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Exploring Phylogenetic Relationships Within the Subgenera of *Bambusa* Based on DNA Barcodes and Morphological Characteristics

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Abstract

The genus *Bambusa* belongs to the subtribe Bambusinae and the subfamily Bambusoideae. The subgenera of *Bambusa* have not been satisfactorily circumscribed, and this remains a major taxonomic problem. Meanwhile, genera such as *Dendrocalamus* and *Gigantochloa* have not been confidently assigned to *Bambusa*. Here, the phylogenetic relationships among subgenera were investigated using five chloroplast deoxyribose nucleic acid (DNA) markers (rpl32-trnL, rpl16, matK, rbcL, and trnH-psbA) for a sample of 50 ingroup species and 16 outgroup species. A total of 186 key morphological descriptors were studied for the 50 ingroup species. The results indicated that rpl 32-trnL and trnH-psbA were more suitable for the identification of *Bambusa* than the other markers. Using these markers, it was possible to distinguish *Bambusa* species from other species and to divide them into several clusters. Phylogenetic analyses conducted using morphological descriptors and a combined marker (rpl32-trnL+rpl16) revealed three and four distinct lineages among the members of the currently recognized *Bambusa*, respectively. The branching pattern of the dendrogram was not completely consistent with the classical taxonomic classification of *Bambusa*. In addition, not all varieties and cultivars were clustered with McClure. As the maximum parsimony topology and morphological analyses were not consistent, some clustering results overlapped. Overall, the results obtained here did not support the current classification of the *Bambusa* subgenera.

Introduction

The genus *Bambusa*, belonging to the subtribe Bambusinae and the subfamily Bambusoideae, is one of the largest genera of woody bamboos, containing over 100 species (Ohrnberger, 1999). The classical botanical classification of bamboo species is based on the morphological characteristics of their culms, branches, and sheaths, because of the rarity at which they blossom. The Flora of China (FOC, Xia *et al*., 2007), the newest authoritative botany book in China, divides *Bambusa* into four subgenera based on the morphologic characteristics of the culm, branch, and sheath: *Bambusa*, *Leleba*, *Lingnania*, and *Dendrocalamopsis*. The subgenus *Dendrocalamopsis* was not included in the former versions of the Flora Reipublicae Popularis Sinicae (FRPS, Jia *et al*., 1996). The FOC has been updated to reflect many new results in bamboo research; however, due to the lack of flowering materials, the definitions of some species are still controversial. Similar to *Bambusa oldhamii*, some studies still use the former name *Dendrocalamopsis oldhamii* (Cao *et al*., 2014).

The distinctive life pattern, infrequent flowering, and predominance of asexual reproduction make bamboo a taxonomically difficult group to classify (Zhang *et al*., 2011). Therefore, there have been many misnamed species. For example, the *Bambusa* species *Bambusa chungii*, *Bambusa guangxiensis*, and *Bambusa cerosissima* have been misnamed *Lingnania chungii*, *Lingnania funghomii*, and *Lingnania cerosissima*, respectively (Xia *et al*., 2007). With the addition of flowering materials, Chinese species were hitherto placed in the *Sasa* subgenus *Sasa*. Qin *et al*. (2021) strongly argued that the monophyly of the Chinese representatives of the *Sasa* subgenus *Sasa* should be a new genus, *Sinosasa*.

Both morphological and molecular systematics have been utilized to solve the problems of bamboo classification. Among the different approaches used in molecular systematics, DNA sequencing has become one of the most widely used methods applied to bamboo (Sungkaew *et al*., 2009; Triplett *et al*., 2010), especially at the genus level (Hilu and Liang, 1997; Kelchner, 2013). Recently, substantial progress has been made towards understanding the evolutionary relationships of *Bambusa* and its allies (*Bambusa*, *Dendrocalamus*, *Gigantochloa*, and *Melocalamus* are classified as a close group, based mainly on their shared characteristics of a solid, thickened, and hairy ovary summit in particular) using molecular data (Yang *et al*., 2010). Yang *et al*. (2010) used nuclear gene (GBSSI) and plastid DNA sequences (psbA-trnH, rpl32-trnL, and rps16), which allowed *Bambusa* and *Dendrocalamopsis* to be formed into one of two clades with reasonable support. Through this approach, 17 *Bambusa* samples were classified into three clades, and this result supported the present subgeneric classification of *Bambusa*. However, other studies have not supported this classification. The phylogeny of bamboo species has also been analyzed using only internal transcribed spacer (ITS) sequences. In this group, each branch was composed of several species of three subgenera (not including the subgenus *Dendrocalamopsis*), and the *Bambusa* and *Dendrocalamus* species formed a group with a bootstrap value of 100 (Sun *et al*., 2005). Goh *et al*. (2010, 2013) used chloroplast DNA markers (rps16-trnQ, trnC-rpoB, and trnD-T) and a nuclear DNA marker (GBSSI) to classify *Bambusa*, *Dendrocalamus*, *Giganto*, and *Chloa* as distinct lineages. This approach identified four *Bambusa* subgenera, which differed from the subgeneric classification. Chloroplast DNA sequences have been extensively used to infer plant phylogeny for uniparental inheritance through comparison with nuclear DNA sequences (Ma *et al*., 2014).

Several DNA markers have been used as core plant barcodes, such as the plastid (chloroplast) markers rbcL, matK, and trnH-psbA. Nuclear ribosomal ITSs have also been used (Kress and Erickson, 2007). Statistical results revealed that these three plastid markers showed high levels of universality (87.1–92.7%) and that the combination of ITS and any of the plastid DNA markers was able to discriminate 69.9–79.1% of species (Li *et al*., 2011). Here, many DNA barcoding primers (trnL-trnF, trnS-trnG, psbB-psbF, rpl16, rpl32-trnL, rbcL, matK, trnH-psbA, and ITS) were used with the aim of amplifying the DNA sequences of bamboo samples. Unfortunately, the plastid DNA markers trnL-trnF, trnS-trnG, and psbB-psbF failed to amplify most specimens, as did the nuclear marker ITS.

In addition to DNA barcoding, researchers have attempted to arrange morphological characteristics into a data matrix, using cladistic analysis (Acevedo-Rosas *et al*., 2004). DAS *et al*. (2007) scored 32 key morphological descriptors for 15 bamboo species and standardized them as qualitative and quantitative interval data to construct a tree graph, using the unweighted pair-group method of arithmetic averages. Regarding other plants, Tilney *et al.* (2009) used morphological and anatomical characteristics as scoring feature matrices for the cladistic analysis of *Lichtensteinia* (Apiaceae). Based on morphological data, Kim *et al.* (2014) conducted principal component analysis and cluster analysis on native chrysanthemum in South Korea.

Here, the phylogenetic relationships among the four subgenera of *Bambusa* were investigated (50 samples) using DNA sequence data and morphological characteristics, employing a much larger taxon

sample than has been previously available. This included representatives from all subgenera of *Bambusa* that have previously been described. DNA sequence data were derived from the plastid markers.

Materials And Methods

Materials

A total of 66 taxa from *Bambusa* and some other bamboo species (Table 1, all Latin names were obtained from the FOC) representing ten genera were sampled for molecular phylogenetic analysis. There were 50 species from *Bambusa* belonging to the four subgenera described in the FOC (Xia *et al*., 2007), including *D. oldhamii* and *Neosinocalamus affinis*, which were not accepted as *Bambusa* in the FRPS, but were moved to *Bambusa* in 2007 (Xia *et al*., 2007); they were named *B. oldhamii* and *Bambusa emeiensis*, respectively. The outgroup taxa included *Dendrocalamus*, *Phyllostachys*, *Pseudosasa*, *Shibataea*, *Drepanostachyum*, *Indosasa*, *Melocanna*, *Oligostachyum*, and *Sasa*. Fifty taxa from *Bambusa* were collected and analyzed for morphological phylogeny.

Table 1
Sixty-six taxa from *Bambusa* and the outgroup.

Name of bamboo	Missing data	Name of bamboo	Missing data
Bambusa albolineata		*Bambusa pachinensis*	
Bambusa arundinacea		*Bambusa pachinensis var. hirsutissima*	
Bambusa blumeana		*Bambusa pervariabilis*	
Bambusa boniopsis		*Bambusa prominens*	
Bambusa cerosissima		*Bambusa sinospinosa*	
Bambusa chungii		*Bambusa surrecta*	
Bambusa chungii vat. velutina		*Bambusa teres*	
Bambusa cornigera		*Bambusa textilis*	
Bambusa contracta		*Bambusa textilis cv. Gracilis*	
Bambusa corniculata		*Bambusa textilis cv. Purpurascens*	
Bambusa distegia		*Bambusa tuldoides*	
Bambusa dolichoclada	trnH-psbA	*Bambusa tuldoides cv. Swolleninternode*	
Bambusa duriuscula	rpl16	*Bambusa ventricosa cv. Nana*	
Bambusa emeiensis/N. affinis		*Bambusa vulgaris*	
Bambusa eutuldoides		*Bambusa vulgaris cv. Vittata*	
Bambusa eutuldoides var. basistriata		*Bambusa vulgaris cv. Wamin*	
Bambusa eutuldoides var. viridi-vittata		*Bambusa xiashanensis*	
Bambusa flexuosa		*Dendrocalamus membranaceus*	*
Bambusa gibba		*Dendrocalamus minor*	* rpl16
Bambusa gibboides	rpl32-trnL, rpl16	*Dendrocalamus minor var. amoenus*	*
Bambusa indigena		*Drepanostachyum scandens*	*
Bambusa lenta		*Indosasa shibataeoides*	* matK
Bambusa longispiculata		*Melocanna baccifera*	*

Name of bamboo	Missing data	Name of bamboo	Missing data
Bambusa macrotis		*Neosinocalamus affinis cv. Viridiflavus*	* trnH-psbA, rpl16
Bambusa multiplex		*Oligostachyum lubricum*	* rbcL
Bambusa multiplex cv. Alphonse-Karr		*Phyllostachys heteroclada*	* matK
Bambusa multiplex cv. Fernleaf		*Phyllostachys heterocycla*	*
Bambusa multiplex cv. Silverstripe		*Phyllostachys praecox*	*
Bambusa multiplex cv. Stripestem Fernleaf		*Pseudosasa amabilis*	* rbcL
Bambusa multiplex var. riviereorum		*Pseudosasa japonica var. Tsutsumiana*	*
Bambusa multiplex var. shimadae		*Sasa auricoma*	*
Bambusa mutabilis		*Shibataea chinensis cv. Aureo-striata*	* trnH-psbA
Bambusa oldhamii / D. oldhamii	*	*Sinobambusa tootsik f. luteolo-albo-striata*	*

Marker name in the missing data column indicates that there was an amplification or sequencing failure; * indicates missing morphological character data.

Dna Isolation, Amplification, Cloning, And Sequencing

Leaves were collected from the Hua'an Bamboo Garden (Fujian Province, China) and Lin'an Taihu Lake Source Bamboo Garden (Zhejiang Province, China). Total DNA was extracted from silica-gel-dried young leaves, using a modification of the method described by Fulton *et al*. (1995). Polymerase chain reaction (PCR) amplification, cloning, and the sequencing of rpl16 were performed according to the forward primer (Cornelia *et al*., 2007) and reverse primer (Downie *et al*., 2000), following the protocol of Cornelia et al. (2007). For rpl32-trnL, the primers rpl32-F and trnL were used, following the protocol of Shaw *et al*. (2007); for rbcL, the primers rbcL-1F and rbcL-724R were used, following the protocol of Fay *et al*. (1997); for matK, the primers matK-ML and matK-MU were used, following the protocol of Zhu et al. (2015); and for the psbA-trnH region, the primers psbA (Tate, 2002) and trnH2 (Sang *et al*., 1997) were used, in accordance with the protocol of Tate and Simpson (2003). All the primer sequences are shown in Table 2.

Table 2
Sequences of the five primers used in this study.

MARK	Prime-F	Prime-R
rpl32-trnL	CTGCTTCCTAAGAGCAGCGT	CAGTTCCAAAAAAACGTACTTC
rpl16	CTATGCTTAGTGTGTGACTC	TCTTCCTCTATGTTGTTTACG
matK	AAACAGAAATCTCGTCAA	AGGGTTCACCAGGTCATT
rbcL	ATGTCACCACAAACAGAGACTAAAGC	TCGCATGTACCTGCAGTAGC
trnH-psbA	CGCGCATGGTGGATTCACAATCC	GTTATGCATGAACGTAATGCTC

PCR was conducted using the TaKaRa Ex™ kit (Takara Biomedical Technology Co., Ltd., Beijing, China) with the following program settings: 5 min at 95.0 ℃; 35 cycles of 30 s at 95.0 ℃, 30 s at annealing temperature, 40 s at 72.0 ℃; 7 min at 72.0 ℃; and then holding at 4.0 ℃. The annealing temperatures used here were 51.0–56.0 ℃. The PCR reaction mixture contained 10 ng of DNA samples, 0.5 μL (10 μM) each of forward and reverse primers, 0.5 μL of deoxyribonucleotide triphosphate (dNTP), 2.5 μL of 10× buffer, and 0.5 μL of deoxyribonuclease (DNase); double distilled water (ddH_2O) was added to make the volume up to 25 μL. PCR products were puri□ed using Promega Wizard® PCR Clean-up System kits (Promega Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions. DNA sequencing was performed commercially by Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China).

Dna Sequence Alignment And Phylogenetic Analyses

DNA sequences were edited with CHROMAS v2.6.5 and aligned by MUSCLE (embedded in MEGAX), with default parameters. They were adjusted manually where necessary. All sequence data were uploaded to the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA706162&o=acc_s%3Aa). Maximum parsimony (MP) analysis was conducted based on the separate rpl32-trnL, rpl16, matK, rbcL, and trnH-psbA datasets and with a combined rpl32-trnL+rpl16 dataset.

MP analysis was performed with MEGAX (https://www.megasoftware.net/); all characteristics were equally weighted, and gaps were coded as missing data. Heuristic searches of 1,000 random addition replicates were conducted using subtree-pruning-regrafting (SPR) branch swapping. This was done to obtain the most parsimonious trees, and ten trees from each random sequence were saved. Estimates of clade robustness were obtained through bootstrap values (BV) calculated from 1,000 replicate analyses, conducted using the heuristic search strategy and through a simple addition sequence of the taxa. The incongruence length difference (ILD) test of Farris *et al*. (1994) was used to evaluate the statistical significance of character incongruence among the rpl32-trnL and rpl16 intron datasets before their combined analysis.

Morphological Characteristic Analyses

Based on the China Industry Standard Guidelines for the conduct of tests for distinctness, uniformity, and stability, 186 key morphological descriptors were used to assess *Bambusa* members. Morphological descriptors were scored as follows: each species was considered as a separate independent operational taxonomic unit (OTU). One hundred and eighty-six key morphological descriptors were used (one root descriptor about aerial root; 22 culm descriptors about powder ring, hair ring, surface cover, color, internode length, diameter, shape, and sheath-node bulge; nine branch descriptors about branch thorn, lowest branch height, and leaf number; six leaf descriptors for length, hair, and base shape; 18 culm descriptors for sheaths about surface cover, hair ring, brim hair, length and color streak; 50 descriptors for sheath auricles about length, the length ratio value of the two auricles, corrugated fold, shape, oral setae length, root location, and extension condition; 54 sheath blade descriptors about shape, reflex, corrugation, hairy, color, tip shape, base length, and length; and 26 sheath ligule descriptors about length, shape, eyelash, and eyelash length). The specific morphological characteristics that were selected are listed in Table 3, which were assessed from each of the 50 OTUs (five replications per OTU) studied in the field. Mean values obtained from five independent replications were used as representative OTU data for each quantitative morphological descriptor. If the sample characteristics conformed to descriptors, they were marked as “0”; if not, they were marked as “1.” The scored qualitative and quantitative interval data were standardized to construct a dendrogram using neighbor-joining (NJ) performed via PowerMarker V3.25.

Table 3
Morphological descriptors.

Organ	characteristics	Comments
Root	Aerial root	
Culm (infancy)	With a ring of white powder below sheath scars	
	Covered with white powder	
	Covered with hairs	
	Covered with tomenta	
	Covered with setae	
	With a ring of tomenta below culm node	
	Color: green	
	Color: green with yellow in concave	
	Color: green and yellow	
	Color: yellow with green in concave	
	Color: yellow with few green streak	
	Color: colorful streak	
	Internodes 0-30 cm long at 2 m high	Include 30 cm
	Internodes 30-50 cm long at 2 m high	
	Internodes over 50 cm long at 2 m high	Include 50 cm
	Internodes terete	
	Sheath-node raised inconspicuously	
	Sheath-node raised slightly	
	Sheath-node raised obviously	
	0-3 cm in diam	Include 3 cm
	3-8 cm in diam	
	Over 8 cm in diam	Include 8 cm
Branch	With thorn	
	With soft thorns	
	With hard thorns	
	Branching from lowest nodes 0-1.3 m above ground	Include 1.3 cm

Organ	characteristics	Comments
	Branching from lowest nodes 1.3-2.6 m above ground	
	Branching from lowest nodes over 2.6 m above ground	Include 2.6 cm
	Ultimate branchlets with 0-5 leaves	
	Ultimate branchlets with 6-9 leaves	
	Ultimate branchlets with over 10 leaves	Include 10 leaves
Leaf	Length: 0-10 cm	Include 10 cm
	Length: 10-20 cm	
	Length: over 20 cm	Include 20 cm
	Abaxially hairy	
	Leaf base shape: wedgy	
	Leaf base shape: olivary	
Culm sheath	Covered with hair	
	Covered with white powder	
	Covered with with sport	
	with a ring of tomenta below sheath scars	
	Brim with hair	
	Length shorter than internodes	
	Length equal with internodes	
	Length over than internodes	
	colorful streak	
Sheath auricle	Length: inconspicuous	
	Length: below 1 cm long	
	Length: 1-3 cm long	
	Length: 3-5 cm long	
	Length: over 5 cm long	
	Two auricles equally	
	Two auricles length ratio value between 1.1-2	
	Two auricles length ratio value between 2-3	

Organ	characteristics	Comments
	Two auricles length ratio value between 3-4	
	Two auricles length ratio value over 4	
	Corrugated fold	
	Shape: half-circle	Only at 1.3 m above ground
	Shape: nearly cone	Only at 1.3 m above ground
	Shape: ovoid	
	Shape: long ovoid	
	Shape: threadiness	
	Oral setae length: 0-0.5 cm	
	Oral setae length: 0.5-1.4	
	Oral setae length: over 1.5 cm	
	Oral setae straight	
	Auricles root and sheath blades separatly	
	Auricles root and sheath blades linked	
	Auricles and sheath blades linked	
	No extension	
	Auricles shouter than half extension	
	Auricles longer than half extension	
Sheath blade	Shape: triangular	
	Shape: lanceolate	
	Erect (<90°)	
	Reflexed (ca. 90°)	
	Reflexed (>90°)	
	Straight without corrugation	
	Corrugation at 1/3 tip	
	Corrugation at 1/3-1/2 tip	
	Corrugation at 1/1-2/3 tip	

Organ	characteristics	Comments
	Corrugation at whole blade	
	Abaxially hairy	
	Ventral hairy	
	Color: green	
	Color: green and purple	
	Color: colorful	
	Color: deadgress	
	Tip shape: long	side over base less than 2
	Tip shape: lacuminate	side over base equal to ca. 2
	Tip shape: equilateral triangle	side over base equal to ca. 1
	Culm sheath top length / sheath blade base length = 1-1.4	
	Culm sheath top length / sheath blade base length = 1.5-2.4	
	Culm sheath top length / sheath blade base length = 2.5-3.4	
	Culm sheath top length / sheath blade base length = 3.5-4.4	
	Culm sheath top length / sheath blade base length > 4.5	
	Sheath clade length / culm sheath length < 1	
	Sheath clade length / culm sheath length = 1	
	Sheath clade length / culm sheath length > 1	
Sheath ligule	Length: 0-2 mm	
	Length: 2-4 mm	
	Length: over 4 mm	
	Entire brim with hair (eyelash)	
	With slender eyelash	
	With thick eyelash	
	Eyelash 0-4 mm long	Include 4 mm

Organ	characteristics	Comments
	Eyelash 4-8 mm long	
	Eyelash over 8 mm long	Include 8 mm
	Entire brim without hair	
	Shape: sunken	
	Shape: flat	
	Shape: bulge	

Characteristics of the culm sheath, sheath auricle, sheath blade, and sheath ligule were recorded at 1.3 and 2.0 m above ground, respectively, as two OTUs, except for the characteristics that have specific comments. Total characteristics comprised 186 OTUs.

Results

Phylogenetic analyses

In this study, all five markers, rpl32-trnL, rpl16, matK, rbcL, and trnH-psbA, were independently detected by MP. Based on the results of these five MP trees and supplemented by information on diversity (performed by DnaSPv5), a combined DNA barcoding (rpl32-trnL+rpl16) was used to perform phylogenetic analyses of *Bambusa*.

The nucleotide diversities (Pi) and haplotype diversities (Hd) of these five DNA barcodes indicated that rbcL and trnH-psbA were not suitable for identifying *Bambusa* because their Pi values were 0.00458 and 0.00406, respectively (i.e., much lower than those of rpl 32-trnL, rpl 16, and matK; see Table 4). The markers rpl32-trnL, rpl16, and matK appeared to be good barcoding candidates for the identification of *Bambusa* species, according to their diversity information. However, matK, rbcL, and trnH-psbA could separate *Bambusa* from other genera, but they grouped over 70% of the *Bambusa* species that were sampled into one cluster. In comparison, rpl32-trnL and rpl16 both divided *Bambusa* into several clusters.

Table 4
Diversity information of DNA sites based on 66 bamboo species.

MARK	Number of the sites	Nucleotide diversity, Pi	Haplotype diversity, Hd
rpl32-trnL	1109	0.07872	0.903
rpl16	1283	0.07573	0.771
matK	1611	0.06624	0.803
rbcL	698	0.00458	0.587
trnH-psbA	636	0.00406	0.581
rpl16 + rpl32-trnL	2392	0.08462	0.961

Table 5
The species list

No.	Latin name	Acquisition number
1	*Bambusa albolineata*	2017080801
2	*Bambusa arundinacea*	2017080802
3	*Bambusa blumeana*	2017080803
4	*Bambusa boniopsis*	2017080804
5	*Bambusa cerosissima*	2017080805
6	*Bambusa chungii*	2017080806
7	*Bambusa chungii vat. velutina*	2017080807
8	*Bambusa cornigera*	2017080808
9	*Bambusa contracta*	2017080809
10	*Bambusa corniculata*	2017080810
11	*Bambusa distegia*	2017080811
12	*Bambusa dolichoclada*	2017080812
13	*Bambusa duriuscula*	2017080813
14	*Bambusa emeiensis/N. affinis*	2017080814
15	*Bambusa eutuldoides*	2017080815
16	*Bambusa eutuldoides var.basistriata*	2017080816
17	*Bambusa eutuldoides var. viridi-vittata*	2017080817
18	*Bambusa flexuosa*	2017080818
19	*Bambusa gibba*	2017080819
20	*Bambusa gibboides*	2017080820
21	*Bambusa indigena*	2017080821
22	*Bambusa lenta*	2017080822
23	*Bambusa longispiculata*	2017080823
24	*Bambusa macrotis*	2017080824
25	*Bambusa multiplex*	2017080825

No.1-50 was collected by Zou Yueguo in Hua'an Bamboo Botanical Garden. No.51-66 was collected by Gu LiJian in Hangzhou Lin’an Taihuyuan Ornamental Bamboo Planting Garden

No.	Latin name	Acquisition number
26	*Bambusa multiplex cv. Alphonse-Karr*	2017080826
27	*Bambusa multiplex cv. Fernleaf*	2017080827
28	*Bambusa multiplex cv. Silverstripe*	2017080828
29	*Bambusa multiplex cv. Stripestem Fernleaf*	2017080829
30	*Bambusa multiplex var. riviereorum*	2017080830
31	*Bambusa multiplex var. shimadae*	2017080901
32	*Bambusa mutabilis*	2017080902
33	*Bambusa pachinensis*	2017080903
34	*Bambusa pachinensis var. hirsutissima*	2017080904
35	*Bambusa pervariabilis*	2017080905
36	*Bambusa prominens*	2017080906
37	*Bambusa sinospinosa*	2017080907
38	*Bambusa surrecta*	2017080908
39	*Bambusa teres*	2017080909
40	*Bambusa textilis*	2017080910
41	*Bambusa textilis cv. Gracilis*	2017080911
42	*Bambusa textilis cv. Purpurascens*	2017080912
43	*Bambusa tuldoides*	2017080913
44	*Bambusa tuldoides cv. Swolleninternode*	2017080914
45	*Bambusa ventricosa cv. Nana*	2017080915
46	*Bambusa vulgaris*	2017080916
47	*Bambusa vulgaris cv. Vittata*	2017080917
48	*Bambusa vulgaris cv. Wamin*	2017080918
49	*Bambusa xiashanensis*	2017080919
50	*Bambusa oldhamii / D. oldhamii*	2017080920
51	Dendrocalamus membranaceus	2018070201

No.1-50 was collected by Zou Yueguo in Hua'an Bamboo Botanical Garden. No.51-66 was collected by Gu LiJian in Hangzhou Lin’an Taihuyuan Ornamental Bamboo Planting Garden

No.	Latin name	Acquisition number
52	Dendrocalamus minor	2018070202
53	Dendrocalamus minor var. amoenus	2018070203
54	Drepanostachyum scandens	2018070204
55	Indosasa shibataeoides	2018070205
56	Melocanna baccifera	2018070206
57	Neosinocalamus affinis cv. Viridiflavus	2018070207
58	Oligostachyum lubricum	2018070208
59	Phyllostachys heteroclada	2018070209
60	Phyllostachys heterocycla	2018070210
61	Phyllostachys praecox	2018070211
62	Pseudosasa amabilis	2018070212
63	Pseudosasa japonica var. Tsutsumiana	2018070213
64	Sasa auricoma	2018070214
65	Shibataea chinensis cv. Aureo-striata	2018070215
66	Sinobambusa tootsik f. luteolo-albo-striata	2018070216
No.1-50 was collected by Zou Yueguo in Hua'an Bamboo Botanical Garden. No.51-66 was collected by Gu LiJian in Hangzhou Lin'an Taihuyuan Ornamental Bamboo Planting Garden		

The combined barcode (rpl 32-trnL+rpl 16) was also used to analyze the phylogeny of 66 taxes, after the ILD test. The P-value of ILD was 0.05 and the combined marker successfully divided bamboo into several clusters (as shown in Figure 1, left side). The tree length, consistency index (CI), and retention index (RI) of the MP analyses for rpl32-trnL+rpl16 were 2392, 0.79, and 0.91, respectively. The BV were mapped on the MP topologies and were shown as figures below the branches.

The analysis based on the rpl32-trnL+rpl16 combined dataset divided the whole group into five major clusters (A [61 BV], B [69 BV], C [55 BV], D [69 BV], and E [67 BV]) and several small, single clades. There was one outgroup cluster (G, the sub-cluster [100 BV] of the major cluster B [Fig. 1] left), constituting members of *Indosasa*, *Sasa*, *Pseudosasa*, *Oligostachyum*, *Sinobambusa*, *Phyllostachys*, *Pseudosasa*, *Shibataea*, and *Drepanostachyum*. However, *Dendrocalamus minor* var. *amoenus*, *Dendrocalamus membranaceus* (in cluster A) and *Melocanna baccifera* (in cluster C, but isolated from the *Bambusa* sub-cluster with 100 BV) were not included and were not separated from the *Bambusa* clusters.

All *Bambusa* taxa were divided into four major clusters (A, C, D, and E), one sub-cluster (F, a sub-cluster (100 BV) of the major cluster B), and a few small, single clades. The branching pattern of the dendrogram was not completely consistent with the classical taxonomic classification of *Bambusa* proposed by the FOC (Xia *et al*., 2007). Several species belonging to the same subgenus were also clustered with a high confidence coefficient in MP analysis. For example, in cluster E, most of the species belonged to the subgenus *Leleba*, except for *B. contracta*, *B. ventricosa* cv. Nana, and *B. indigena*. In cluster A, most species belonged to the subgenus *Leleba*, except *for B. macrotis* and *D. membranaceus*. However, species of the subgenus *Bambusa* were dispersed in each cluster and those of the subgenus *Lingnania* were dispersed in small, single clades (except for *B. chungii* in F and *B. oldhamii* in D).

Varietas and cultivarietas did not always stay with their McClure. For example, *B. chungii* and *B. chungii* var. *velutina* were separated into two clusters. Two varieties of *B. eutuldoides* were assigned to cluster A, but were separated from *B. eutuldoides* (in F). Meanwhile, cultivarietas of *B. textilis* were assigned to cluster E but were separated from *B. textilis* (in F), *B. vulgaris*, and *B. vulgaris* cv. Vittata formed a small cluster (90 BV), but was separated from *B. vulgaris* cv. Wamin. *B. multiplex* and its varietas and cultivarietas (except *B. multiplex* cv. Fernleaf and *B. multiplex* cv. Stripestem Fernleaf) formed a sub-cluster of cluster E with 93 BV.

Morphological Characteristics Analyses

In the absence of flower or fruit characteristics, the culm sheaths (Raizada and Chatterjee, 1956) and characteristics were treated as two major taxonomic keys to classify *Bambusa*. According to 186 key morphological descriptors, the whole dendrogram (Fig. 1, right) was split into three clusters (K, I, and J). One main cluster (H) was divided into two sub-clusters (K and L). These four clusters (K, L, I, and J) did not completely conform to the existing classification.

B. textilis and *B. teres* were totally isolated in a small cluster (J) belonging to the subgenus *Leleba.* Species of the subgenus *Lingnania* sampled here were all in one subclade of cluster L. The subgenera *Bambusa* and *Leleba* were not separated from one another. Although there were some subgenera, *Leleba* taxa formed a few clades in the main clusters. For example, *B. eutuldoides*, *B. eutuldoides* var. *viridi-vittata*, and *B. pervariabilis* formed a clade. Furthermore, *B. vulgaris* and its cultivarietas formed a clade in cluster I.

Otherwise, varietas and cultivarietas, such as *B. vulgaris* and *B. multiplex*, were more likely to stay with their McClure. *B. vulgaris* and its two cultivars formed a small clade in cluster I. Meanwhile, *B. chungii* and *B. chungii* var. velutina were grouped into cluster L. The varietas and cultivarietas of *B. multiplex* were placed into cluster K. *B. textilis*, *B. textilis* cv. Purpurascens, and *B. textilis* cv. Gracilis were split into clusters J, L, and K, respectively. *B. tuldoides cv. Swolleninternode* and *B. tuldoides* were split into clusters I and L.

Topological Congruences

The MP topology analyses were largely inconsistent with morphological analysis, but cluster E in the MP analysis was largely consistent with cluster K in the morphological analysis. This highly consistent cluster included *B. textilis* cv. Gracilis, *B. indigena*, *B. pachinensis* var. *hirsutissima*, *B. ventricosa* cv. Nana, *B. corniculata*, *B. multiplex*, *B. multiplex* cv. Alphonse-Karr, *B. multiplex* cv. Silverstripe, *B. multiplex* var. *shimadai*, *B. multiplex* var. *riviereorum*, and *B. multiplex* cv. Stripestem Fernleaf. These species share some of the same characteristics: no aerial root, wedge-shaped leaf base, sheath clade length/culm sheath length < 1, sheath blade erect, and hairy ventral.

Discussion

Bambusa and its allies

In the present study, the DNA barcode rpl32-trnL+rpl16 identified *Bambusa* from other genera that were close to *Bambusa*, although it struggled to distinguish *Bambusa* from *Dendrocalamus*. Previous molecular studies have not convincingly shown that *Bambusa* is a monophyletic genus when related genera have also been considered. Sun *et al*. (2005) used ITS and random amplified polymorphic DNA (RAPD) and found that three *Dendrocalamus* species (*D. latiflorus*, *D. membranaceus*, and *D. strictus*) were nested among the *Bambusa* taxa. Yang *et al*. (2008) used the combined ITS+GBSSI+trnL-F combinatorial regions to show that eight *Bambusa* taxa (including *B. oldhamii*) were resolved as a single clade in a phylogenetic tree supported by the posterior probability of Bayesian analysis. However, the sister grouping of *Dendrocalamus* received unequivocal support. Goh et al. (2013) used the combined plastid DNA rps16-trnQ+trnC-rpoB+trnD-T and sampled 53 kinds of bamboo. They determined that *Dendrocalamus* and *Gigantochloa* were embedded in *Bambusa* taxa; however, the nuclear DNA marker (GBSSI) allowed *Dendrocalamus* to exist as a subclade that was departed from *Bambusa*, but it was still its sister. DAS *et al*. (2007) attempted to construct a phylogenetic tree using 32 morphological characteristics for 15 bamboo species, but failed to separate *Bambusa*, *Dendrocalamus*, and *Gigantochloa* successfully. Here, *Dendrocalamus* was completely embedded in *Bambusa* taxa. *B. emeiensis* and *B. oldhamii* were also intermixed with *Bambusa*; they were classified as new members of *Bambusa*, having previously been named *N. affinis* and *D. oldhamii*, respectively.

Morphological characteristics analyses and subgeneric classification

According to the FOC, the genus *Bambusa* has four subgenera: *Lingnania*, *Dendrocalamopsis*, *Bambusa*, and *Leleba*. The subgenus *Lingnania* was found to share the following typical characteristics: a culm sheath with a narrow blade, a base only one-third of the width of the sheath apex; culm internodes that are usually longer than 30 cm, and thin walls (often < 8 mm). Three other subgenera shared the following characteristics: a culm sheath with a broad blade, a base 1/2–3/4 of the width of the sheath apex; culm internodes shorter than 30 cm, and thick walls (up to 2 cm). Meanwhile, the subgenus *Dendrocalamopsis* shared the following typical characteristics: culm sheath auricles and small, rounded

spikelets that are dense at maturity. The rest of the subgenera shared the following characteristics: culm sheath auricles that are large, rounded, irregular, or absent and spikelets that are loose at maturity, with broad florets on short rachilla segments. Otherwise, the characteristics of the subgenus *Bambusa* were found to be branchlets of lower branches specialized into tough or weak leafless thorns, and with culm sheaths with persistent blades. The subgenus *Leleba* had branchlets in their lower branches that were normal and leafy; and their culm sheath blade was deciduous.

To the best of our knowledge, this study represents the first attempt to distinguish *Bambusa* subgenera by using 186 morphological descriptors to sample more than 50 *Bambusa* taxa. As mentioned in the traditional classification above, eight to 14 morphological characters were used to identify a subgenus, which are fewer than the number of morphological characters used in this study. Therefore, it is not surprising that the morphological phylogenetic tree generated here did not coincide exactly with the existing *Bambusa* subgenus classification. Establishing a phylogenetic tree based on morphological characteristics is a new way to explore bamboo classification. According to the findings of this approach, we described more than 39 morphological features as 186 key morphological descriptors. This meant that the results were focused more on the overall characteristics of each species, rather than on one or several obvious or easily identifiable features.

Controversial bamboo species

The FRPS classified *B. arundinacea* as a member of the subgenus *Bambusa*. However, Xia *et al*. (2007) pointed out that *B. vulgaris* was incorrectly named by Aiton as *B. arundinacea* and that *B. auriculata* and *B. striata* were also the same species as *B. vulgaris*. DAS *et al*. (2007) did not support this point based on morphological characters and molecular analysis. Instead, they found that, from a morphological aspect, these four bamboo species (*B. arundinacea*, *B. vulgaris*, *B. auriculata*, and *B. striata*) were different from one another, and *B. striata* and *B. vulgaris* were more similar to one another than the others in RAPD analysis. Here, *B. auriculata* and *B. striata* were not sampled, and the data of morphological characteristics and DNA sequence between *B. arundinacea* and *B. vulgaris* were different in this study.

B. chungii var. *velutina* is a new variant of *B. chungii* that, to date, has only been found in the Fujian province of China. It was previously considered be a member of the genus *Lingnania*, but is now considered to be a sub-genus of *Bambusa*. Here, *B. chungii* and *B. chungii* var. *velutina* were found to be similar in both MP and morphological characteristic analyses.

Application of the codes

Using DNA barcodes to classify or identify species will be more widely applied after the molecular biology technology matures. However, neither the nucleus nor the chloroplast genome barcodes could perfectly align with traditional botanical classification. Nevertheless, DNA barcodes can provide auxiliary data or evidence to help scientists identify new species or resolve some disputes.

Based on the morphological features, morphological codes were used as a classification method to evaluate whether they can match traditional classification. However, after statistical operation, the results showed that it could not appropriately be explained in relation to morphological classification. Maybe a new operating model of morphological code needs to be developed for application of botanical classification.

Declarations

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Author Contributions

Y.F.B. conceived and designed the study. Y.F.B. and A.K.W. carried out the experiments and wrote the manuscript. Z.X.Z. exulted the experiment at outdoor, H.Z., and Q.F.L. analyzed the data and prepared the tables and figures, H.J.C., S.H.L, and X.H.D. proofed the manuscript.

Compliance with ethical standards

Conflict of interest: All authors declare that they have no potential competing interests.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Herbarium vouchers: Hua'an Bamboo Botanical Garden and Hangzhou Lin’an Taihuyuan Ornamental Bamboo Planting Garden supported the research work of this article. The Materials were collected by Zou Yueguo(Hua'an Bamboo Botanical Garden) and Gu LiJian(Hangzhou Lin’an Taihuyuan Ornamental Bamboo Planting Garden) in accordance with related management rules without damaging the growth of bamboo, and the relevant herbarium vouchers were kept in Hua'an botanical garden and Hangzhou Lin’an Taihuyuan Ornamental Bamboo Planting Garden. The list of specific species is attached in the annex. The list of specific species is attached in the annex. The specific species are listed below:

References

1. Acevedo-Rosas, Sosa V, Lorea F G. Phylogenetic relationships and morphological patterns in Graptopetalum (*Crassulaceae*). The New York Botanical Garden Press, 2004, 56 (2): 185-194.
2. Cao S, Ma X, Lin L, et al. Morphological and Chemical Characterization of Green Bamboo (*Dendrocalamopsis oldhami* (Munro) Keng f.) for Dissolving Pulp Production. Bioresources, 2014, 9

(3): 4528-4539.

3. Cornelia L, Borsch T and Wiersema J H. Phylogenetic analysis of Nymphaeales using fast-evolving and noncoding chloroplast markers. Botanical Journal of the Linnean Society, 2007, 154 (2): 141-163.
4. Das M, Bhattacharya S, Basak J, et al. Phylogenetic relationships among the bamboo species as revealed by morphological characters and polymorphism analyses. Biologia Plantarum, 2007, 51 (4): 667-672.
5. Downie S R, Kzta-Downie D S and Watson M K. A phylogeny of the flowering plant family apiaceae based on chloroplast DNA rpl16 and rpoC1 intron sequences: towards a suprageneric classification of subfamily apioideae. American Journal of Botany, 2000, 87 (2): 273-293.
6. Farris J S, Källersjö M, Kluge A G et al. Testing significance of incongruence. Cladistics,1994, 10: 315-319.
7. Fay M F, Swensen S M and Chase M W. Taxonomic affinities of *Medusagyne oppositifolia (Medusagynaceae)*. Kew Bulletin, 1997, 52 (1): 111-120.
8. Fulton, T M, Chunwongse J and Tanksley S D. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Molecular Biology Reporter. 1995, 13 (3), 207-209.
9. Goh W L, Chandran S, Franklin D C, et al. Multi-gene region phylogenetic analyses suggest reticulate evolution and a clade of Australian origin among paleotropical woody bamboos (Poaceae: Bambusoideae: Bambuseae). Plant Systematics and Evolution, 2013, 299 (1): 239-257.
10. Goh W L, Chandran S, Lin R S, et al. Phylogenetic relationships among Southeast Asian climbing bamboos (Poaceae: Bambusoideae) and the *Bambusa* complex. Biochemical Systematics and Ecology, 2010, 38 (4): 764-773.
11. Hilu K W and Liang H. The matK Gene: Sequence Variation and Application in Plant Systematics. American Journal of Botany, 1997, 84 (6): 830-839.
12. Jia L Z, Feng X L, Dai Q H. Flora Reipublicae Popularis Sinicae-BAMBUSOIDEAE. Science Press, Beijing, Vol. 9 (1): 48-130.
13. Kelchner S A. Higher level phylogenetic relationships within the bamboos (Poaceae: Bambusoideae) based on five plastid markers. Molecular Phylogenetics and Evolution, 2013, 67 (2): 404-413.
14. Kim S J, Lee C H, Kim J, et al. Phylogenetic analysis of Korean native Chrysanthemum species based on morphological characteristics. Scientia Horticulturae, 2014, 175: 278-289.
15. Kress W J and Erickson D L. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. PLoS ONE, 2007, 2 (6): e508.
16. Li D Z, Gao L M, Li H T, et al. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proceedings of the National Academy of Sciences of the United States of America, 2011, 108 (49):19641-19646.
17. Ma P F, Zhang Y X, Zeng C X, et al. Chloroplast phylogenomic analyses resolve deep-level relationships of an intractable bamboo tribe *Arundinarieae* (Poaceae). Systematic Biology, 2014, 63

(6): 933-950.

18. Ohrnberger, D. The bamboos of the world: Annotated nomencla-ture and literature of the species and the higher and lower taxa. Amsterdam: Elsevier BV. 1999: 450-522.
19. Qin Q, Tong Y, Zheng X, et al. *Sinosasa* (Poaceae: Bambusoideae), a new genus from China. Taxon, 2021, 70 (1): 27-47.
20. Sang T, Crawford D J and Stuessy T F. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). American Journal of Botany, 1997, 84 (9):1120-1136.
21. Shaw J, Lickey E B, Schilling E E, et al. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. American Journal of Botany, 2007, 94 (3): 275-288.
22. Sun Y, Xia N and Lin R. Phylogenetic analysis of *Bambusa* (Poaceae: Bambusoideae) based on internal transcribed spacer sequences of nuclear ribosomal DNA. Biochemical Genetics, 2005, 43 (11-12): 603-612.
23. Sungkaew S, Stapleton C M A, Salamin N, et al. Non-monophyly of the woody bamboos (Bambuseae; Poaceae): a multi-gene region phylogenetic analysis of Bambusoideae s.s.. Journal of Plant Research, 2009, 122 (1): 95-108.
24. Tate J A and Simpson B B. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. Systematic Botany, 2003, 28(4):723-737.
25. Tate J A. Systematics and evolution of *Tarasa* (Malvaceae): an enigmatic Andean polyploid genus. Ph.D. dissertation. The University of Texas at Austin. 2002.
26. Tilney P M, Wyk B, Downie S R, et al. Phylogenetic relationships in the genus Lichtensteinia (Apiaceae) based on morphological, anatomical and DNA sequence data. South African Journal of Botany, 2009, 75(1): 64-82.
27. Triplett J K and Clark L G. phylogeny of the temperate bamboos (Poaceae: Bambusoideae: Bambuseae) with an emphasis on Arundinaria and allies. Systematic Botany, 2010, 35 (1):102-120.
28. Xia N H, Jia L Z, Li D Z, et al. 2007. Flora of China-Tribe BAMBUSEAE, Science Press, Beijing, vol. 22: 7-38.
29. Yang J B, Yang H Q, Li D Z, et al. Phylogeny of *Bambusa* and its allies (Poaceae: Bambusoideae) inferred from nuclear GBSSI gene and plastid psbA-trnH, rpl32-trnL and rps16 intron DNA sequences. Taxon, 2010, 59 (4): 1102-1110.
30. Yang, H Q, Yang J B, Peng Z H et al. A molecular phylogenetic and fruit evolutionary analysis of the major groups of the paleotropical woody bamboos (Gramineae: Bambusoideae) based on nuclear ITS, GBSSI gene and plastid trnL-F DNA sequences. Molecular Phylogenetics and Evolution, 2008, 48 (3):809-824.
31. Zhang Y J, Ma P F and Li D Z. High-throughput sequencing of six bamboo chloroplast genomes: phylogenetic implications for temperate woody bamboos (Poaceae: Bambusoideae). PLoS ONE, 2011, 6 (5): 1-16.

32. Zhu F M, Du J W, Zhou G X, et al. Construction phylogenetic tree in phyllostachys based on the DNA sequence analysis [J]. Journal of West China Forestry Science, 2015. 44 (2): 63-68 (In Chinese with English abstract).

Figures

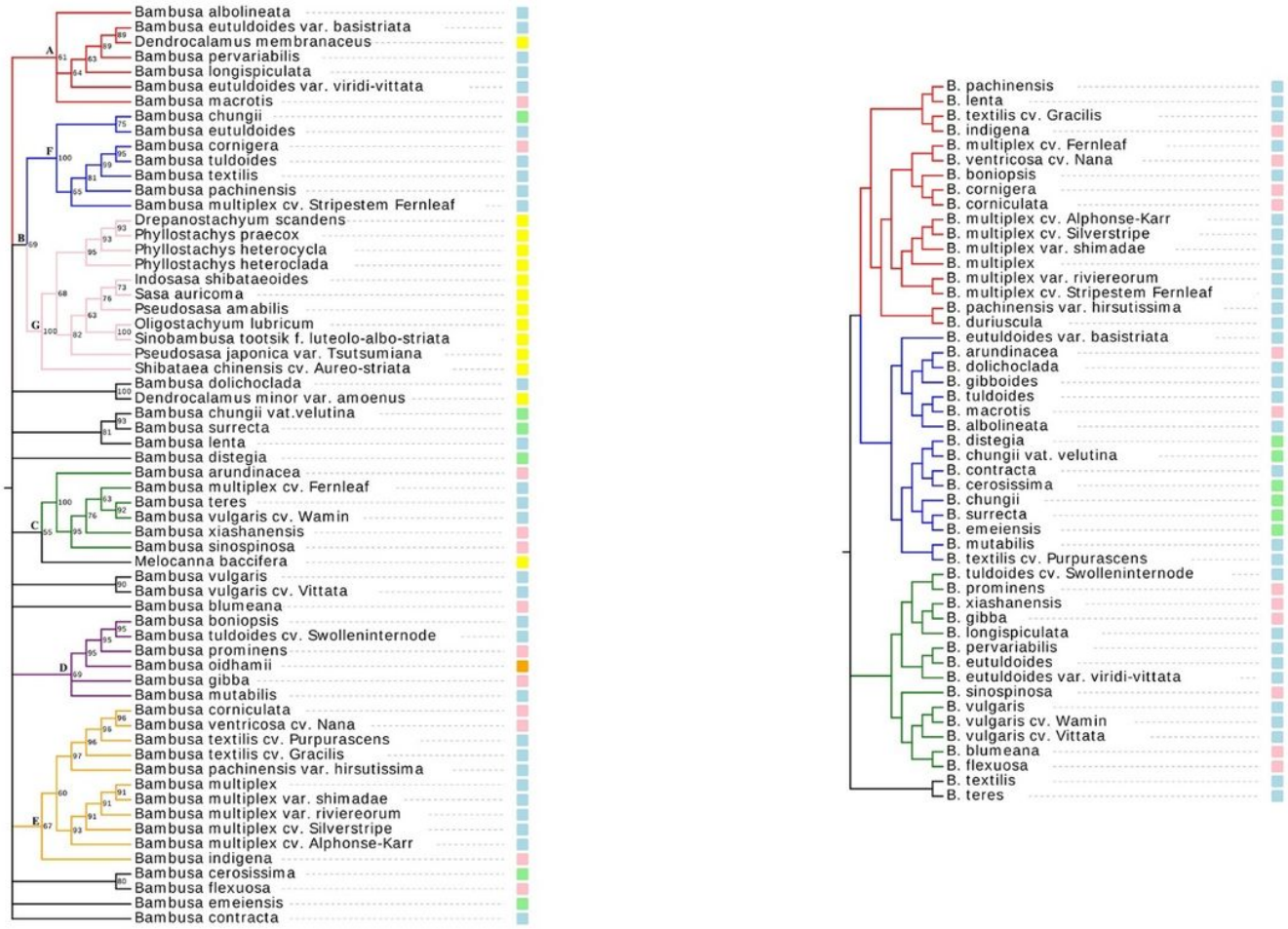

Figure 1

Dendrogram derived from NJ cluster analysis based on 186 morphological descriptors of 50 bamboo species (right); strict consensus of the most parsimonious trees based on two cpDNA datasets (left).