, branching patterns, inflorescence, flowers, and fruits. This approach has been problematic because there are limited and peculiar morphological characters in bamboo, particularly its flowers, and the flowering period

may vary between 15 – 120 years, and some species have never known to flower (Janzen, 1976). Although traditional taxonomy depends heavily on inflorescence and floral morphology, Usui (1957) revealed the importance of branch and bud characters, and McClure (1973) studied the morphology of the rhizome, branching patterns, and culm sheath for bamboo species identification. Soderstrom and Ellis (1988) also considered leaf morphology characters for subfamilial and subtribal level identification, but they failed to apply it at the generic level.

Vegetative characters are often influenced by the environment, making them less constant for systematic purposes (Yeasmin et al., 2015). Various studies have misclassified bamboo species into different taxonomic groups by considering the morphological features that have not been resolved to date. It is not always easy, for example, to define the inflorescence types in bamboos, and in many cases, several conflicting interpretations have been noticed. Chao and Renvoize (1989) recognized the inflorescence of *Racemobambos* as 'iterauctant', while Dransfield (1992) described it as 'semelauctant'. For its allied genus *Neomicrocalamus*, related confusion has also been observed (Dransfield, 1992; Stapleton, 1994). Furthermore, basic knowledge of bamboo biology and genetics is still severely lacking. Since morphological identification has not proven successful, there is an urgent need to implement alternative techniques to recognize the taxonomic complexities of bamboo.

DNA FINGERPRINTING IN BAMBOOS

The use of molecular markers has increased in many branches and subdisciplines of biology as an alternative method for identifying DNA polymorphisms between individuals, studying genetic diversity, and analyzing the genetic distance between species. Determining the appropriate taxonomic level at which it is most informative and correlating it with morphological taxonomic grouping is the major challenge for molecular markers. The application of molecular techniques for studying genetic diversity in bamboo, however, has still been limited to date. The present study aims to review different molecular tools currently used to assess genetic diversity and infer phylogenetic relationships in the bamboos.

Several generations of molecular markers have become increasingly reliable in the past decades. DNA products such as isozymes and secondary compounds, including phenolics, were initially used to explore the phylogenetic relationship between taxa and assess infraspecific polymorphism for species identification (Alam, Sarker, & Hassan, 1997; Biswas, 1998; Chou & Hwang, 1985; Pattanaik & Hall, 2011). Later on, the focus of the research was on variation in the DNA structure, providing two different types of data, namely restriction fragment data (DNA fingerprinting) and gene sequence data (DNA barcoding). In previous phylogenetic analyses of temperate bamboos, especially *Phyllostachys* and *bambusa*, molecular markers based

on restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) were used (Table 1; Barkley, Newman, Wang, Hotchkiss, & Pederson, 2005; Das, Bhattacharya, Basak, & Pal, 2007; Friar & Kochert, 1994; Kobayashi, 1997). More recently, DNA sequences have also been used to deduce bamboo phylogenetic relationships, but only limited sequence variations were found in the genes studied (Attigala, Wysocki, Duvall, & Clark, 2016; Hodkinson, Renvoize, Chonghaile, Stapleton, & Chase, 2000; Ma, Zhang, Zeng, Guo, & Li, 2014; Zhang, 2000).

Table 1 Molecular techniques for classification of bamboo species

Techniques*	Species tested	References
RFLP	*Phyllostachys* spp.	Friar and Kochert (1994)
RAPD	*Bambusa* spp. *Cephalostachyum pergracil* *Chimonobambusa* spp. *Dendrocalamus* spp. *Dinocloa m'Clellandi* *Gigantochloa atroviolacea* *Neosinocalamus affini* *Phyllostachy* spp. *Pseudobambusa kurzii* *Qiongzhuca tumidinoda* *Sasa* sp. *Yushania* sp.	Biradar et al. (2005) Das et al. (2007) Nayak et al. (2003) Zhang et al. (2011)
AFLP	*Bambusa* spp. *Chimonobambusa marmorea* *Dendrocalamus* spp. *Gigantochloa* spp. *Neomicmlamus andropcgonifolius* *Phyllostachys* spp. *Shibataea chinensis* *Sinobambusa tootsik* *Thyrsostachys siamensis*	Hodkinson et al. (2000) Loh et al. (2000)
SCAR	*Bambusa* spp.	Das et al. (2005)
ISSR	*Bambusa* spp. *Dendrocalamus* spp. *Melocanna baccifera* *Oxytenanthera nigrociliata* *Phyllostachys* spp. *Pleioblastus* spp. *Sasa auricoma* *Schizostachyum pergracile* *Thyrsostachys oliveri*	Lin et al. (2010) Mukherjee et al. (2010) Nilkanta et al. (2017) Tian et al. (2012) Yang et al. (2012)

EST-SSR	*Arundinaria* spp. *Bambusa* spp. *Brachystachyum densiflorum* *Dendrocalamus* spp. *Hibanobambusa tranquillans* *Indocalamus* spp. *Melocanna baccifera* *Ochlandra* spp. *Phyllostachys* spp. *Pseudosasa* spp. *Sasa* spp. *Semiarundinaria fastuosa* *Shibataea* spp. *Sinobambusa* spp.	Barkley et al. (2005) Cai et al. (2019) Sharma et al. (2008)

*RFLP = Restriction fragment length polymorphism; RAPD = Random amplified polymorphism DNA; AFLP = Amplified fragment length polymorphism; SCAR = Sequence characterized amplified region; ISSR = Inter-simple sequence repeat; EST-SSR = Expressed sequence tag-simple sequence repeat

Restriction Fragment Length Polymorphism (RFLP)

Friar and Kochert (1994) were the first researchers to use RFLP to identify 61 accessions and 20 species of the genus *Phyllostachys*. RFLP is a polymorphism that results from sequence variation in the genomic DNA recognized by restriction enzymes. The earlier findings of the presence of two distinct sections (*Phyllostachys* and *Heteroclada*) in the *Phyllostachys* species pool were confirmed by RFLP analysis. However, the analysis disagreed with a previous study on putting *Phyllostachys nigra* under the section of *Heteroclada* (Wang et al., 1980). Due to the requirements of a large amount of DNA along with the use of radioactive isotopes, the routine use of RFLP in plant genotyping as well as in bamboo has been limited.

Random Amplified Polymorphism DNA (RAPD)

The RAPD markers are the most straightforward, fastest, and easiest assay to assess the genetic diversity and variation between populations and within them (Kumari & Pande, 2010). The technique has been successfully employed in determining genetic relationships in various plant species. Nayak, Rout, and Das (2003) assessed the genetic variability using RAPD markers in twelve bamboo species and found a wide range of variability among them with ten random primers. Subsequent cluster analysis showed that the twelve taxa belonging to different bamboo genera formed two major clusters based on similarity indices and three minor clusters from each of the major clusters. In

a separate study using 80 random primers on the clones of *Dendrocalamus strictus* and *Bambusa bambos*, a total of 42 and 32 RAPD markers produced polymorphic patterns in *D. strictus* and *B. bambos*, respectively (Biradar, Patil, Kuruvinashetti, Biradar, Patil, & Yaradoni, 2005). Das et al. (2007) showed a phylogenetic relationship between 15 bamboo species using 32 key morphological descriptors and 120 polymorphic loci of the genomic DNA generated by the RAPD primers. The dendrogram and principal component analysis revealed that their phylogenetic relationships were consistent with the commonly referred bamboo classification system. Still, discriminatory was shown by the cluster pattern generated from the similarity matrix of the key morphological characters. More recently, Zhang, Yang, and Liu (2011) had conducted the RAPD analysis using chloroplast DNA of 22 bamboo species to assess the polymorphisms, similarities, and relationships between them. These studies concluded that molecular evidence based on RAPD markers needs to be accompanied by morphological characteristics to confirm the relationships between taxa.

Amplified Fragment Length Polymorphism (AFLP)

The AFLP method involving a combination of restriction digestion and PCR amplification was first developed by Vos et al. (1995) and employed by Loh, Kiew, Set, Gan, and Gan (2000) to identify bamboo species and determine their genetic relationships. The study was carried out using a combination of eight primers on 15 bamboo species belonging to four genera in the subtribe Bambusinae. The results showed that the species were divided into different clusters based on 13 unique banding patterns. Molecular markers of AFLP were also used to compare *Phyllostachys* species (Hodkinson et al., 2000), *Bambusa* species, and their closely related genera (Loh et al., 2000) for phylogenetic analysis. Compared with RAPD, the use of AFLP and RFLP techniques can produce more polymorphic fragments (bands). However, AFLP is often more expensive, time-consuming, complicated in interpretation, and technically demanding than other alternatives.

Sequence Characterized Amplified Region (SCAR)

With higher annealing temperatures, SCAR has been developed as an extension of the RAPD analysis with better reproducibility (Paran & Michelmore, 1993). Later on, two species-specific SCAR markers have been developed for *Bambusa balcooa* and *Bambusa tulda*, the two commercially important species for plantations, to aid the paper and pulp industry in the accurate species diagnosis, especially when key morphological features are indistinguishable during the seedling stage (Das, Bhattacharya, & Pal, 2005). Specifically, the markers were developed from the Bb836

and Bt609 sequences of the respective species, using primers from both flanking ends of the RAPD primers. They successfully amplified the DNA of individuals representing both *B. balcooa* and *B. tulda* species. The findings concluded that the two molecular markers were particularly useful for the regulatory establishment of sovereign rights of the *B. balcooa* and *B. tulda* germplasms.

Inter-Simple Sequence Repeat (ISSR)

The molecular markers of ISSR have been used to identify the genetic diversity of many plant species, including bamboos. Due to their more extended primer sequence and higher annealing temperature, ISSR markers are more efficient and reliable than RAPD, resulting in higher stringency (Tikendra, Amom, & Nongdam, 2019). They have reportedly been used to determine genetic variation and the relationship between various bamboos, including *Phyllostachys* (Lin et al., 2010), *Dendrocalamus* (Tian, Yang, Wong, Liu, & Ruan, 2012; Yang, An, Gu & Tian, 2012), and *Melocanna* (Nilkanta, Amom, Tikendra, Rahaman, & Nongdam, 2017). Lin et al. (2010) crossbred two species of *Phyllostachys* and successfully identified three hybrids produced by the cross with eight ISSR primers. Furthermore, 25 ISSR markers were used by Mukherjee et al. (2010) to investigate genetic diversity among 22 bamboo taxa, of which 12 resulted in reproducible and scorable bands.

Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR)

Combining different molecular markers in identifying the genetic diversity among the population species has also been carried out. Barkley et al. (2005) used 25 EST-SSR markers derived from cereal crops including maize, wheat, sorghum, and rice in evaluating the genetic diversity of 92 bamboos classified under 11 genera and 44 species to find out which markers produced well-resolved polymorphic bands. Considering that grass genomes have co-evolved and share large-scale synteny, Sharma et al. (2008) tested 98 rice SSR primers and 20 sugarcane EST-SSR primers on 23 bamboo species. They reported 44 rice and 15 sugarcane SSR primers for transferability to at least one species of bamboo. More recently, Cai et al. (2019) have developed abundant EST-SSR resources from Lei bamboo transcriptome data useful for genetic diversity analysis and molecular verification of bamboo, suggesting that the markers are more effective and reliable than ISSR and other alternatives.

DNA BARCODING IN BAMBOOS

As reviewed in this section, DNA barcoding or sequence-based methods for phylogenetic analysis on bamboo and grass have been recorded in several studies to date. DNA barcoding is a way of identifying species based on a short sequence of DNA that varies from species to species but is conserved within species (Hebert, Cywinska, Ball, & deWaard, 2003). Two plastid gene regions, *rbcL* and *matK*, were selected as the core DNA barcodes for all land plants (CBOL Plant Working Group 1 et al., 2009). These plastid regions, which played an essential role in the phylogenetic reconstruction of land plants, were used because of their strong phylogenetic signals in both *rbcL* and *matK* (Chase et al., 1993). In previous studies, for both *rbcL* and *matK* barcodes, the success rate of generic and species-level identification was around 70%. With a supplementary marker, the identification success improved to 98%.

Besides, Kress, Wurdack, Zimmer, Weigt, & Janzen (2005) also proposed the nuclear internal transcribed spacer region and plastid *trnH-psbA* intergenic spacer as potentially usable DNA barcodes for flowering plants. In their study, the two plastid genomes of tobacco and deadly nightshade (belladonna) were compared with closely related species in seven plant families and a group of species collected from a local flora comprising 50 plant families, suggesting that the sequences in this pair of loci have the potential to discriminate between the largest number of plant species for barcoding purposes. Although mitochondrial DNA barcoding is well developed in animals using *COI* regions, mitochondrial DNA, however, has low substitution rates and rapidly changing gene content and structure, making it unsuitable for barcoding in plants (Sinha, Kumari, & Singh, 2012).

Chloroplast DNA Barcode

The chloroplast genome has long been used to assess the phylogeny of the grass family and resolve systematic problems at the subfamilial and tribal levels (Hilu & Johnson, 1991). Nadot, Bajon, and Lejeune (1994) used the chloroplast *rps4* gene to construct a phylogenetic tree comprising 28 Poaceae species and resolve the position of Bambusoids with other groups to demonstrate how rice and bamboo were closely related. Meanwhile, the chloroplast *ndhF* gene was also used to address phylogenetic relationships between 45 grass (i.e. the ingroup) and two outgroup taxa (Clark, Zhang, & Wendel, 1995). The analysis resulted in the two neotropical herbaceous bamboo tribes, the Streptochaeteae and Anomochloeae, being resolved as the most basal clade within the family, conforming to the primitive features of their unique inflorescences and spikelets. Other researchers also used chloroplast genes, such as *matK* and *rbcL*, to establish the phylogeny of Poaceae at the subfamilial and tribal levels and study glass systematics and evolution (Cai, Zhang, Zhang, Gao, & Li, 2012; Hilu, Alice, & Liang, 1999; Liang & Hilu, 1996; Yang et al., 2008). Based on a more recent comparative

analysis using 6.7 kb of coding and noncoding sequence data and 37 microstructural characters from the chloroplast genome, four main lineages, i.e., temperate woody, paleotropical woody, neotropical woody, and herbaceous bamboos, were recognized in a phylogeny estimation of bamboo tribes and subtribes (Kelchner & Bamboo Phylogeny Group, 2013).

Nuclear DNA Barcode

In most cases, nuclear DNA barcodes, mainly ITS regions, were used in conjunction with chloroplast DNA for barcoding plants, including bamboo species. Yang et al. (2008) employed the nuclear ITS and GBSSI gene apart from chloroplast *trnL-F* DNA sequences to establish a phylogenetic framework of the paleotropical woody bamboos involving 53 species and 17 genera. Using four woody bamboos of the North Temperate Zone as outgroups, the analysis revealed that the ingroup species clustered into three clades: the Bambusinae clade (including *Thyrsostachys*, *Neosinocalamus*, *Racemobambos*, *Molecalamus*, *Dendrocalamopsis*, *Bambusa*, *Gigantochloa*, *Oxytenanthera* s. str., *Dendrocalamus*, *Bonia* and *Neomicrocalamus*), the Dinochloa clade, and the Melocanninae clade (including *Pseudostachyum*, *Leptocanna*, *Cephalostachyum*, *Melocanna* and *Schizostachyum* s. str.); and further grouped into two monophyletic clades, i.e., the Bambusinae + Dinochloa clade and the Melocanninae clade. Moreover, Cai et al. (2012) suggested that the discriminatory power of the species was substantially enhanced as the nuclear ITS region integrated with a single or combination of plastid markers. Although the ITS alone could identify up to 66.7% of the examined Bambusoideae taxa, combining ITS and chloroplast *rbcL* could exhibit the highest species identification power and serve as a potential DNA barcode for temperate woody bamboos.

Multilocus DNA Barcoding

A combined analysis of multi-gene regions is often useful for improving phylogenetic resolution and support in plant taxonomy. Qiang et al. (2005) compared the genus *Arundinaria* with other related genera, such as *Pleioblastus*, *Pseudosasa*, *Oligostachyum*, *Bashania*, and *Clavinodum*, to determine their phylogenetic relationships by using the nuclear ITS and chloroplast *trnL-F* intergenic spacer. Sungkaew, Stapleton, Salamin, and Hodkinson (2009) reported phylogenetic groupings within Bambusoideae and resolved several previously unrecognized and poorly supported phylogenetic patterns using five chloroplast DNA regions, *trnL* intron, *trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, *rps16* intron, and *matK*. Cai et al. (2012) analyzed four barcoding markers, namely *matK, rbcL, trnH-psbA*, and ITS, in species identification of temperate woody bamboos (Bambusoideae) and recommended *rbcL* + ITS as a potential barcode region for species discrimination. Furthermore, Sosa, Mejía-

Saules, Cuéllar, and Vovides (2013) created a DNA barcode library with some leading candidate plastid DNA regions, i.e. *matK*, *rbcL*, and *psbI-K* spacer, as an essential tool for phytosanitary authorities to classify species belonging to groups that command high horticultural trade prices in Mexico. Their study showed that the *psbI-K* spacer retrieved more polymorphic sites in bamboos and suggested *matK* + *psbI-K* as the discriminant barcode loci to identify temperate bamboos to at least their generic level. Lately, in the absence of discriminatory features at the juvenile stage, multiple sequence alignment of *psbA-trnH* barcode has been used to certify planting materials, such as micropropagated plantlets, rhizome transplants, and culm cuttings, of the commercial bamboo species (including *Bambusa*, *Dendrocalamus*, *Melocanna*, *Oxytenanthera*, and *Ochlandra*) in bamboo nurseries in India (Dev, Sijimol, Prathibha, Sreekumar, & Muralidharan, 2020).

CONCLUSION

The bamboo taxonomy is currently in a state of flux, and more phylogenetic studies are required to help resolve the remaining systematic issues. In this paper, we reviewed the availability of numerous molecular-level methods and techniques for achieving bamboo species identification and how the barcoding markers help to provide insights into species-level taxonomy in bamboos. While morphological taxonomy still plays a crucial role in the classification of various bamboo species, molecular markers are undoubtedly valuable tools for addressing phylogenetic questions in the easily confused or cryptic species and can assist traditional approaches in making the identification process more rapid and effective. The availability of the bamboo genome database (http://www.bamboogdb.org/) in the post-genomic era will provide global researchers with a key genomic resource and an extensible analytical platform to better understand the bamboo genome and thus promote future studies based on previous achievements, especially in advanced analysis of phylogenetic and genetic diversity in bamboos.

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