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ORIGINAL ARTICLE

# Molecular evidence for polyphyly in the woody bamboo genus *Dendrocalamus* (subtribe Bambusinae)

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**Abstract** *Dendrocalamus* is an economically important woody bamboo genus from the Old World tropics. The traditional circumscription of the genus is not satisfactory owing to overlapping limits between *Bambusa* and *Dendrocalamus*. Limited earlier studies in the genus, using molecular techniques, have reported wide genetic variation, and there is disagreement among published infrageneric classifications in the genus. Thus, lack of sound taxonomy is hindering scientific conservation and management of the woody bamboos belonging to this genus. In the present study, amplified fragment length polymorphism markers (AFLPs), generated using five primer combinations, were used to investigate relationships among ten *Dendrocalamus* (*D. strictus*, *D. hamiltonii*, *D. membranaceus*, *D. brandisii*, *D. sikkimensis*, *D. asper*, *D. giganteus*, *D. calostachyus*, *D. sahnii* and *D. somdevai*) and five outgroup species (*Bambusa balcooa*, *Dinochloa macclellandii*, *Melocalamus compactiflorus*, *Oxytenanthera abyssinica* and *Thyrsostachys siamensis*) from subtribe Bambusinae. Neighbour-joining and maximum-parsimony analyses of AFLP dataset provided evidence for polyphyly in the current circumscription of *Dendrocalamus*. All *Dendrocalamus* taxa, except *D. strictus*, clustered into three monophyletic groups. The type species *Dendrocalamus strictus* was found to be genetically distant from the rest of the *Dendrocalamus* and did not cluster into any of these groups. Furthermore, *Bambusa balcooa* was recovered in a cluster containing *D. hamiltonii* and *D. sikkimensis*. The study did not find support for the various earlier infrageneric classifications within *Dendrocalamus*. The implications of the findings are discussed.

**Keywords** *Dendrocalamus* · Bambusinae · AFLP · Molecular phylogeny

## Introduction

*Dendrocalamus* is a woody bamboo genus placed in the subtribe Bambusinae and tribe Bambuseae (Ohrnberger 1999). Species referred to the genus are characterized by their sympodial rhizomes and large-sized, dense clumps. The genus contains over 50 species, naturally distributed in tropical and subtropical regions of the Old World, many of which are economically exploited by communities in south and southeast Asia. The original description of the genus was based on the type species *D. strictus*. The description was subsequently expanded to include pericarp characters, which were used to distinguish between *Dendrocalamus* and *Bambusa* (Munro 1868; Bentham 1883; Gamble 1896). While at present it is taxonomically convenient for *Dendrocalamus* to be recognized in a broad sense, the limits between *Bambusa* and *Dendrocalamus* are not satisfactorily defined, thus creating confusion in their systematic classification. Lack of sound taxonomy is hindering scientific conservation and management of the woody bamboos belonging to this genus.

Since *Dendrocalamus* was separated from *Bambusa* by Nees von Esenbeck in 1834, over 70 species names have

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been assigned to the genus, although Ohrnberger (1999) retains only 51 of these. Most of the species that have not been maintained by Ohrnberger have been reduced to synonymy or to infraspecific rank. A few are transferred to, or sunk into, other genera: *Ampelocalamus* (subtribe Thamnocalaminae); *Gigantochloa* and *Pseudoxytenanthera* (Bambusinae).

Various infrageneric classifications of *Dendrocalamus* have been adopted by Chinese botanists. Hsueh and Li (1988) proposed the first infrageneric classification of *Dendrocalamus* by recognizing two subgenera and five sections, limiting the assignments to only those species reported from China. Ohrnberger (1999) assigned species only to sections *Dendrocalamus*, *Bambusoidetes*, *Sinocalamus* and *Draconicalamus*. Out of the 51 taxa recognized by Ohrnberger, 22 were assigned to particular sections, while 29 taxa were unplaced. A more recent taxonomic revision of Chinese *Dendrocalamus* (Li and Stapleton 2006) retains the subgenera proposed by Hsueh and Li (1988) but disregards sectional assignments, merging sections *Dendrocalamus* and *Bambusoidetes* as subgenus *Dendrocalamus*, and sections *Sinocalamus* and *Draconicalamus* as subgenus *Sinocalamus*. Li and Stapleton (2006) transferred 11 taxa previously referred to subgenus *Sinocalamus* to subgenus *Dendrocalamus*. The major problem faced in the infrageneric classification of *Dendrocalamus* is the paucity of published morphological character information for many of the species. Twenty-seven species do not appear to have been referred to any subgenus or section under any of the proposed schemes.

Bamboos have always been a taxonomically challenging group of plants because, while the classification of flowering plants depends largely on the characteristics of reproductive organs, flowering is rare in many bamboo species. Some bamboo species flower at intervals as long as 120 years, and for some there is no report of flowering to date. Because of apparent paucity of morphological characters in bamboos, taxonomists have long sought different sources of taxonomically informative data. The availability of molecular data in the final decade of the 20th century enabled taxonomists to review phylogenetic concepts of the Poaceae more objectively. Initially DNA products, viz. isozymes and secondary compounds such as phenolics, were used in exploring relationships among taxa (Chou and Hwang 1985), species identification (Alam et al. 1997) and assessment of infraspecific polymorphism (Biswas 1998). In a study involving five *Dendrocalamus* taxa, *Arthrostylidium naibunensis* W.C. Lin and *Chimonobambusa quadrangularis* Makino, a *Dendrocalamus* cluster could be differentiated from the other two genera using phenolic compounds and isozyme patterns of esterase and peroxidase. Within *Dendrocalamus* two sub-clusters were recognized; *Dendrocalamus asper* associated with *D. giganteus*, while *D. latiflorus* associated with its variety *D. latiflorus* var. *mei-nung*. However, *Dendrocalamus strictus* was distant from these two sub-clusters. Later on, variation in DNA itself was the subject of investigations. The more pertinent studies involving named *Dendrocalamus* taxa are those of Loh et al. (2000) and Sun et al. (2005), but these studies entailed only limited sampling of the genus. In the first, two *Dendrocalamus* taxa were sampled, with *D. brandisii* clustering with taxa from *Bambusa*, and *D. giganteus* appearing genetically distant from all other taxa included. In the second study three *Dendrocalamus* taxa were sampled. These three taxa did not form a separate *Dendrocalamus* cluster but were found in two distinct sub-clusters within *Bambusa*. *D. membranaceus* showed close affinity to *D. strictus* and both were placed within one *Bambusa* cluster, whereas *D. latiflorus* was associated with the other *Bambusa* cluster. The study reported wide genetic variation within *Dendrocalamus* and raised questions about its monophyly. These earlier molecular studies included limited samples from *Dendrocalamus* and did not throw much light on the infrageneric relationships within the genus. In the present investigation there was wider provision within *Dendrocalamus* with ten putative taxa, and five outgroup taxa from the subtribe Bambusinae. Amplified fragment length polymorphism (AFLP) markers were used with the objectives to (1) test the monophyly of *Dendrocalamus*, and (2) assess the molecular support for various infrageneric assignments proposed in *Dendrocalamus*.

## Materials and methods

### Site description

Genetic material was collected from the Bambuseta of five botanical gardens in India: Forest Research Institute (FRI), Dehra Dun; National Botanical Garden of Botanical Survey of India (NBG), Howrah; State Forest Research Institute (SFRI), Chessa; ICAR research complex for northeastern hill region (ICAR), Basar; and Rain Forest Research Institute (RFRI), Jorhat. The genetic material of the monotypic African bamboo *Oxytenanthera abyssinica* was available as the result of previous research work at Bangor University, UK (Inada 2004).

### Genetic material

Unopened terminal leaves were collected from ten *Dendrocalamus* and five outgroup taxa from subtribe Bambusinae. The leaves were dried using silica gel as per the procedure of Chase and Hills (1991) and then transported from the field sites in India to the laboratory at CAZS

**Table 1** Voucher specimens deposited at the Forest Research Institute (DD) herbarium, Dehra Dun, India

| Species | Subgenus | Accession code | Origin | Accession ID at FRI herbarium |
|---|---|---|---|---|
| *Bambusa balcooa* Roxburgh | Outgroup | S23 | RFRI, Jorhat | 1/2/156840 |
| *Dendrocalamus asper* K. Heyne | *Dendrocalamus* | S6 | FRI, Dehra Dun | 2/2/156810 |
| *Dendrocalamus brandisii* Kurz | *Dendrocalamus* | S9 | SFRI, Chessa | –[a] |
| *Dendrocalamus calostachyus* Kurz | *Sinocalamus* | S30 | FRI, Dehra Dun | 1/2/156809 |
| *Dendrocalamus giganteus* Munro | *Sinocalamus* | S10 | SFRI, Chessa | 1/3/156842 |
| *Dendrocalamus hamiltonii* Munro | *Dendrocalamus* | 31 | RFRI, Jorhat | 1/3/156830 |
| *Dendrocalamus membranaceus* Munro | *Dendrocalamus* | S3 | FRI, Dehra Dun | 1/2/156807 |
| *Dendrocalamus sahnii* H.B. Naithani and Bahadur | Not defined | S15 | ICAR, Basar | 1/2/156819 |
| *Dendrocalamus sikkimensis* Oliver | *Dendrocalamus* | S11 | ICAR, Basar | 1/4/156799 |
| *Dendrocalamus somdevai* H.B. Naithani | Not defined | S4 | FRI, Dehra Dun | 1/3/156786 |
| *Dendrocalamus strictus* Nees | *Dendrocalamus* | S52 | RFRI, Jorhat | 1/4/156847 |
| *Dinochloa macclellandii* Kurz | Outgroup | S13 | NBG, Howrah | 1/2/156797 |
| *Melocalamus compactiflorus* Bentham | Outgroup | S27 | FRI, Dehra Dun | 1/2/156793 |
| *Oxytenanthera abyssinica* Munro | Outgroup | S32 | Lilongwe, Malawi | –[a] |
| *Thyrsostachys siamensis* Gamble | Outgroup | S14 | NBG, Howrah | 1/2/156781 |

The subgeneric assignments follow the classification of Li and Stapleton (2006)

[a] No vouchers deposited

Natural Resources, Bangor University, for DNA extraction and analysis. Six of the *Dendrocalamus* taxa (*D. strictus*, *D. hamiltonii*, *D. membranaceus*, *D. brandisii*, *D. sikkimensis* and *D. asper*) represented subgenus *Dendrocalamus* and two other taxa (*D. giganteus* and *D. calostachyus*) represented subgenus *Sinocalamus*, in the infrageneric classification of *Dendrocalamus* by Li and Stapleton (2006). No information was available regarding the infrageneric assignment of *D. sahnii* and *D. somdevai*. The outgroup taxa were from the genera *Bambusa*, *Melocalamus*, *Oxytenanthera*, *Dinochloa* and *Thyrsostachys*, all of which belong, like *Dendrocalamus*, to subtribe Bambusinae as recognized by Ohrnberger (1999). For each collected taxon, except *Oxytenanthera abyssinica* and *Dendrocalamus brandisii*, a voucher specimen was prepared and deposited at the herbarium of the Forest Research Institute, Dehra Dun, India (DD) (Table 1).

## DNA extraction

DNA was extracted from 50 mg of dried leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) protocol of Doyle and Doyle (1990). Modifications of the protocol included an increased incubation period in the CTAB buffer (2 h), increased concentration of $\beta$-mercaptoethanol (1%) and two clean-up cycles with chloroform and isoamyl alcohol. The tissue was homogenized mechanically in a tissue lyser (Roche). About 700 µl pre-warmed CTAB buffer [2% CTAB, 1.4 M NaCl, 20 mM ethylenediamine tetraacetic acid (EDTA), 100 mM Tris, 1% polyvinylpyrrolidone (PVP) and 1% $\beta$-mercaptoethanol] was added to the homogenate and incubated at 65°C for 2 h. Immediately after incubation, about 700 µl chloroform:isoamyl alcohol (24:1) was added to the incubated sample and thoroughly mixed by inverting the sample tube. Following a centrifugation step, the supernatant was transferred to a fresh tube. DNA present in the supernatant was precipitated by adding ice-cold isopropanol (0.4 × volume) and 5 M NaCl (0.5 × volume). The DNA pellet was washed with 70% ethanol and resuspended in Tris-EDTA (TE). To degrade RNA, the enzyme RNAseA was added to the suspended DNA and incubated at 37°C for 1 h. A second cycle of clean-up and DNA precipitation, as described earlier, was repeated. In the final step, the DNA pellet was washed with 70% ethanol, dried and resuspended in TE. The quality of extraction was checked by running a 1% agarose mini-gel (run at 50 V for 30 min) in Tris–borate–EDTA (TBE buffer, 1×) containing 0.5 µg/ml ethidium bromide and visualizing under a ultraviolet light source. Genomic DNA concentration was determined in a Fluostar Galaxy Fluorometer using the fluorescent dye Pico green.

## Generation of AFLP markers

The AFLP assay was performed following the protocol of Vos et al. (1995), adapted for the Beckman Coulter Sequencer. The process was carried out in four steps. In the first step, 250 ng genomic DNA was double-digested in a final volume of 40 µl at 37°C for 3 h with *Eco*RI (5 U) and *Mse*I (*Tru*9I) (5 U). In the second step, a ligation reaction was set up by adding 10 µl of a master mix (containing *Eco*RI adapter, *Mse*I adapter, T4 DNA ligase and ligase

buffer) to the products of earlier digestion reaction. The mixture was incubated overnight at 16°C. In the third step, a pre-selective polymerase chain reaction (PCR) was performed with a final volume of 20 µl, containing 5 µl restriction-ligation reaction, 10 µl Reddy mix (Abgene) and 5 µl universal primer mix (E00—30 ng and M00—30 ng). The thermocycler conditions for the pre-selective PCR included 30 cycles consisting of 30 s denaturation at 94°C, 60 s annealing at 56°C, 60 s extension at 72°C and 600 s extension at 72°C. In the fourth step, a selective PCR was performed with a final volume of 20 µl, containing 5 µl pre-selective products, 10 µl Reddy mix (Abgene) and 5 µl selective primer mix (E00 + 3—25 ng and M00 + 3—30 ng). The selective primer E00 + 3 was end-labelled with a fluorescent dye instead of the radioactive labelling described in the original protocol of Vos et al. (1995). The thermocycler conditions for the selective PCR included 13 touchdown cycles to avoid amplifying non-specific sequences (30 s denaturation at 94°C, 30 s annealing at 65°C which was then reduced by 0.7°C per cycle, 60 s extension at 72°C), 23 normal cycles (30 s denaturation at 94°C, 30 s annealing at 56°C, 60 s extension at 72°C) and 420 s final extension at 72°C.

A primer screening experiment was set up with two samples and 20 primer pairs (four *Eco*RI primers and five *Mse*I primers) to screen primer pairs having ability to generate most polymorphic loci. A locus was treated as polymorphic when a peak was detected in one sample but not in the other. Detection of peaks at the same locus in both samples made the locus monomorphic. Five primer pairs generating most polymorphic loci were short-listed and used in amplifying AFLP markers from all 15 operational taxonomic units (OTUs) used in the present investigation. The primer sequences are provided in Table 2. A negative control without template DNA was run in each batch of PCRs to confirm that no contamination had occurred. The reproducibility of AFLP peaks was checked by repeating the whole process a number of times.

## Separation and scoring of AFLP markers

The selective PCR products were separated through capillary gel electrophoresis in the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.) and analysed with the fragment analysis software. During fragment analysis the separated fragments were sized with the use of internal size standards (PA400). Following this, the sized fragments were subjected to an AFLP binning analysis that converted the AFLP peak profiles into binary matrix. The presence of a peak was scored as 1, and its absence was scored as 0. Peaks of size ranging from 60 to 400 bp were scored.

## Data analysis

The AFLP dataset was analyzed both phenetically and cladistically. For phenetic analysis, the binary matrix ($470 \times 15$) of multilocus peak patterns was converted to a matrix of pairwise distances between OTUs, expressed as Jaccard's (1908) distance coefficient using the software package NTSYSpc 2.11X (Rohlf 2000). Cluster analysis was carried out using the neighbour-joining (NJ) algorithm of Saitou and Nei (Saitou and Nei 1987) in the same software package using Jaccard's distance matrix as the input file. The generated phenogram was rooted using the outgroup option, taking *Oxytenanthera abyssinica* as the outgroup taxon. Statistical support for the internal branches was assessed by performing a bootstrap analysis with 1,000 replicates in the software package FREETREE (Pavlicek et al. 1999). A Mantel test was performed to test how well the phenogram represented the inter-OTU distances, following the procedure described by Koopman et al. (2001). Initially, the NJ tree was transformed to a tree-distance matrix using the COPH module in NTSYSpc 2.11X (Rohlf 2000), then the MXCOMP module was used to calculate the co-phenetic correlation coefficient ($r$), which indicated the goodness of fit of the cluster analysis to the distance matrix.

Cladistic analysis of the AFLP dataset was performed under maximum-parsimony criterion with PAUP 4.0b10 (Swofford 2002). The large number of included taxa (>12) ruled out an exhaustive search. So, heuristic search was used to identify the most parsimonious tree. Heuristic search was performed with the following criteria: 1,000 replicates, random additions of sequence, tree-bisection-reconnection (TBR) branch swapping, character optimizations using accelerated transformation (Perrie and Brownsey 2005). Output trees were rooted using the outgroup option with *Oxytenanthera abyssinica* as the outgroup taxon.

**Table 2** Primer sequences of selective primers used in the present investigation to generate AFLP markers in the 15 OTUs from subtribe Bambusinae

| Primer pair | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| E33_M47 | 5′ GACTGCGTACCAATTC **AAG** 3′ | 5′ GATGAGTCCTGAGTAA **CAA** 3′ |
| E35_M47 | 5′ GACTGCGTACCAATTC **ACA** 3′ | 5′ GATGAGTCCTGAGTAA **CAA** 3′ |
| E32_M47 | 5′ GACTGCGTACCAATTC **AAC** 3′ | 5′ GATGAGTCCTGAGTAA **CAA** 3′ |
| E33_M59 | 5′ GACTGCGTACCAATTC **AAG** 3′ | 5′ GATGAGTCCTGAGTAA **CTA** 3′ |
| E33_M60 | 5′ GACTGCGTACCAATTC **AAG** 3′ | 5′ GATGAGTCCTGAGTAA **CTC** 3′ |

Bold-type letters indicate +3 nucleotide extensions

Statistical support for internal branches was assessed using the bootstrap analysis (Felsenstein 1985) in PAUP 4.0b10 with the following criteria: 1,000 replicates, heuristic search and a 50% confidence level.

## Results

The five primer pairs used in the present investigation generated 609 AFLP marker loci, out of which 99.2% (604) loci were polymorphic and only 0.8% (5) loci were monomorphic across the 15 OTUs. The dataset also contained 134 (22.0%) loci where only one peak was detected. These loci are uninformative as far as relationships among taxa are concerned and were excluded from subsequent analysis. The number of AFLP marker loci generated by the individual primer sets varied from 101 to 133 with a mean of 121.8 (Table 3).

The genetic distance estimates based on Jaccard's measure varied from 0.47 to 0.92 (Table 4). Although referred to the Bambusinae, the monotypic African bamboo *Oxytenanthera abyssinica* shared very few peaks with other taxa included in the study and was found genetically distant from them (distance ranged from 0.77 to 0.92). However, even within *Dendrocalamus* there was wide genetic variation. *Dendrocalamus strictus* appeared isolated with a minimum distance of 0.77 (from *D. somdevai*) and a maximum distance of 0.85 (from *D. asper*). Among the outgroups included in the present investigation, *Bambusa balcooa* was found to be the closest to *Dendrocalamus* sensu lato with a mean distance of 0.60.

Cluster analysis with the NJ algorithm resulted in a single tree (Fig. 1) with high co-phenetic correlation coefficient ($r = 0.971$). A well-supported (85% bootstrap support) major cluster was recovered, containing all *Dendrocalamus* taxa (except *D. strictus*) with *Melocalamus compactiflorus*

**Table 3** Summary statistics of AFLP markers obtained from 15 OTUs belonging to subtribe Bambusinae, with five selective primer pairs

| Primer pair | Number of monomorphic loci | Number of polymorphic loci | Total |
|---|---|---|---|
| E32_M47 | 2 (1.5) | 131 (98.5) | 133 |
| E33_M47 | 0 (0.0) | 133 (100.0) | 133 |
| E33_M59 | 1 (0.9) | 100 (99.1) | 101 |
| E33_M60 | 0 (0.0) | 129 (100.0) | 129 |
| E35_M47 | 2 (1.8) | 111 (98.2) | 113 |
| Total | 5 (0.8) | 604 (99.2) | 609 |
| Mean | 1.0 | 120.8 | 121.8 |

Values in parenthesis indicate percentage of total loci

**Table 4** Half-matrix of pairwise Jaccard's distance between 15 OTUs from subtribe Bambusinae

| Operational taxonomic units | S3 | S4 | S9 | S10 | S11 | S15 | S30 | S13 | S14 | S23 | S27 | S52 | S6 | 31 | S32 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. membranaceus* (S3) | 0 | | | | | | | | | | | | | | |
| *D. somdevai* (S4) | 0.51 | 0 | | | | | | | | | | | | | |
| *D. brandisii* (S9) | 0.55 | 0.55 | 0 | | | | | | | | | | | | |
| *D. giganteus* (S10) | 0.66 | 0.65 | 0.60 | 0 | | | | | | | | | | | |
| *D. sikkimensis* (S11) | 0.68 | 0.59 | 0.59 | 0.64 | 0 | | | | | | | | | | |
| *D. sahnii* (S15) | 0.61 | 0.56 | 0.57 | 0.58 | 0.56 | 0 | | | | | | | | | |
| *D. calostachyus* (S30) | 0.63 | 0.60 | 0.53 | 0.57 | 0.57 | 0.55 | 0 | | | | | | | | |
| *Dinochloa* (S13) | 0.66 | 0.63 | 0.61 | 0.69 | 0.64 | 0.62 | 0.58 | 0 | | | | | | | |
| *Thyrsostachys* (S14) | 0.69 | 0.68 | 0.70 | 0.75 | 0.74 | 0.73 | 0.71 | 0.73 | 0 | | | | | | |
| *Bambusa* (S23) | 0.65 | 0.59 | 0.57 | 0.61 | 0.51 | 0.54 | 0.54 | 0.63 | 0.74 | 0 | | | | | |
| *Melocalamus* (S27) | 0.69 | 0.67 | 0.68 | 0.66 | 0.67 | 0.63 | 0.63 | 0.70 | 0.73 | 0.66 | 0 | | | | |
| *D. strictus* (S52) | 0.79 | 0.77 | 0.84 | 0.84 | 0.85 | 0.84 | 0.82 | 0.82 | 0.83 | 0.87 | 0.85 | 0 | | | |
| *D. asper* (S6) | 0.70 | 0.69 | 0.59 | 0.62 | 0.65 | 0.64 | 0.63 | 0.73 | 0.76 | 0.66 | 0.71 | 0.85 | 0 | | |
| *D. hamiltonii* (31) | 0.65 | 0.54 | 0.58 | 0.62 | 0.52 | 0.53 | 0.55 | 0.66 | 0.72 | 0.47 | 0.68 | 0.83 | 0.60 | 0 | |
| *Oxytenanthera* (S32) | 0.83 | 0.83 | 0.82 | 0.83 | 0.82 | 0.81 | 0.80 | 0.84 | 0.84 | 0.83 | 0.83 | 0.92 | 0.82 | 0.77 | 0 |

Rows are labelled with taxon names and columns are labelled with OTU codes only

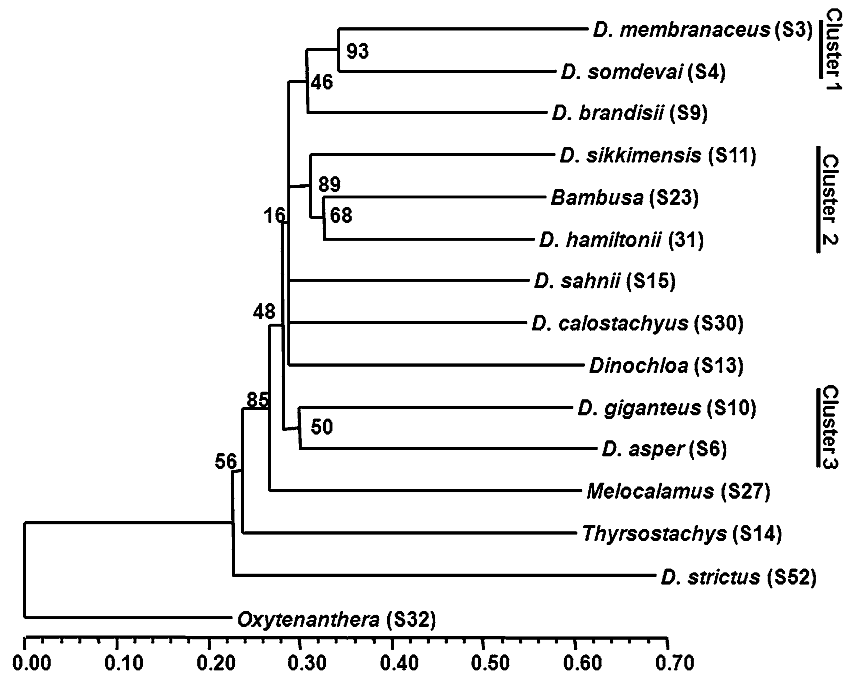


**Fig. 1** Neighbour-joining phenogram depicting phenetic relationships among 15 OTUs sampled from subtribe Bambusinae. *Numbers* at the nodes indicate bootstrap (%) support for the respective clusters. OTU codes are indicated within parentheses. *Scale bar* indicates additive distance between pairs of taxa

as sister lineage. *D. strictus* was recovered near the root of the tree, distant from the cluster containing other *Dendrocalamus*. Three clusters that had varying degree of bootstrap support (≥50%) could be recognized within the major cluster: cluster 1: *Dendrocalamus membranaceus* and *D. somdevai* (93% bootstrap support); cluster 2: *Dendrocalamus sikkimensis*, *Bambusa balcooa* and *D. hamiltonii* (89% bootstrap support); cluster 3: *D. giganteus* and *D. asper* (50% bootstrap support). However, the relative relationship among these three clusters was not clear due to polytomy. Furthermore, the affinity of *D. sahnii*, *D. calostachyus* and *Dinochloa macclellandii* remained unresolved. Also, the affinity of *D. brandisii* to cluster 1 was unreliable owing to poor bootstrap support (46%).

Maximum-parsimony analysis of the AFLP dataset (consisting of 470 characters, of which 460 were parsimony informative) with heuristic search yielded two equally parsimonious trees (Fig. 2a, b), giving two alternate but equally likely hypotheses of evolutionary relationships among the OTUs. Each tree was 1,468 steps (character state changes) long with consistency index (CI) = 0.320, retention index (RI) = 0.298 and rescaled consistency index (RC) = 0.095. These most parsimonious trees were congruent for most part of the trees but differed in the placement of *Dendrocalamus calostachyus*. Strict consensus of the most parsimonious trees resulted in a polytomy (Fig. 3). Three groups were recovered that were identical to the clusters in NJ tree. Because of polytomy, the relationship among these three groups was not clear. Bootstrap analysis with 1,000 bootstrap replicates showed varying degree of support for these groups.

## Discussion

The ability of AFLP markers to detect polymorphisms rapidly without prior knowledge of the target genome is an advantage that has been widely exploited in plants in a range of applications including genetic identification through fingerprinting, quantitative trait loci mapping, population genetics and systematics (Mueller and Wolfenbarger 1999). The use of AFLPs in the present investigation is justified on the grounds that woody bamboos have evolved recently and have undergone little molecular evolution, as a result of which sequencing studies (nuclear gene-internal transcribed spacer) in other genera have found very little sequence variation among closely related taxa (Hodkinson et al. 2000). Similarly, sequencing of plastid gene (*ndhF*) has not been successful below tribal level (Clark 1997; Zhang and Clark 2000). The AFLP assay was successful in detecting polymorphism across the 15 OTUs included in the present investigation. A mean number of 120.8 polymorphic loci were detected using fluorescence-labelled primers in combination with gel analysis in an automated DNA sequencer, a number comparable to finding in earlier study of bamboos (Hodkinson et al. 2000).

The two different approaches adopted for data analysis in the present investigation, viz. NJ cluster analysis and maximum-parsimony analysis, yielded partially resolved trees. Even though the basal lineages were well resolved, there was internal polytomy in both trees. Three identical monophyletic internal groups were recovered; however, the degree of statistical support for these groups differed in

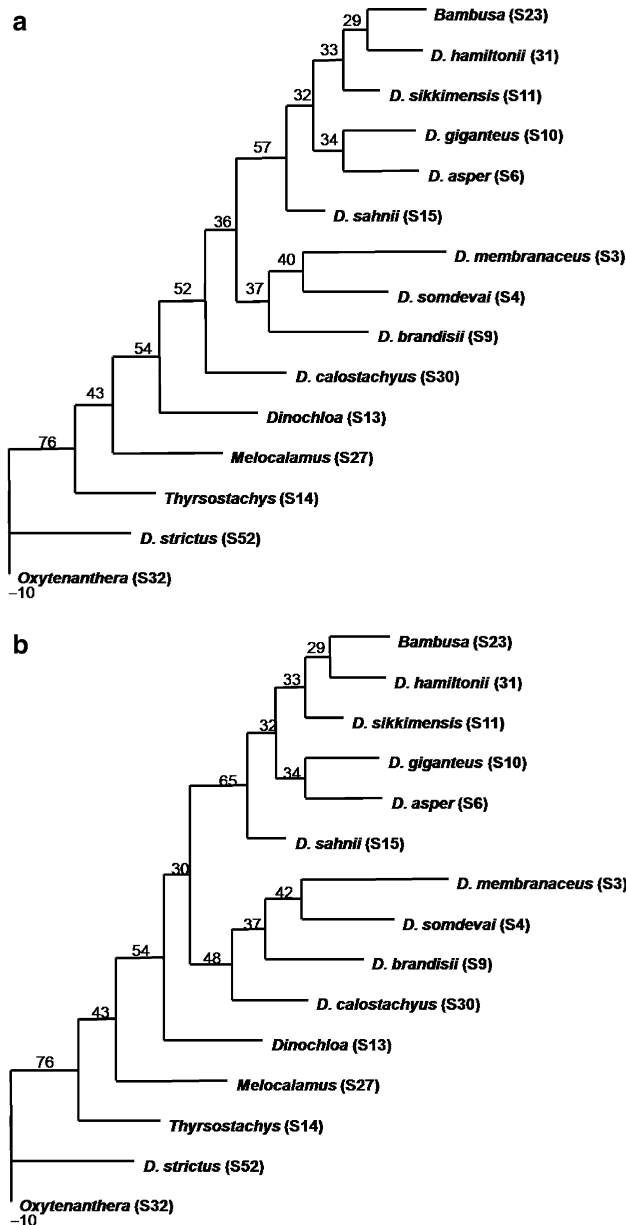


**Fig. 2** Phylograms depicting two equally parsimonious trees resulting from maximum-parsimony analysis of a character matrix of 470 AFLP markers. Each tree has a length of 1,468 steps, CI = 0.320, RI = 0.298, RC = 0.095. *Values above segments* indicate character state changes (gains/losses of AFLP bands) supporting respective nodes. OTU codes are indicated within parentheses. *Horizontal bars* below trees represent 10 character state changes

both analyses. Only one group containing *Dendrocalamus membranaceus* and *D. somdevai* had high bootstrap support (93% and 82% in NJ and parsimony analysis, respectively) in both analyses. The affinity of *Dendrocalamus sahnii*, *D. calostachyus* and *D. brandisii* were inconclusive in both analyses. Similarly, the affinity of *Dendrocalamus sikkimensis* to the group containing *Bambusa balcooa* and *Dendrocalamus hamiltonii* was not

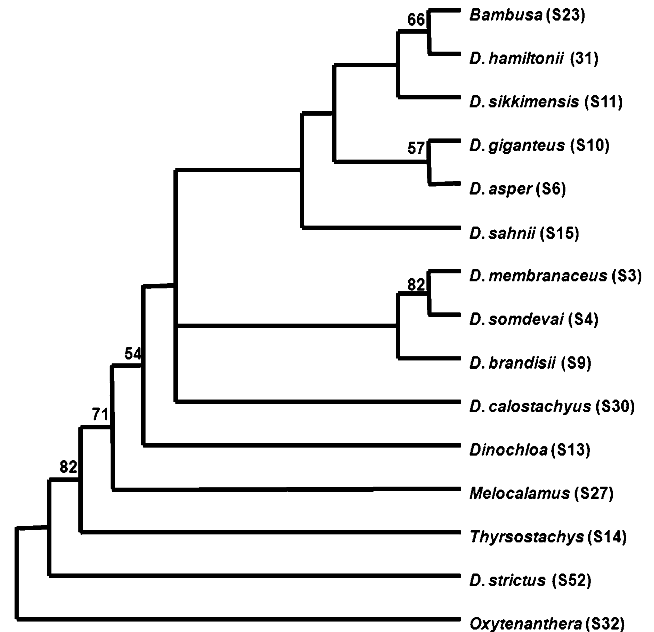


**Fig. 3** Cladogram depicting strict consensus of the two parsimonious trees obtained in maximum-parsimony analysis of a character matrix of 470 AFLP markers. Length = 1,480 steps (character state changes), CI = 0.317, RI = 0.289, RC = 0.092. *Values above segments* indicate bootstrap support for the respective nodes. Bootstrap support for nodes with <50% support and which collapse under the 50% majority rule tree is not shown. OTU codes are indicated within parentheses

supported in the maximum-parsimony analysis, though it was well supported (89% bootstrap support) in the NJ analysis. Koopman et al. (2008) argue that, as the AFLP markers are randomly sampled from across different parts of the genome which may have different evolutionary history and hence may contain conflicting signals, phylogenetic analysis will result in low support values for those parts of the tree containing the species involved. Nevertheless, even the poorly supported parts of the phylogenetic tree showed congruence with that of the NJ phenogram. The three monophyletic groups did not agree with any of the sectional assignments within *Dendrocalamus* sensu lato circumscribed by Hsueh and Li (1988), Ohrnberger (1999) and Li and Stapleton (2006).

Neither the phenetic nor the phylogenetic analysis adopted in the present investigation supported monophyly of the genus *Dendrocalamus* as circumscribed by Hsueh and Li (1988), Ohrnberger (1999) and Li and Stapleton (2006), based on morphological data. The placement of *Bambusa balcooa* and *Dendrocalamus strictus* suggested otherwise. *Bambusa balcooa*, instead of forming a separate lineage, was recovered in cluster 2 shared with *Dendrocalamus hamiltonii* and *D. sikkimensis* (Fig. 1). This supports the findings of Stapleton (1994), who had reported the closeness of *Bambusa balcooa* to *Dendrocalamus* species

on morphological grounds, stressing similarity in the profuse aerial roots at the culm nodes, the large rhizomatous branch bases and the culm wax. Further similarities between *Bambusa balcooa* and *Dendrocalamus hamiltonii* can be found in the reproductive parts, with both species having three stigmas each. The placement of *Dendrocalamus strictus* near the root of the tree away from rest of the *Dendrocalamus* sensu lato was not entirely unexpected considering the findings of Chou and Hwang (1985), who had reported the isolation of *D. strictus* from other *Dendrocalamus* taxa based on studies involving isozymes and phenolics.

Morphologically, the isolation of *D. strictus* could be explained by presence of inflorescence comprising fascicular pseudospikelets (2.2–2.5 cm in diameter) on each node of flowering branches, distinguishing them from other species included in the study (Yang et al. 2008). Ecologically also, *D. strictus* is very distinct from the other *Dendrocalamus* taxa included in the present investigation. *Dendrocalamus strictus* naturally occurs in dry deciduous open forests, receiving as little as 750 mm annual rainfall and exposed to low relative humidity. The other *Dendrocalamus* taxa included in the present investigation are confined to moister areas (moist deciduous to wet evergreen forests), with annual rainfall in excess of 1,500 mm.

Bambusinae as circumscribed by Ohrnberger (1999) is an Old World tropical subtribe with its centre of diversity in southeast Asia. It contains 17 genera, the relationships among which are not fully understood. In the present investigation *Bambusa balcooa* was placed within *Dendrocalamus* sensu lato, supporting the closeness, or even inseparability, of these two genera. *Melocalamus* and *Thyrsostachys* were recovered as sister lineages to *Dendrocalamus* and *Bambusa*. Watanabe et al. (1994), the first to study phylogenetic relationships among Asian bamboos using restriction fragment length polymorphism of chloroplast DNA, recovered a clade representing subtribe Bambusinae *sensu* Ohrnberger (1999), containing *Bambusa*, *Dendrocalamus*, *Gigantochloa* and *Thyrsostachys*. Internally, however, Watanabe's clade was poorly resolved in terms of relationships among *Bambusa*, *Gigantochloa* and *Dendrocalamus*, suggesting close relationships among these genera. *Thyrsostachys* had emerged as a sister lineage to the other genera included in Watanabe's study. The study of Loh et al. (2000) and Ramanayake et al. (2007), using AFLPs and random amplified polymorphic DNAs (RAPDs), respectively, also indicated a close relationship between *Bambusa* and *Gigantochloa*. The combined evidence from these earlier molecular studies and the present investigation suggests that taxa belonging to *Bambusa*, *Dendrocalamus*, *Dinochloa* and *Gigantochloa* form a close complex but are relatively distant from *Melocalamus*, *Thyrsostachys* and *Oxytenanthera*.

## Conclusions

The phenetic and phylogenetic trees generated in the present study are plausible hypotheses pertaining to relationships within *Dendrocalamus*. The low statistical support for two of the three identified monophyletic groups might improve with inclusion of more informative characters, which could be generated by using more selective primer sets. The study confirms that the current taxonomic treatment of *Dendrocalamus* is unsatisfactory and needs revision. A broader study encompassing a wider selection of taxa from *Bambusa*, *Dendrocalamus*, *Dinochloa* and *Gigantochloa*, and inclusion of evidence from multiple data sources (including AFLP and sequencing of fast-evolving genes) might be expected to produce a robust phylogenetic tree for this suite of closely related taxa.

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