DNA barcoding: An emerging tool for precise identification and certification of planting stock in taxonomically challenging bamboo species

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Abstract: Bamboos are a group of woody arborescent grasses that provides for the livelihood of a large number of people living in rural areas in many parts of the world. Owing to the increased demand for bamboos for traditional as well as new commercial uses, there is an urgent need for undertaking plantation of the commercially important bamboo species. To ensure higher productivity it is also important that the plantations be established using superior quality planting materials. From the taxonomic standpoint, bamboos are a challenging group of plants. Species identification in bamboos is mainly based on morphological characters and the unpredictable flowering behavior coupled with environmental plasticity leads to taxonomic complexities. This is particularly confounding when the plants are in the juvenile stage as in the bamboo nurseries. This poses certain hurdles in the certification of superior planting materials owing to the lack of distinguishable morphological characters. Mixing up of species with similar morphological features is rather common in bamboo nurseries and mistakes are realized only when the plants attain maturity after few years. Hence a well defined molecular tool like DNA barcoding is envisaged to tackle these taxonomic complexities in species identification as well as for the certification of planting materials. This article brings out the significance of DNA barcoding in species identification and to resolve taxonomic complexities pertaining to bamboo. The ongoing research in the use of DNA markers in taxonomy and biosystematics of bamboo and the challenges faced is also reviewed.

Key words: Bamboo, taxonomy, species identification, certification, DNA barcoding

INTRODUCTION

Bamboo, belonging to the subfamily Bambusoideae within the grass family, Poaceae, holds an important position among the non-timber forest products, supporting the livelihood potential of the people living in rural areas. Given the multifarious purposes to which it is put to use, both in socio-economical as well as ecological aspects, they are commonly known as 'Poor man's timber' and 'Green Gold of India' (Orhnberger and Georrings, 1986; Tewari, 1992). Bamboos are naturally distributed throughout the tropical and subtropical regions of the world except in Europe (Bystriakova, 2003). Worldwide, there are 1400 species of temperate and woody bamboos belonging to 115 genera (BPG, 2012). The distribution of bamboos extends from 51˚ N latitude in Japan to 47˚ S latitude in South Argentina, where as South America and East Asia are

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considered to be the centre of bamboo diversity. In India, 130 bamboo species under 18 genera are distributed in the Western Ghats, North Eastern India and Andaman and Nicobar Islands (Kumar, 2011) and has the second largest reserve of bamboo resources in the world next to China.

Taxonomic complexities in bamboo

Traditional identification of plant species is mainly based on vegetative as well as floral characters. In bamboos most of the vegetative characters are not stable as well as not uniformly applicable to all species. Hence their identification and classification poses certain problems for the taxonomists. The incongruent flowering behavior resulting in unavailability of floral parts as well as vegetative parts also found to be hurdle in identification. Apart from this, the morphological characters commonly employed in the traditional taxonomic classification of bamboos, show close affinities among some species. Moreover, identification and classification of bamboo using anatomical features also didn't prove to be successful (Goyal *et al.,* 2013). Various studies reported that the same bamboo species has been classified by different taxonomists in different genera or same species were described in different names and consequently led to many revisions in various genera over the past few decades. Bahadur and Jain (1981) described *Bambusa arundinaceae* var. *gigantea,* later on, Bennet and Gaur (1990) assigned this variety as *Bambusa bambos* var. *gigantea,* and based on morphological similarity synonymised under *Bambusa bambos.* Several revisions have also been happened in the genus *Dendrocalamus* in which Gamble (1890) pointed out the resemblance of morphological characters of *Oxytenanthera stocksii* Munro (1868) with the genus *Dendrocalamus* and placed *O. stocksii* under the genus *Dendrocalamus.* Munro (1868) initially described the genus *Oxytenanthera* with type specimen, *Oxytenanthera abyssinica.* Another species, *Oxytenanthera monadelpha* has been transferred to a new genus *Pseudoxytenanthera* (Sodestrom and Ellis, 1987) based on vegetative as well as floral characters, while Majumdar (1989) merged the genus *Pseudoxytenanthera* into *Oxytenanthera* due to its resemblance with type specimen, *Oxytenanthera abyssinica.* But, Sharma (1996) pointed out that morphological characters are not sufficient enough to separate the genus *Pseudoxytenanthera* from *Oxytenanthera Munro* and the generic name *Oxytenanthera* is retained. Kumar and Remesh (2008) merged *Oxytenanthera ritchiei* (Munro) Blatt. & McCann to the genus *Munrochloa* Kumar & Remesh and a new combination *Munrochloa ritchiei* (Munro) Kumar & Remesh was added to the genus. Similar confusions also happened in the genus *Ochlandra* Thw., an endemic reed bamboo, described with 11 species and three varieties (Orhnberger and Georring, 1986) which was reduced to ten species after several taxonomic treatments by different authors (Kumar, 1995; Seethalakshmi and Kumar, 1998; Kumar *et al.,* 1999; Kumar *et al.,* 2001; Unnikrishnan, 2003 and Kumar, 2011). Another example of generic treatment is between *Arundinaria* and *Sinarundinaria*; Chao and Renvoize (1989) after several revisions transferred all species from the temperate woody bamboo genus, *Arundinaria* to *Sinarundinaria,* whereas Nakai (1925) treated some *Arundinaria* species under the genus *Indocalamus.* Seethalakshmi and Kumar (1998) also supported the generic concept of Chao and Renvoize (1989). However, Attigala *et*

al. (2016) conducted a thorough investigation of the tribe Arundinarieae based on molecular as well as morphological data and treated South Indian and Sri Lankan *Arundinaria* genera as separate entity and the genus *Arundinaria* is now recognized as *Kuruna* representing the twelfth major linage of Arundinarieae distinguished by a combination of vegetative and reproductive characters. The genus *Teinostachyum* originally described by Munro (1868) under the subtribe Melocanninae. Gamble (1896) and Dransfield (1980) treated *Cephalostachyum, Teinostachyum, Pseudostachyum, Schizostachyum* as separate genera of this subtribe, while Kumar (2011) supported the concept of Gamble (1896) and Dransfield (1980). The taxonomy of bamboos thus appears to be in a constant state of fluidity due to the complexities arising out of the dependence on morphological characters and hence a supplementary molecular tool is often envisaged to tackle the existing complexities in the species identity in many bamboo genera.

Identification and certification of bamboos

Unlike, other grass species of Poaceae, bamboos have some exceptional morphological features like culm sheath, ligules, well developed branching system, rhizome pattern, among others. These morphological characters played an important role in taxonomic identification and systematics of bamboo species. But the morphological characters and terminology used for the identification are interpreted differently by taxonomists all over the world. Usui (1957) studied the morphology of rhizome, culm sheath and inflorescence for the identification of bamboos while McClure (1966), Sodestrom and Ellis (1988) considered the leaf anatomy for the species identification. Conventionally it is the flower parts that play an important role in taxonomy of plants, however the long and often unpredictable flowering behaviour characteristic of most bamboo is the major hurdle for species identification. The field identification of some bamboo species is thus extremely difficult even for an expert in taxonomy due to the high degree of morphological similarities.

The planting materials commonly used for the establishment of bamboo plantations *viz*. seedlings, rooted culms, branch cuttings, rhizome transplants and micropropagated plantlets lack all these specific features associated with the mature culms. Identification of species for the purpose of certification of these planting materials produced in the nurseries before large scale establishment of plantations is thus often difficult. Bamboo seeds of dubious identity are often offered for sale by unscrupulous traders. In the current scenario in many parts of the world, like in India where several species are cultivated, the possibility of misidentified clumps being mass multiplied inadvertently or at times even intentionally, is of great concern. Thus a supplementary tool which imparts more precision than the conventional methods has been a need for a long time to sort out the complexities existing in the traditional bamboo taxonomy.

Molecular markers in bamboo taxonomy

With the advent of PCR technology, PCR based molecular markers, ranging from

RAPD, ISSR, AFLP and SSR, among others have been extensively used for multifarious purposes due to their abundance, precision and reproducibility irrespective of the environment. Extensive progress has also been achieved in bamboo research using molecular markers starting with the precise identification of bamboo genotypes, for the assessment of genetic variation as well as to address the various issues in bamboo taxonomy including systematics and evolution. The use of RFLP markers were the first of its kind, in investigating genetic variability and phylogenetic relationships among different *Phyllostachys* species (Friar and Kochert, 1991). RAPD has been used for the genetic profiling of *Yushania* (Hsiao and Rieseberg, 1994), *Phyllostachys* (Gielis *et al.,* 1997; Ding and Zhao, 1998), *Bambusa* (Nayak *et al.,* 2003) and *Dendrocalamus* (Eevera *et al.,* 2008). Molecular analysis using RAPD has been employed for *Ochlandra travancorica* of the Western Ghats (Nag *et al.,* 2013) and *O. stridula* of Sri Lanka (Ramanayake *et al.,* 2006) to assess the genetic diversity of the species. Shalini *et al.* (2010) investigated the genetic relationship of bamboo species belonging to three genera, *Bambusa, Dendrocalamus* and *Guadua* using RAPD. AFLP based identification has been carried out in *Phyllostachys, Bambusa, Fargesia* species and their cultivars (Bennet and Gaur, 1990). AFLP has been used as the basis for the early assessment and the construction of genetic maps in *Dendrocalamus strictus* and *D. giganteus* (Gielis *et al.,* 1997) as well as to evaluate the genetic relationships of four genera within the subtribe Bambusinae (Loh *et al.,* 2000). Marulanda *et al.* (2002) employed AFLP markers to establish the genetic relationship between multiple accessions and biotypes of *Guadua angustifolia*. Mukherjee *et al.* (2010) evaluated the genetic relationships among 22 taxa of bamboo using ISSR markers as well as expressed sequence tag (EST) based random primers. Yang *et al.* (2012) reported the use of ISSR markers in genetic diversity assessment of *D. membranaceus* in Yunnan. Six microsatellites were also isolated from *B. arundinacea (B. bambos)* and their cross species amplification was tested in 18 other bamboo species (Nayak and Rout, 2005). Marulanda *et al.* (2007) evaluated the genetic diversity of *Guadua augustifolia* using microsatellite sequences of rice and sugarcane species. A total of 98 mapped SSR primers representing 12 linkage groups of rice and 20 EST derived sugarcane SSR primers were evaluated for the transferability to 23 bamboo species (Sharma *et al.,* 2008). Yasodha (2010) identified microsatellites in the tribe Bambuseae for the analysis of morphologically indistinguishable bamboo species and investigated their genetic diversity pattern. Lin *et al.* (2014) analyzed the association between SSR and transposable elements and estimated the outcrossing rates in *Phyllostachys pubescens*. Zhao *et al.* (2015) presented the first genome wide microsatellites in moso bamboo (*Phyllostachys edulis*) and their results showed that microsatellite markers are valuable for investigating the genomic studies in bamboo and related grass species.

Extensive research have also been carried out using sequences of coding as well as non-coding regions of DNA (*viz. ndhF, rps16* intron, *rpl16* intron, *trnD-T* intergenic spacer, *trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, etc.) for tracing the origin and evolution of major lineages in Poaceae (Watanabe *et al.,* 1994; Clark *et al.,* 1995; Kelchner and Clark, 1997; Clark *et al.,* 2007; Yang, 2008; Sunkaew *et al.,*

2009; Triplett *et al.,* 2010; Zheng *et al.,* 2010; Zhang *et al.,* 2011; BPG, 2012; Kelchner and BPG, 2013). Even though molecular markers have been widely used across various genera and species, so far they have not been employed for conclusive species discrimination and identification in bamboos.

DNA barcoding for species identification and biosystematics

The concept of using short stretch of DNA sequences as barcode for species identification has been proposed using *CO1* barcode region in animals (Hebert *et al*., 2003). Subsequently, based on the discrimination power across various angiosperm genera, a core barcode comprising *matK+rbcL* together with *psbA-trnH* as well as the nuclear ribosomal internal transcribed spacer region (*ITS2*) has been recommended for barcoding in plants (CBOL, 2009; Hollingsworth *et al*., 2009) since it became apparent that *COI* is ineffective for the purpose in plants. Several studies have been reported on barcoding in plants using these CBOL recommended barcode regions either alone or in combinations (Newmaster *et al.,* 2006; Kress and Erickson, 2007; Chase *et al.,* 2007; Hollingsworth *et al.,* 2009; Yao *et al.,* 2010; Hollingsworth *et al.,* 2011, Clement *et al., 2012*). This multilocus core barcode system envisages to tackle the taxonomical complexities related to species identification in plants. DNA barcoding in closely related or recently diverged species is found to be a great challenge in some instances, both in terms of a suitable combination of barcoding regions as well as universality of primers used. Similarly, in some taxonomically complex groups, due to the problematic levels of hybridization, introgression or incomplete lineage sorting the exact identification of species using a barcoding approach is a difficult task (Ennos *et al.,* 2005; Hollingsworth *et al.,* 2011). Collins and Cruickshank (2012) reported seven possible setbacks that were commonly observed in barcoding and recommended some potential improvements for more reliable and accurate outcomes in DNA barcoding works.

DNA barcoding is gaining more attention nowadays, because of its accuracy as compared to taxonomy species identifications in plants. DNA barcoding has been extensively used in wide range of angiosperm taxa such as *Allium* (Anvarkhah *et al.,* 2013), *Phyllanthus* (Awomukwu *et al.,* 2014), *Ocimum* (Anbhazhagan *et al.,* 2014), Sapotaceae (Vivas *et al.,* 2014), *Dendrobium* (Xu *et al.,* 2015), figs (Castro *et al.,* 2015), among others and also found to be an effective tool for the biodiversity assessment and conservation of temperate (Liu *et al.,* 2014) as well as tropical tree species (Huang *et al.,* 2015). DNA barcoding has also been effectively utilized in other plant groups *viz*. Gymnosperms (Sass *et al.,* 2007; Li *et al.,* 2011), Bryophytes (Li *et al.,* 2009; Liu *et al*., 2010; Yan *et al*., 2011), Ferns (Nitta, 2008; Ebihara *et al*., 2010; Ma *et al*., 2010; de Groot *et al.,* 2011). At present, DNA barcoding has been highly exploited in species identification of medicinal plants as well as for tracing the adulteration (Techen *et al.,* 2014; Palhares *et al.,* 2015). Trivedi *et al*. (2015) reviewed the progress of DNA barcoding in marine plant species and nuclear *ITS* for *Halophila* (Waycott *et al*., 2002; Uchimura *et al*., 2008), *trnK* introns and *rbcL* for *Zostera* (Les *et al*., 2002), *rbcL* and *matK* for seagrasses (Lucas *et al*., 2012) have been reported. Buchheim *et al*. (2011) proposed *ITS2* as a potential barcode for species confirmation

in algae. DNA barcoding has been suggested as a powerful tool for species identification in one of the largest marine green algal genera, *Codium* (Lee *et al.,* 2015). DNA barcoding has also been reported in aquatic duckweeds (Wang *et al.,* 2010), Nymphaea (Biswal *et al.,* 2012) and *Potamogeton* (Ito *et al.,* 2014). DNA barcoding has also reported to be an effective tool for the efficient distinction of invasive aquatic plant species from non-invasive related species (Ghahramanzadeh *et al.,* 2013).

DNA barcoding in the family Poaceae

DNA barcoding has been developed in many of the taxonomic challenging lineages in the grass family Poaceae. Drumwright *et al.* (2011) surveyed and tested grass species across the family for locating a DNA barcode and suggested *matK+rbcL* as the core barcode for species identification with 95% accuracy. Lopez-Alvarez *et al.* (2012) employed widely accepted barcoding regions *trnL-F* and *ITS2* to clearly discriminate among *Brachypodium distachyon*, *B. stacei* and *B. hybridum.* Analysis has been carried out using five barcode regions in the genus *Leymus* of the tribe Triticeae but was unable to locate a discriminant barcode (Liu *et al.,* 2014). The discrimination power of *matK* in the family of Poaceae has also been reported (Jethra *et al.,* 2014). High frequency of cpDNA inversions leading to huge amount of intraspecific variations has been demonstrated in the tribe Triticaceae using three barcode regions (*matK, rbcL* and *psbA- trnH*) (Bieniek *et al.,* 2014). Divergence dating of *Poa* in the Australasian region has been investigated using three plastid gene regions (*rbcL, matK, rpl32-trnL* spacer) as well as two nuclear gene regions (*ITS* and *ETS)* (Birch *et al.,* 2014).

DNA barcoding in bamboos

DNA barcoding research has started gaining momentum in the taxonomically challenging species of various bamboo genera only recently. Cai *et al.* (2012) tested the feasibility of the four proposed barcoding loci *viz. matK*, *rbcL*, *psbA-trnH*, *ITS2* in temperate woody bamboos and suggested the combination of *rbcL+ITS2* as a potential barcode for species discrimination. The recommended barcode region, *matK* failed to discriminate *Bambusa* species due to interspecific hybridization and polyploidy (Das *et al.,* 2013). The low discriminatory power of the core barcode (*rbcL+matK*) as well as greater discriminatory power of *trnG-trnT* spacer in bamboos has also been reported (Zhang *et al.,* 2013). Sosa *et al.* (2013) evaluated the efficiency of *matK, rbcL* and the intergenic spacer *psbK-psbI* and *matK+psbK-psbI* has been reported as the discriminant barcode loci in temperate bamboos.

Preliminary studies carried out by the authors on the potential of the CBOL recommended barcodes in the bamboo genera of importance to the Indian subcontinent and in the endemic bamboo genera of the Western Ghats region, indicate that the five recommended barcode regions (*rbcl, matK, trnH-psbA, rpoB, rpoC)* tried singly failed to show species discrimination. The barcode region *trnH-psbA* in combination with other spacer regions was ideal for discriminating the commercially

important species mainly belonging to the genera *Bambusa* and *Dendrocalamus.* Further studies to address the problems of species identification and biosystematics prevailing in bamboo taxonomy are clearly required. In commercial bamboos, where precise identification of species at the juvenile stage is difficult, DNA barcoding is now seen as an essential and dependable tool for confirming the species identity of the multiplied planting material in the nursery before the establishment of large scale plantations. Thus DNA barcoding has a critical role to play in the process of certification of quality planting material of bamboo which is being implemented in India by the National Bamboo Mission.

CONCLUSION

Species identification in bamboo, based solely on morphology, is a challenging task due to the environmental plasticity as well as dearth of distinguishable characters, primarily due to the absence of flowers during much of the lifecycle. The identification of planting material derived from superior clones needs accurate identification of the species. Due to the lack of sufficient technical expertise for morphology based identification, coupled with absence of discriminating characters at the juvenile phase, some misidentification is unavoidable. A major hurdle in the certification of quality planting stock is the inability to precisely identify species at the juvenile phase and in nurseries. A well defined molecular tool like DNA barcoding has been found as an alternative or a supplementary tool to address the various taxonomic complexities prevailing in the taxonomically challenging bamboo species. The species specific barcode if developed can serve as a species identification tool for the certification of planting stock materials at the nursery level before the establishment of large scale plantations. DNA barcoding has the potential to serve as a novel molecular technique to complement the conventional methods in the identification of taxonomically challenging bamboo species.

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