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Phylogenetics of the *Thamnocalamus* group and its allies (Gramineae: Bambusoideae): inference from the sequences of GBSSI gene and ITS spacer

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Abstract

Phylogenetics of 33 species (35 species in the ITS analysis) of the *Thamnocalamus* group and its allies inferred from partial sequences of the nuclear GBSSI gene and from those of the nuclear ribosomal ITS spacer was discussed in the present paper. The analyses of two separate data and combined data sets were performed using the parsimony method. Two species from *Arundinaria* and *Acidosasa* were used as outgroups. All three analyses supported the monophyly of the *Thamnocalamus* group and its allies, which have pachymorph rhizomes and semelauctant synflorescences with three stamens. The two sampled species of *Chimonocalamus* were resolved as a strongly supported monophyletic group and as basal in the *Thamnocalamus* group and its allies in the ITS and combined analyses. The resolution of the *Thamnocalamus* group and its allies in the GBSSI-gene-based tree was generally poor, while the gene still identified some clades with strongly internal supports, i.e., the *Chimonocalamus* clade, the *Ampelocalamus* clade, the clade of *Thamnocalamus spathiflorus* and its variety, that of *Fargesia porphyrea* and *Yushania bojieana*, and the clade of *Fargesia edulis* and *Fargesia fungosa*. The topology resulting from the GBSSI and ITS combined data analysis had a better resolution than those from the two separate data sets. *T. spathiflorus* and its variety comprised another strongly supported basal clade and may be next to the *Chimonocalamus* clade. The positions of the African *Thamnocalamus tessellatus* and *Arundinaria* (*Yushania*) *alpina*, and the monotypic Chinese endemic *Gaoligongshania* were problematic. The *Thamnocalamus* group per se was resolved as polyphyletic. Most species of *Fargesia* and *Yushania* formed a group with no bootstrap support. This assemblage was heterogeneous according to the morphological characters and further investigation is needed. This study implicated that the current limitation of three genera of *Thamnocalamus*, *Fargesia* (incl. *Borinda*) and *Yushania* may not reflect the true phylogenetic relationships of the complex. The phylogenetic utility of GBSSI gene in closely related woody bamboos was also evaluated.

Keywords: *Thamnocalamus* group; Thamnocalaminae; Phylogenetics; GBSSI gene; Combined data sets

1. Introduction

The *Thamnocalamus* group and its allies were defined to include the genera of *Thamnocalamus*, *Fargesia* (*Borinda*, *Sinarundinaria*), and *Yushania*, and their closely related genera of *Ampelocalamus*, *Chimonocalamus*, *Drepanostachyum* (*Himalayacalamus*), and *Gaoligongshania* (Li, 1997b). They are distributed in mountainous areas in mainland China and the adjacent Himalayas, with some species in Africa, Sri Lanka, India, and eastwards to the islands of Taiwan and the Philippines (Guo et al., 2001, 2002). The *Thamnocalamus* group and its allies have pachymorph rhizomes, semelauctant synflorescences with three stamens and appear to have very large microhairs and dumb-bell-shaped silica bodies although some lack fusoid cells (Soderstrom and Ellis, 1982; Soderstrom and Ellis, 1987; Wu, 1960, 1962). These bamboos are of great ecological and economic importance as they include the main food bamboos for the giant pandas and other rare fauna of the Himalayas and adjacent areas (McNeely, 1999; Yi, 1985). Collectively, these genera are placed in the subtribe

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Thamnocalaminae Keng f., with the exception of *Gaoligongshania* (Ohrnberger, 1999). They were included in subtribe Arundinariinae which belongs to the temperate clade of woody bamboos (Clark, 1997; Clayton and Renvoize, 1986; Soderstrom and Ellis, 1987; Zhang, 1996). Wang (1997) included this generic complex and the New World *Arthrostylidium* in the Arthrostylidiinae due to their similarity in morphological characters; however, molecular evidence proved that the New World bamboos and Old World bamboos likely evolved independently (Clark et al., 1995; Kelchner and Clark, 1997; Zhang, 1996).

As briefly reviewed by Guo et al. (2001, 2002), the generic delimitation in this group was highly controversial as different authorities emphasized one or another morphological characters. Soderstrom (1979a,b) and Soderstrom & Ellis (1982) and Chao et al. (1980) recognized two genera, *Thamnocalamus* with bracteate racemiform synflorescences and *Sinarundinaria* with open panicles. This was followed by Clayton and Renvoize (1986), Hsueh and Li (1987), and Chao and Renvoize (1989). On the basis of floral and rhizome morphology, Wang and Ye (1980), Keng (1983), and Yi (1985, 1988) accepted three genera, i.e., *Thamnocalamus*, *Fargesia*, and *Yushania*. This was adopted in *Flora Reipublicae Popularis Sinicae* (as *FRPS* thereafter) (Keng and Wang, 1996). Stapleton (1994a) separated a new genus, *Borinda* from Yi's *Fargesia*. Because of confusion surrounding these genera, Soderstrom and Ellis (1988) later suggested a "very widest" *Arundinaria* to accommodate the Sri Lanka members of the *Thamnocalamus* group. The relationships of their closely related genera, *Ampelocalamus*, *Chimonocalamus*, *Drepanostachyum* (*Himalayacalamus*), and *Gaoligongshania* were also obscure. Soderstrom and Ellis (1987) recognized *Chimonocalamus*, *Ampelocalamus*, and *Drepanostachyum*, whereas Clayton and Renvoize (1986) and Chao and Renvoize (1989) included them in *Sinarundinaria*. *Himalayacalamus* was recognized by Stapleton (1994b), but was merged with *Drepanostachyum* by Soderstrom and Ellis (1987) and Campbell (1991). *Gaoligongshania* was a newly published genus whose relationship needed to be elucidated (Li et al., 1995). For a summary of different opinions, see Li (1997b).

Recently, we explored the molecular phylogeny of the *Thamnocalamus* group and its allies based on the ITS sequence data (Guo et al., 2001, 2002). Due to the relatively slower molecular evolution and low substitution rate of the ITS spacer in the woody bamboos, there are still many phylogenetic problems which had not been resolved and the general supports for most clades were low. In this study, we explored the nuclear GBSSI gene encoding granule-bound starch synthase (alternatively "Waxy" gene; see Fig. 1), consisting 14 exons (with the first untranslated) and 13 introns with about 3 kb in total length. The gene exists in single copy in Poaceae and many other taxa in which it has been studied (Mason-Gamer et al., 1998), although it appears to be duplicated in the Rosaceae (Evans et al., 2000). The GBSSI gene is used in relatively fewer phylogenetic studies (Evans et al., 2000; Mason-Gamer et al., 1998; Peralta et al., 1997) than the ITS region. However, in those studies, the introns of this gene showed higher genetic divergence than the ITS region among very closely related species. So we selected this gene for phylogenetic studies on woody bamboos expecting to get more resolution and we also evaluated its utility in these special group compared with other previously used DNA sequences.

Our main objectives are: (1) to examine the adequacy of GBSSI sequences for phylogenetic reconstruction in the *Thamnocalamus* group and its allies; (2) to explore phylogenetic relationships of the *Thamnocalamus* group and its allies; and (3) to compare the resolutions of the ITS and GBSSI gene separate analyses and that based on combined data sets.

2. Materials and methods

2.1. Materials

A total of 35 species was sampled (Table 1). For the GBSSI gene and combined data, we were only able to get the sequences of 33 species as two ingroup species, *Fargesia murielae* and *Ampelocalamus actinotrichus* with several failed attempts. In the ITS data set, we used the data set of 33 species in the previous papar (Guo et al., 2002) with two additional species, *Arundinaria alpina* (as *Yushania alpina*, or as *Sinarundinaria alpina*, see Chao and Renvoize, 1989) and *Chimonocalamus fimbriatus*. We used the same outgroup species as in the previous paper (Guo et al., 2002). For practical reasons, we followed the classification scheme of the *Thamnocalamus* group and its allied genera in *Flora Reipublicae Popu-*

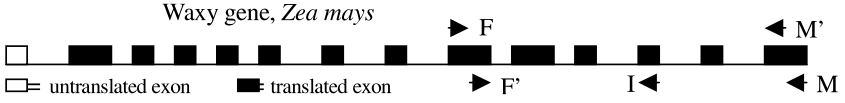

Fig. 1. Schematic diagram of the GBSSI gene of *Zea mays*. Letters and arrows indicating designations, locations, and directions of the primers (modified from Mason-Gamer et al., 1998).

Table 1
Taxa and vouchers for species sequenced

Taxon	Taxon abbr.	Voucher Nos.	GenBank Nos.	
			GBSSI	ITS
Arundinaria gigantea (Walter) Muhlenberg	ARGIG	ZHG20001	AF445159	AF305726
Acidosasa purpurea (Hsueh et Yi) P.C. Keng	ACPUR	ZHG109	AF445158	AF305727
Ampelocalamus scandens C.J. Hsueh and W.D. Li	AMSCA	ZHG013	AF445164	AF280993
A. patellaris (Gamble) C.M.A. Stapleton	AMPAT	ZHG170	AF445163	AF280984
Am. actinotrichus (Merr. and Chun) S.L. Chen et al.	AMACT	DZL199904		AF280992
Chimnocalamus pallens C.J. Hsueh and T.P. Yi	CPALL	ZHG039	AF445161	AF445160
C. fimbriatus C.J. Hsueh & T.P. Yi	CFIMB	ZHG020	AF305724	AF454509
Drepanostachyum hookerianum (Munro) P.C. Keng	DHOOK	DZL199903	AF445165	AF305725
Gaoligongshania megalothyrsa (Hand.-Mazz) D.Z. Li et al.	GMEGA	JRX9401	AF445162	AF305728
Thmnocalamus spathiflorus (Trin.) Munro	TSPAT	Mcbeath19901722	AF445166	AF305729
T. tessellates (Nees) T.R. Soderstrom and R.P. Ellis	TTESS	DZL199901	AF445168	AF280988
T. spathiflorus var. *crassinodus* (Yi) C.M.A. Stapleton	TSPAV	DZL199902	AF445167	AF280989
Arundianria alpina K.Schumann	ARALP	ZHZ200101	AF445171	AF454508
Fargesia altior T.P. Yi	FALTI	ZHG008	AF445172	AF280982
F. porphyrea T.P. Yi	FPORP	ZHG144	AF445180	AF280969
F. yunnanensis T.P. Yi	FYUNN	ZHG014	AF445185	AF280987
F. sylvestris T.P. Yi	FSYLV	ZHG017	AF445182	AF280971
F. fractiflexa T.P. Yi	FFRAC	ZHG018	AF445175	AF280972
F. yulongshanensis T.P. Yi	FYULO	ZHG019	AF445184	AF280973
F. frigida T.P. Yi	FFRIG	ZHG004	AF445176	AF280976
F. yuanjiangensis C.J. Hsueh and T.P. Yi	FYUAN	ZHG002	AF445183	AF280977
F. edulis C.J. Hsueh and T.P. Yi	FEDUL	ZHG011	AF445174	AF280978
F. fungosa T.P. Yi	FFUNG	ZHG016	AF445177	AF280979
F. communis T.P. Yi	FCOMM	ZHG010	AF445173	AF280981
F. hygrophila C.J. Hsueh and T.P. Yi	FHYGR	ZHG003	AF445178	AF280983
F. spathacea Franch.	FSPAT	JRX96049	AF445169	AF280985
F. nitida (Mitford) P.C. Keng and T.P. Yi	FNITI	TPY99215	AF445170	AF280990
F. lushuiensis T.P. Yi	FLUSH	ZHG012	AF445179	AF302723
F. setosa T.P. Yi	FSETO	YF&HS323	AF445181	AF280991
F. murielae (Gamble) T.P. Yi	FMURI	DZL19950106		AF280986
Yushania bojieana T.P. Yi	YBOJI	ZHG040	AF445186	AF280968
Y. falcatiaurita C.J. Hsueh and T.P. Yi	YFALC	ZHG006	AF445187	AF280970
Y. oblonga T.P. Yi	YOBLO	ZHG019	AF445189	AF280974
Y. polytricha C.J. Hsueh and T.P. Yi	YPOLY	ZHG6	AF445190	AF280975
Y. niitakayamensis (Hayata) P.C. Keng	YNIIT	MZ98–291	AF445188	AF280980

laris Sinicae (*FRPS*) (Keng and Wang, 1996) with a few exceptions: e.g., *Acidosasa purpurea* (as *A. hirtiflora*, see Li, 1997a), *Ampelocalamus scandens*, and *Ampelocalamus patellaris* (as *Drepanostachyum scandens* and *Dendrocalamus patellaris*, see Stapleton, 1994b), *Thamnocalamus spathiflorus* var. *crassinodus* (as *Fargesia crassinoda*, see Stapleton, 1994a). Five series of two sections of *Fargesia* and the two sections of *Yushania* (Yi in Keng and Wang, 1996) were well represented.

2.2. DNA isolation

Total DNA was extracted from leaves with the exception of *Gaoligonshania* (from synflorescences) using a modified CTAB procedure (Doyle and Doyle, 1987). We used silica-gel-dried or fresh tissues for most accessions although leaf materials from herbarium specimens were used in some cases. Sterilization was applied to leaves with 75% alcohol prior to DNA extration.

2.3. PCR amplification

Double-stranded DNA of GBSSI gene was directly amplified by symmetric PCR amplification with the F-for and M-bac primer of Mason-Gamer et al. (1998). Reaction volumes were 20 μL and contained 1.5 U Ampli*Taq* DNA polymerase, Replitherm buffer, 1.5 mmol/L $MgCl_2$, 1 mmol/L dNTP, 0.2 μmol/L primer, and 25–60 ng sample DNA. PCR was performed in a GeneAmp 9600 thermal cycler (Perkin–Elmer, Norfolk, CT) and consisted of an initial denaturation at 94 °C for 5 min, followed by 5 cycles of 1.5 min at 94 °C for template denaturation, 2 min at 65 °C for primer annealing, and 1 min at 72 °C for primer extension, then additional 30 cycles of 94 °C of 1 min, 65 °C for 1 min, and 72 °C for 1 min, finally followed by an extension of 20 min at 72 °C. PCR products were purified using Watson's purification kit. Cleaned PCR products were cloned into Promega's pGEM-T System I vector. Ligation,

transformation and plating were carried out following the recommendations of the manufacturer with some modifications. One clone of each species was obtained and plasmid preparations were carried out following the Watson's plasmid mini-columns precipitation protocols. The same procedures were applied to the two additional species for the amplification of the ITS region (Guo et al., 2001).

2.4. DNA sequencing

Plasmid DNA was sequenced using dideoxy chain termination method with an ABI PRISM Bigdye Terminator Cycle Sequencing Ready Reaction Kit with Ampli*Taq* DNA polymerase FS (Perkin–Elmer, Norfolk, CT). Reactions and programs were chosen according to the recommendations of the handbook, with slight modification in some cases. Samples were electrophoresed in an ABI310 genetic analyzer. Because the annealing temperate of the amplification primers is too high to perform sequencing reactions, three sequencing primers, F′-for, I-bac, and M′-bac (Table 2) were designed in this study according to the available sequences of rice and fours species of woody bamboos obtained in this study using the primers located in the vector (T7 and Sp6). The waxy-f and waxy-b were also used for amplification for some species. The same procedures were applied to the two additional species for the sequencing of the ITS region (Guo et al., 2001).

2.5. Phylogenetic analyses

Base determination was complete and unambiguous in all cases and no cells were treated as missing. DNA sequences were edited using SeqMan (DNASTAR Package), aligned by Clustal X and adjusted manually where necessary. Substitutions and indels were used as equally probable events and potentially informative indels that were located in regions of unambiguous alignment were scored following the "simple indel coding" method (Simmons and Ochoterena, 2000) and added to the matrix as extra gap characters. One portion of overlapping gaps that were too complicated to be coded was treated as missing.

Maximum parsimony (MP) analysis was performed with PAUP version 4.0b8 (Swofford, 2000). Searches were conducted on the separate ITS and GBSSI data sets and on the combined data set and the option of collapse branches if minimum length is zero ("amb-") was selected. A successive weighting strategy (SW) (Farris, 1969) was implemented in the analyses. SW is useful tool employed to globally reduce the effect of highly homoplasious base positions on the resulting topologies (Meerow et al., 2000). The initial tree search was conducted under equal and unordered weights criterion used the heuristic search option with stepwise data addition (1000 random replications, start seed = 1) and TBR branch-swapping, but permitting only 20 trees to be held at each step; the shortest trees found were used as input trees for TBR branch-swapping with 1000 random-addition replicates to search for shorter "islands" of trees. The characters were then reweighted according to the maximum value of their rescaled consistency indices (RC), with a base weight of 1000. To assess the relative support for each clade, bootstrap values were calculated with both unweighted and reweighted character matrices from 1000 replicate analyses with the heuristic search strategy and simple addition sequence of the taxa, respectively. Additionally, the sensitivity analysis was conducted to revalue the stability of resulted topologies.

3. Results

3.1. The GBSSI gene analysis

The 3′ end GBSSI gene sequence from 33 species of ingroups and outgroups were obtained. The length of the partial sequences ranged from 1148 to 1221 bp, including five exons and five introns. Among them, exons ranged from 656 to 657 bp in length and introns from 491 to 564 bp in length. The mean G + C contents of the total sequences, the exons and the introns were 50.2, 58.9, and 39.5%, respectively. There was a single poly-T hypervariable region from position 115 to position 183 in the first intron. Many indels were detected in this region, and the longest indel was up to 61 bp. This region abundant with gaps was too complicated to be coded and due to the multiple states in 33 species, it is also difficult to devise the implemented step matrix, so the gaps in this region were treated as missing data. Two hundred and eleven variable substitutions out of 1241

Table 2
List of GBSSI gene primers

Primer	Sequence	Used for
F-for	TGCGAGCTCGACAACATCATGCG	Amplification (Mason-Gamer et al., 1998)
M-bac	GGCGAGCGGCGCGATCCCTCGCC	Amplification (Mason-Gamer et al., 1998)
F′-for	TGCGAGCTCGACATCATCATG	Sequencing, amplification (this study)
M′-bac	TAATGTTCTCCCAGTTCTTTGC	Sequencing, amplification (this study)
I-bac	GCCTACTTCGACACTGAGAC	Sequencing (this study)

positions in the total sequence were detected with 84 in exons and 127 in introns, 54 (including three informative indels) potential informative sites in the total sequence with 13 in exons and 41 in introns. The divergence within ingroups ranged from 0.43 to 2.24% in total sequences, from 0 to 2.44% in exons, and from 0.38 to 3.04% in introns.

The strict consensus of all 261 most parsimonious trees is shown with bootstrap values in Fig. 2. Each of these trees had a minimal length of 255 steps, a CI of 0.859, an RI of 0.625 before character reweighting and a CI of 0.966 and an RI of 0.865 after character reweighting. The resolution of the *Thamnocalamus* group and allies in the GBSSI gene based tree was generally poor. The *Thamnocalamus* group and its allies were resolved as monophyletic with a moderate bootstrap value of 78% (after reweighting, same thereafter if not specified) and the ingroups were highly collapsed with eight groups and seven species were resolved from base line. However, the gene still identified several strongly supported monophyletic groups, i.e., the *Chimonocalamus* subclade (*Chimonocalamus pallens* and *C. fimbriatus*) with a bootstrap support of 99%, the *Ampelocalamus* subclade (*A. patellaris* and *A. scandens*) (100%) (bootstrap value, same thereafter), the clade of *T. spathiflorus* and its variety (98%), that of *Fargesia yuanjiangensis* and *Fargesia lushuiensis* (89%, but only 62% before reweighting), that of *F. porphyrea* and *Yushania bojieana*,

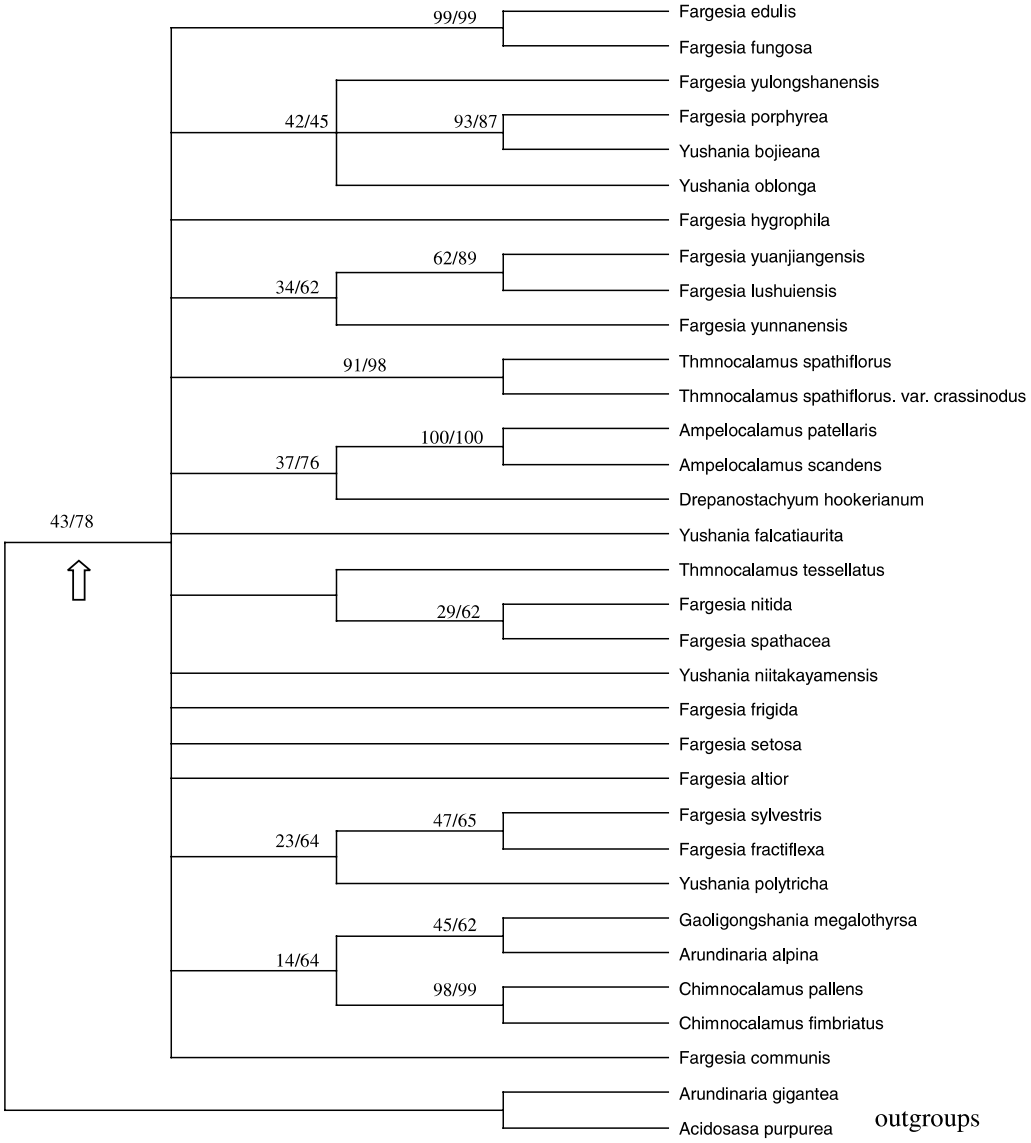

Fig. 2. Strict consensus tree based on GBSSI gene. Tree length = 255, CI = 0.859, and RI = 0.625 before character reweighting; CI = 0.966 and RI = 0.865 after character reweighting. Arrow indicates the Thamnocalaminae. Bootstrap values are indicated above branches (before/unweighting; after/reweighting).

(87%) and the clade of *Fargesia edulis* and *Fargesia fungosa* (99%). Other moderately supported groups in the GBSSI gene based tree included a subclade of *Gaoligongshania* and *A. alpina* (62%, 45% before reweighting), and their sister relationships with the two species of *Chimonocalamus* (64%, only 14% before reweighting); the subclade of *Fargesia sylvestris* and *Fargesia fractiflexa* (65%, 47% before reweighting) and their sister relationship with *Y. polytricha* (64%, but 23% before reweighting); the subclade of *Fargesia nitida* and *Fargesia spathacea* (62%, but 29% before reweighting); the *Ampelocalamus* subclade and its sister relationship with *Drepanostachyum hookerianum* (76%, but 37% before reweighting); the *F. yuanjiangensis* and *F. lushuiensis* subclade and its sister relationship with *Fargesia yunnanensis* (62%, 34% after reweighting).

3.2. The ITS analysis

Of 611 characters used, 45 (including four informative indels) were informative. The strict consensus tree for three most parsimonious trees is shown in Fig. 3 with tree length = 148, a CI = 0.595, an RI = 0.712 before character reweighting and a CI = 0.812, an RI = 0.837 after character reweighting).

The bootstrap values for the clades are generally lower than that of GBSSI-gene-based tree. The *Thamnocalamus* group and its allies were again resolved as monophyletic with a higher bootstrap value of 88%. The basal position of the monophyletic *Chimonocalamus* was resolved, and *T. spathiflorus* var. *crassinodus* was resolved as basal next to the *Chimonocalamus* clade with a bootstrap value of 84%

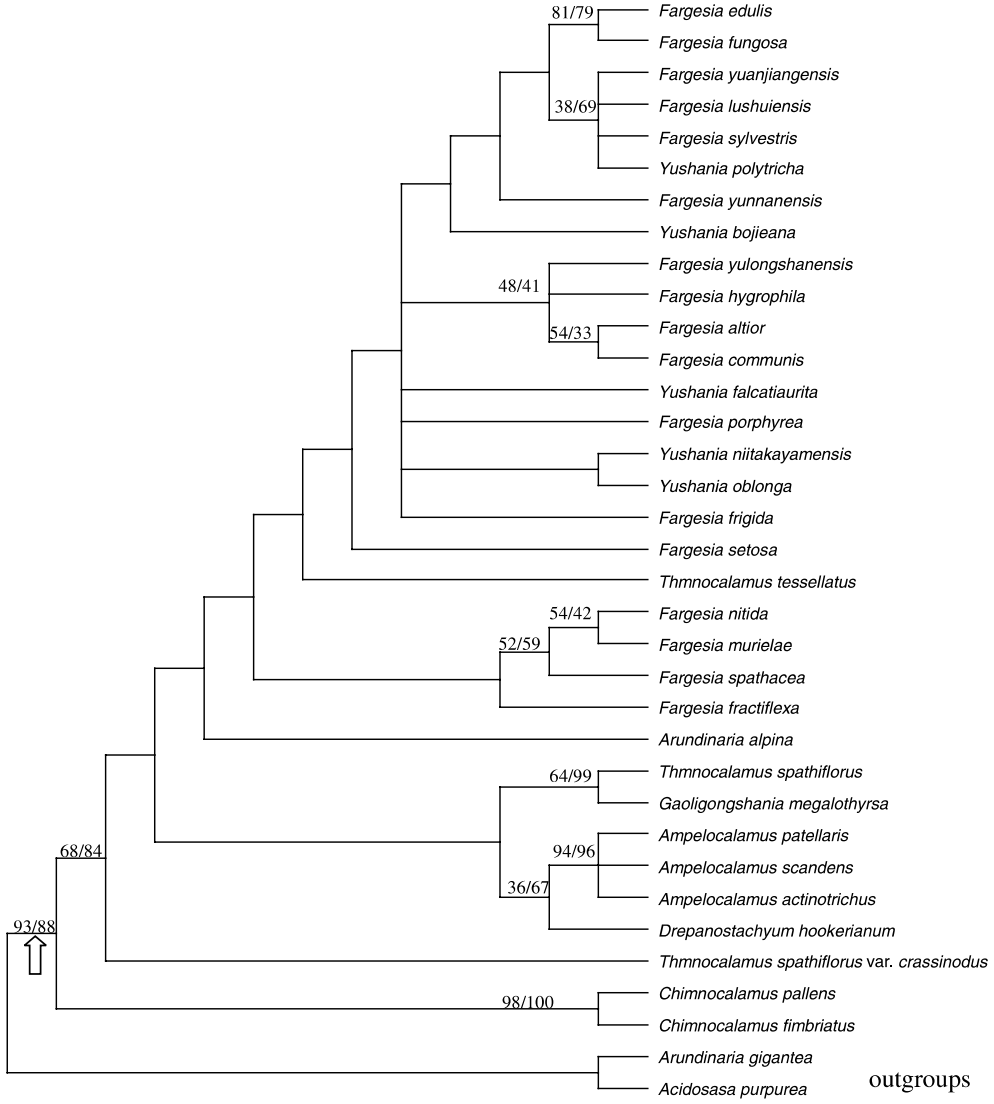

Fig. 3. Strict consensus tree based on ITS region. Tree length = 148, CI = 0.595, and RI = 0.712 before character reweighting; CI = 0.812 and RI = 0.837 after character reweighting. Arrow indicates the Thamnocalaminae. Bootstrap values are indicated above branches (before/unweighting; after/reweighting).

(68% before character reweighting). Within this topology, the resolution was poor except a few monophyletic groups. The three *Ampelocalamus* species formed a strongly supported subclade (96%) and may be sister to *D. hookerianum* (67%, 36% before reweighting). *T. spathiflorus* and *Gaoligongshania* formed a monophyletic group with a bootstrap value of 99% (64% before reweighting). *F. edulis* and *F. fungosa* formed a moderately supported group (79%). *Y. polytricha*, *F. sylvestris*, *F. lushuiensis*, and *F. yuanjiangensis* formed another moderately supported clade (69%, 38% before reweighting). The strongly supported groups were in consensus with the previous analysis of 33 species (Guo et al. 2002), while other grouping with no or low bootstrap support was still variable.

3.3. The combined analysis

Of 1852 characters used, 99 were informative. A single most parsimonious tree was yielded (Fig. 4) with tree length = 420, a CI = 0.736, an RI = 0.630 before character reweighting and a CI = 0.920, an RI = 0.819 after character reweighting.

The resolution and bootstrap value for the clades were higher than both of the GBSSI gene and ITS separate analyses. The *Thamnocalamus* group and its allies were resolved as monophyletic with a high bootstrap value of 100%. The monophyletic *Chimonocalamus* with a bootstrap value of 100%, was resolved as basal. The remaining species formed a moderately supported monophyletic group with a bootstrap value of 75% (52% before reweighting). Within this topology, *T. spathiflorus*

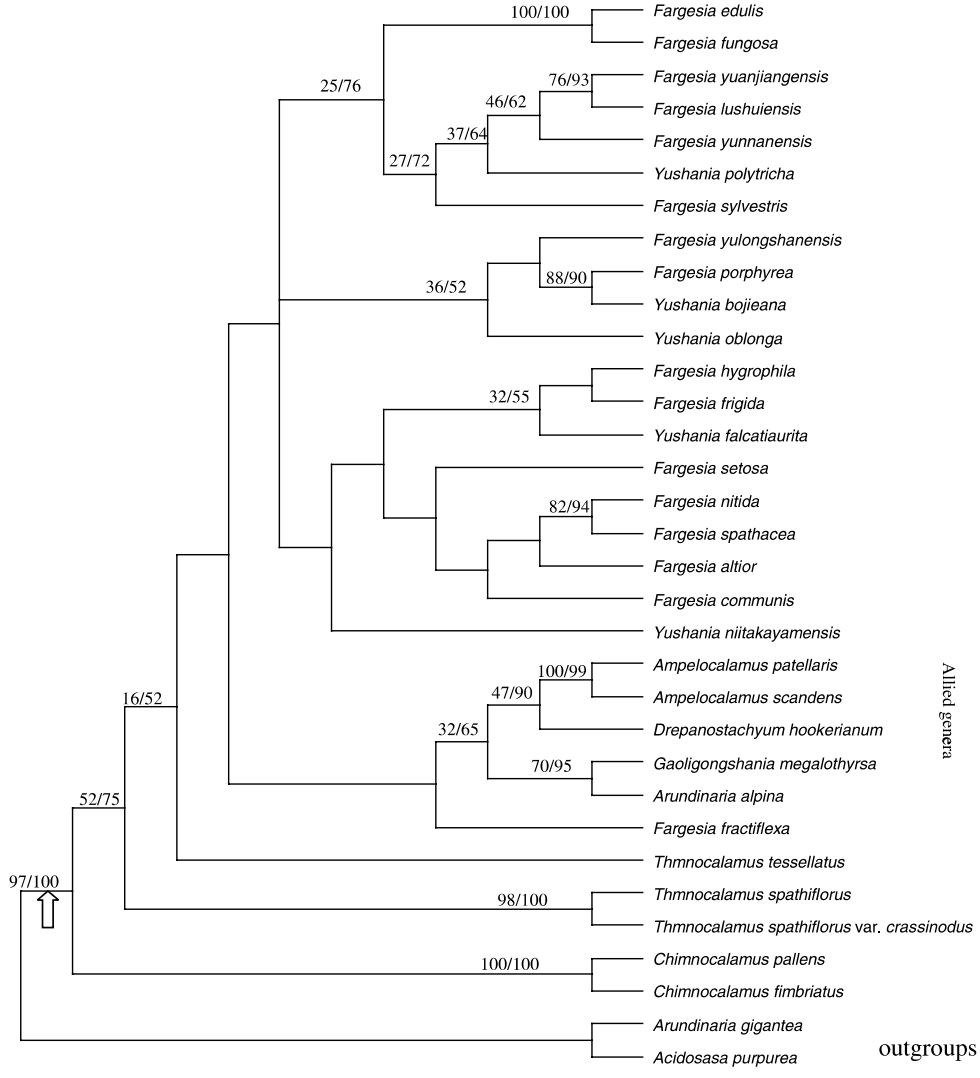

Fig. 4. The single most parsimonious tree based on the combined data. Tree length = 420, CI = 0.736, and RI = 0.630 before character reweighting; CI = 0.920 and RI = 0.819 after character reweighting. Arrow indicates the Thamnocalaminae. Bootstrap values are indicated above branches (before/unweighting; after/reweighting).

and its variety formed a strongly supported monophyletic basal clade (100%). The rest species formed a clade with low internal support (52%). *Thamnocalamus tessellatus* may be sister to remainders that were divided into two assemblages with no internal supports. The first one included the other allies (except the basal *Chimonocalamus*, and *Thamnocalamus* per se), i.e., a strongly supported *Ampelocalamus* and *D. hookerianum* subclade (90%, but 47% before reweighting), a strongly supported *Gaoligongshania* and *Arundinaria* (*Yushania*) *alpinia* subclade (95%, but moderately before reweighting) plus *Fargesia* (*Drepanostachyum*) *fractiflexa*. The other one included all species of *Fargesia s. l.*, (incl. *Borinda*) and *Yushania* with low bootstrap support, which was subdivided into three subclades with low internal supports. Firstly, the *F. yunnanensis* subclade was resolved with a bootstrap value of 76% (25% before reweighting), within which *F. edulis* and *F. fungosa* was strongly supported as monophyletic (100%). Secondly, *F. porphyrea* and *Y. bojieana* was monophyletic with strong internal support (90%), together with *F. yulongshanensis* and *Y. oblonga*, they formed another subclade with low bootstrap support (52%). Within the third weakly supported grouping, *Fargesia hygrophila*, *Fargesia frigida*, and *Yushania falcatiaurita* were weakly supported as monophyletic (55%), and *Fargesia s. s.* (*F. spathacea* and *F. nitida*) was strongly supported as monophyletic (94%). *F.* (*Borinda*) *setosa* and *Y. niitakayamensis* were also nested in the third subclade, but again, with no bootstrap support.

All the aligned matrices are available upon request form the corresponding author.

4. Discussion

4.1. Phylogenetic utility of the GBSSI gene in closely related woody bamboos

The GBSSI gene is a nuclear genomic gene which encodes granule-bound starch synthase (GBSSI; Fig. 1), consisting of 13 translated exons and 13 introns with about 3 kb in total length in most plants. In this study, we obtained 3′ end partial sequence of this gene, including five exons and five introns with about 1.2 kb in length. These partial GBSSI gene sequences provided 211 variable substitutions and 54 (including four informative indels) potentially informative sites with 13 in exons and 42 in introns. The previous studies of GBSSI gene (Peralta et al., 1997; Mason-Gamer et al., 1998) proved that the introns of this gene showed a higher genetic divergence than the ITS region among very closely related species of *Solanum* and of Triticeae. In the present analysis, the divergence within ingroups ranged from 0.43 to 2.24% in total sequences, from 0 to 2.44% in exons, and from 0.38 to 3.04% in introns. Compared with ITS region, GBSSI gene can provide more variable and informative sites although with slightly lower genetic divergence. However, the level of pairwise sequence divergence of GBSSI gene among very closely related species is higher than that of ITS, such as *F. hygrophila* and *F. yulongshanensis*, *F. porphyrea* and *Y. falcatiaurita*, these two paired species have identical ITS sequences, respectively, while their genetic divergences were 0.77 and 0.96% in the GBSSI gene, respectively. This difference in apparent rate of molecular evolution between GBSSI gene and ITS at different levels of the taxonomic hierarchy was also detected in the *cycloidea* (*cyc*) gene and ITS region in Gesneriaceae (Möller et al., 1999) and may exist in most of the single copy gene and multiple-copy gene families. The reasonable explanation is that fixation of genetic changes in the multi-copied ribosomal DNA constrains evolutionary rate at lower levels of divergence, whereas the single copy nuclear gene does not have this problem. However, at high levels of divergence, this constraint appears to have little influence (Möller et al., 1999). This constraint may be the main reason that the ITS spacer sometimes cannot identify the relationship between very close species. Therefore, the single copy genes, such as the GBSSI gene, display their advantages. In comparison with the *rpL*16 intron, the GBSSI gene showed a higher divergence (26.4%) than that of *rpL*16 intron (6.1%) between two species of the American woody bamboo genus *Chusquea* (Mason-Gamer et al., 1998). Consequently, the GBSSI gene and its introns can provide more variable and informative characters compared with other DNA sequences that were previously used in woody bamboos. Besides, GBSSI gene exists in a single copy in the genome of bamboos and can avoid the problem of paralogy and orthology that has been frequently encountered with the ITS region (Soltis et al., 1998). By combining the data sets in this study, better resolution of taxa was accomplished. However, because of the need for cloning when sequencing the GBSSI gene, this marker (or any nuclear marker) will be more expensive and more time-consuming than plastid markers. Additionally, the usefulness of combining multiple data sets extends to the plastid genome, and may allow identification of hybrids, which really highlights the need to use both nuclear and plastid sequences. There was a single poly-T hypervariable region from position 115 to position 183 in the first intron. Many indels were detected in this region, and the longest indel is up to 61 bp. This region abundant with gaps is too complicated to be coded following either of the indel coding methods proposed by Simmons and Ochoterena (2000), therefore, the gaps in this region have to be treated as missing data although it contains some systematic information. Nevertheless, alignment of the remainder of the sequences except this highpervariable region was easy to complete.

Although all evidence to date points to GBSSI gene being single copy in grasses, the woody bamboos are all, as far as we know, ancient polyploid (Soderstrom, 1981). Thus, the potential for multiple copies exists, which could affect phylogenetic inference. Only one clone per species was obtained, but generating multiple clones in future study would be necessary to help establish the assumption that GBSSI is single-copy gene in the bamboos.

4.2. Phylogenetics of the Thamnocalamus group and its allies

The topologies from separate analyses of the ITS, and the GBSSI sequences of the *Thamnocalamus* group and its allies were generally congruent in the main robust clades that were resolved with high bootstrap support. Given the lack of resolution obtained in other studies, the GBSSI gene provided more phylogenetic information than other plastid genes or introns. However, the bootstrap values of separate analyses were generally low, especially in the ITS-based analysis. This may be due to the slow molecular evolution in woody bamboos (Guo et al., 2001, 2002; Hodkinson et al., 2000). It was again proved that a single DNA region can not provided enough informative characters in separate analyses. In the combined analysis, relatively high and better resolution was obtained, in comparison with the two separate analyses, and provided some valuable information. The following is some points that could be made based on the topologies and internal supports obtained through the three analyses.

4.2.1. The monophyly of the Thamnocalamus group and its allies

All of the ITS, GBSSI gene and combined data analyses supported the *Thamnocalamus* group and its allies as a monophyletic clade with a bootstrap value of 88, 78 (43% before reweighting), and 100%, respectively, when *Arundinaria gigantea* and *A. purpurea* were used as outgroups. Morphologically, this clade can be also distinguished by the presence of pachymorph rhizomes and three stamens. However, the other genera of the Arundinariinae, such as *Arundinaria* and *Acidosasa* (with six stamens) have leptomorph rhizomes. Though both types of rhizomes are produced in the New World genus of *Chusquea*, and presumably rhizome may be an ecological strategy for habitats (Clark, 1997), the rhizome type may be a good criterion in defining some bamboo genera or clades.

4.2.2. The monophyly and the basal position of Chimonocalamus

All three analyses strongly supported the monophyly of two sampled species of *Chimonocalamus* and its basal position was resolved in ITS and combined analyses with a bootstrap value of 88 and 100%. This genus appeared to be sister to the remainder of the *Thamnocalamus* group and its allies.

Chimonocalamus was published by Hsueh and Yi (1979) to include some Sino-Himalayan species previously placed in *Arundinaria*. This genus bears three branches at each node with a ring of spiny roots, which is similar to *Chimonobambusa* of the *Shibataeinae*, and bears the same synflorescence type as *Yushania*. Soderstrom and Ellis (1987) and Keng and Wang (1996) in *FRPS* recognized it as a good genus, whereas Chao and Renvoize (1989) treated it as a section of *Sinarundinaria*. Our molecular analysis supports the former opinion. *Chimonocalamus* has a synflorescence similar to *Yushania*; meanwhile, it is distributed at higher elevations and has similar warm temperate habitats to those of some genera in the *Arundinaria* group (Li, 1997b).

4.2.3. The phylogenetic relationships of the other allies of the Thamnocalamus group

The other allies of the *Thamnocalamus* group, i.e., *Ampelocalamus*, *Drepanostachyum*, *Gaoligongshania*, together with *Arundinaria* (*Yushania*) *alpinia* formed a weakly supported clade which may be sister to *Fargesia* (*Drepanostachyum*) *fractiflexa* in the combined analysis. The monophyly of *Ampelocalamus* and its sister relationship to *D. hookerianum* were generally supported by the three analyses, especially in the GBSSI gene analysis (76%) and the combined analysis (90%). These results were congruent with their morphological characters. *Ampelocalamus* has a lax synflorescence and sometimes scrambling culms with a reiterative central branch, which is able to replace the main culm. Soderstrom and Ellis (1987) and Stapleton (1994b) recognized *Ampelocalamus* although Clayton and Renvoize (1986) and Chao and Renvoize (1989) included it in *Sinarundinaria*. Keng (1986) and Yi (1993) restricted *Ampelocalamus* to the type and *A. calcareus*, referring the other six species to *Drepanostachyum*. Our analyses confirmed *Ampelocalamus* as a good genus and *A. patellaris* certainly belongs to *Ampelocalamus* although it differs in vegetative characters from the other scrambling species. *Drepanostachyum* has a lax falcate synflorescence and occurs in similar habitats to *Ampelocalamus*, which is regarded as a related genus (Li et al., 1996).

The position of the newly-published *Gaoligongshania* is problematic. It seemed to be sister to *T. spathiflorus* in the ITS-base analysis. However, in the GBSSI and combined analyses, the genus was resolved as sister to the African *Arundinaria* (*Yushanaia*) *alpina* with low support. Since the bootstrap values for the relationships before reweighting were not more than 70%, there are still some uncertainty. *Gaoligongshania* is a monotypic genus distributed in northwest Yunnan. Its semelauctant synflorescences, pachymorph rhizomes, solitary midculm branch and epiphytic habitat, distinguishes it from the other genera. As to a defined position of

Gaoligongshania and its relationship with other genera, more molecular analyses with broader sampling and morphological studies are still required.

4.2.4. The polyphyly of the Thamnocalamus group per se

The *Thamnocalamus* group as currently defined is polyphyletic and heterogeneous inferred from three analyses, and the internal supports of most species were generally low. In this group, the *Thamnocalamus* clade except the African *T. tessellatus*, and the species of *Fargesia s. l.*, (incl. *Borinda*) and *Yushania* assemblage were generally resolved as monophyletic in the combined analysis. However, None of three genera, as currently defined, was well resolved as monophyletic.

(1) *The separation of Thamnocalamus s. s.* The clade of *T. spathiflorus* and its variety was strongly supported as monophyletic in GBSSI gene analysis as well as in the combined analysis. However, in the GBSSI-based analysis, *T. spathiflorus* formed a clade with *Gaoligongshania*. Their separation with other species of the *Thamnocalamus* group appeared to be clear in the ITS analysis although they seemed to be paraphyletic because of the odd position of *Gaoligongshania*. In the combined analysis, this clade was basal to the rest of the *Thamnocalamus* group and allis next to the basal *Chimonocalamus*. The sensitivity analysis also indicated this separation was stable. This result supported the separation of *Thamnocalamus* and *Fargesia s.s* which consisted of *F. spathacea*, *F. nitida* and *F. murielae*. Furthermore, it seemed to be interesting that conflicting position of *T. spathiflorus*, the type species of *Thamnocalamus* in the separated analyses.

The African species of *T. tessellatus* was nested in a weakly supported clade together with sister *Fargesia s. s.* in the GBSSI gene analysis. In the ITS-based tree, it appeared to be sister to the majority *Fargesia* (except *Fargesia s. s.*) and all sampled species of *Yushania* with low bootstrap support. In the combined analysis, it seemed to be sister to the rest species the *Thamnocalamus* group and allies except the basal *Chimonocalamus* and *Thamnocalamus s. s.* In all analyses, *Thamnocalamus* became paraphyletic when *T. tesselatus* was considered. Though this species similar to *T. spathiflorus* morphologically (Soderstrom and Ellis, 1982), its final position should be ascertained by further comprehensive studies.

(2) *The position of the problematic F. fractiflexa*. It may be sister to *F. sylvestris* in the GBSSI gene analysis. In the ITS-based tree, *F. fractiflexa* appeared to be sister to a weakly supported *Fargesia s. s.* clade with low internal support. It seemed to be sister to the other allies of *Thamnocalamus* plus *Arundinaria* (*Yushania*) *alpinia* in the combined analysis although the topology may collapse. Morphologically, *F. fractiflexa* has many subequal culm branches with underdeveloped secondary branches, which is very different from other species of *Fargesia* but similar to *Drepanostachyum.* Li (1997b) even transfered it to this genus due the similar vegetable characters since its synflorescence is unknown. Because the position of this problematic species is not in consensus in different analyses, further research based on more comprehensive data is necessary to clarify its definite position.

(3) *The assemblage of Fargesia s. l. (incl. Borinda) and Yushania.* This assemblage was resolved in combined analysis but with low bootstrap value. It was heterogeneous based on their gross morphology. Within this topology, a few monophyletic groups were identified. In all analyses, the genera *Fargesia* and *Yushania*, as accepted in the *FRPS* (Keng and Wang, 1996), and *Borinda*, as accepted in the *Flora of Bhutan* (Noltie, 2000), were paraphyletic.

The subclade consisted of *F. edulis* and *F. fungosa* was strongly supported in all three analyses with high bootstrap values implying their robust relationship. This subclade was sister to a subclade consisted of *F. sylvestris*, *Y. polytricha*, *F. yunnanensis*, *F. lushuiensis*, and *F. yuanjiangensis* with a moderate bootstrap support in the combined analysis (76%). This was the *F. yunnanensis* clade identified in the previous ITS analysis (Guo et al., 2002). However, the ITS-based tree was only weakly supported. This clade is very heterogeneous in morphological characters. *F. yunnanensis* and *Y. polytricha* have very open paniculate synflorescences with no bract and long-necked rhizomes while *F. edulis*, *F. fungosa*, and *F. yuanjiangensis* have slightly open paniculate synflorescences with some bracts at the base and shorter-necked rhizomes. The common character this clade has is two stigmas regardless of they were placed in *Fargesia*, *Borinda*, or *Yushania*. This implied that the number of stigmas may not be a good character for the delimitation of this clade.

Fargesia porphyrea and *Y. bojieana* formed a strongly supporte subclade in the GBSSI-based tree and the combined analysis. Together with *F. yulongshanensis* and *Y. oblonga*, they formed a weakly supported group in GBSSI gene analysis (45%) and combined analysis (52%). The lengths of culm-necks of this group greatly varied from species to species although and their synflorescence was unknown.

A weakly supported clade included *F. hygrophila*, *F. frigida*, and *Y. falcatiaurita* was resolved in the combined analysis with a bootstrap value of 55%. This clade may not reflect the natural relationship among these species considering its high sensitivity. A *Fargesia s. s.* clade was more or less resolved in all three analyses, with various internal supports (62–94%). This clade consisted of *F. spathacea*, *F. nitida* and *F. murielae*. The three species are very closely related and their interrelationship was confusing (see Li, 1997b; Soderstrom, 1979b). Morphologically, all three species of *Fargesia s. s.* have bracteate racemiform synflorescences and three stigmas, differing from the general condition of two stigmas in other species of *Fargesia* (or *Borinda*) and *Yushania*. Soderstrom

(1979b) regarded *F. spathacea* and *F. murieliae* as conspecific and named them *Thamnocalamus spathaceus* treating *Fargesia* as the synonym of *Thamnocalamus* because of their similarity of synflorescence and pachymorph rhizome. In addition, Stapleton (1995) considered *F. nitida* as conspecific with *F. spathacea* when it flowered in 1993. Wang and Ye (1980) and Yi (1988) distinguished three species due to its series of bracts and racemiform synflorescence as opposed to one large bracts and panicle synflorescence at the nodes of flowering branches of *Thamnocalamus*. It seemed clear that *Fargesia s. s.* should not be merged in *Thamnocalamus* although the relationship of the *Fargesia s. s.* clade was still obscure. In the combined analysis, this clade was nested with various *Fargesia s. l.* (incl. *Borinda*) and *Yushania* species, including *F. setosa*, a species placed in *Borinda* by Stapleton (1994a), and very similar to (probably the same as) *B. macclureana*, the type species of *Borinda*, and *Y. niitakayamensis* (the type species of *Yushania*) with low internal supports.

The length of rhizomes and the synflorescences are the main characters for delimiting the genera, however, there are many intermediate species between them although Yi (1986, 1988) and Stapleton (1994a) delimitated *Fargesia* as having rhizome necks shorter than 20–25 cm and a synflorescence subtended by a series of large or small bracts at the base and without pulvini, while *Yushania* has rhizome necks longer than 20–25 cm and a synflorescence without bracts and with many pulvini, and *Borinda* being intermediate between *Fargesia* and *Yushania*. Although this result remains to be confirmed by other data, it is suggested that the delimitation between Yi's (1988) *Fargesia* and *Yushania* based on length of culm-necks, and Stapleton's (1994a,b) *Borinda* and *Yushania* on the same basis, were not stable and the limitation of these three genera may not reflect the true phylogenetic relationships.

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