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Phylogenomic analyses reveal intractable evolutionary history of a temperate bamboo genus (Poaceae: Bambusoideae)

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ABSTRACT

Shibataea is a genus of temperate bamboos (Poaceae: Bambusoideae) endemic to China, but little is known about its phylogenetic position and interspecific relationships. To elucidate the phylogenetic relationship of the bamboo genus *Shibataea*, we performed genome-scale phylogenetic analysis of all seven species and one variety of the genus using double digest restriction-site associated DNA sequencing (ddRAD-seq) and whole plastid genomes generated using genome skimming. Our phylogenomic analyses based on ddRAD-seq and plastome data congruently recovered *Shibataea* as monophyletic. The nuclear data resolved *S. hispida* as the earliest diverged species, followed by *S. chinensis*, while the rest of *Shibataea* can be further divided into two clades. However, the plastid and nuclear topologies conflict significantly. By comparing the results of network analysis and topologies reconstructed from different datasets, we identify *S. kumasasa* as the most admixed species, which may be caused by incomplete lineage sorting (ILS) or interspecific gene flow with four sympatric species. This study highlights the power of ddRAD and plastome data in resolving complex relationships in the intractable bamboo genus.

1. Introduction

Shibataea is a small genus of the temperate bamboo tribe Arundinarieae (Poaceae: Bambusoideae), members of which are shrubby and usually cultivated as ornamentals in gardens. All members of *Shibataea*, which comprises seven species and two varieties, are naturally distributed in southeastern China, and one species has been introduced to southwestern Japan (Li et al., 2006). Although *Shibataea* has been recognized as a distinct genus in its own subtribe, Shibatataeinae, in many traditional classification systems of bamboos (Soderstrom and Ellis, 1987; Dransfield and Widjaja, 1995; Keng and Wang, 1996; Li, 1997; Ohrnberger, 1999), little is known about its phylogenetic position and interspecific relationships.

Molecular analyses of the phylogenetic placement of *Shibataea* have yielded conflicting results. Studies based on plastid genes have supported the monophyly of *Shibataea*, and formed clade IV (*Shibataea* clade) with *Gelidocalamus*, *Ferrocalamus*, *Sasa*, and *Indocalamus* (Triplett and Clark, 2010; Zeng et al., 2010; Attigala et al., 2016; Zhang et al., 2016; Ma et al., 2017). However, the sampling of *Shibataea* in these studies was sparse and several relationships among this genus were poorly resolved. A study that used a single nuclear gene, *GBSSI*, recovered *Shibataea* as monophyletic, but clade IV collapsed; furthermore, although poorly supported, this study suggests that *Shibataea* is closely related to *Phyllostachys* (Zhang et al., 2012), the most economically important genus of bamboos in China. Recently, the close relationship between *Shibataea* and *Phyllostachys* was strongly supported by restriction-site associated DNA sequencing (RAD-seq) data employed to infer phylogenetic relationships for *Fargesia*, *Yushania* and their close relatives in the temperate bamboos (Wang et al., 2017). However, this study only sampled one *Shibataea* species. To the best of our knowledge, no phylogenomic studies have yet to focus on the genus *Shibataea*, and the interspecific relationships within *Shibataea* remain unclear.

One potential approach to improving phylogenetic reconstructions is the use of the double digest restriction-site associated DNA (ddRAD-seq) sequencing protocol. ddRAD-seq samples thousands of genomic regions across the nuclear genome for

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phylogenetic inference (Baird et al., 2008; Peterson et al., 2012). In addition, genome skimming can be used to characterize complete plastid genomes. These approaches generate high-efficiency data sets that can reveal the underlying reticulate pattern of evolution (Vargas et al., 2017).

The aim of this study is to resolve the phylogenetic placement and interspecific relationships of *Shibataea*. For this purpose, we performed phylogenetic analysis of all seven species and one variety of *Shibataea* using ddRAD-seq data and plastid genomes generated from genome skimming. We were also able to compare the genealogies generated from nuclear ddRAD and plastome sequences to explore the reticulate history of this genus.

2. Materials and methods

2.1. Taxon sampling

Seven species and one variety of *Shibataea* were sampled for ddRAD sequencing, in addition to four closely related species (two species from clade IV, *Ferrocalamus rimosivaginus* and *Gelidocalamus tessellatus*, and two *Phyllostachys* species, *P. edulis* and *P. varioauriculata*) which were chosen based on the studies of plastid genes in Zhang et al. (2012) and the RAD tree in Wang et al. (2017), respectively. *Fargesia nitida* was selected as an outgroup based on the studies above. Of the 13 bamboos sampled here, the complete plastid genomes of four bamboos were previously published and downloaded from GenBank database, the remaining nine genomes were newly sequenced for this study (Appendix: Table S1).

2.2. DNA extraction, library preparation and data generation

DNA was extracted from ~100 mg dried leave tissue with a modified CTAB method (Doyle, 1987). Our ddRAD sequencing library was prepared using the *Ava*II and *Msp*I restriction enzymes following the methods of Yang et al. (2016). Paired-end reads of 2 × 150 bp were generated on an Illumina HiSeq X Ten System (San Diego, CA, USA). Total genomic DNA was also fragmented to build short-insert libraries (500 bp) according to the manufacturer's protocol (Illumina, San Diego, CA, USA) for genome skimming. Paired-end sequencing of 150 bp was conducted on an Illumina HiSeq 2000 at BGI-Shenzhen, generating ~2 Gb data per sample.

2.3. Data assembly

For ddRAD-seq data, paired-end raw reads were demultiplexed, trimmed and filtered using the program '*process_radtags*' of *Stacks* v1.41 (Catchen et al., 2013) to 140 bp. Then the sequence quality of filtered reads was assessed using FASTQC v.0.11.5 (Andrews, 2016). Because the results revealed low quality over the restriction overhang regions and a portion of the R2 reads, we excluded R2 reads from subsequent analyses. Data were assembled into loci using *de novo* assembly method in *ipyrad* version 0.7.28 (http://ipyrad.readthedocs.io/), a toolbox for assembly and analysis of RAD-seq data sets based on the *pyRAD* pipeline (Eaton, 2014). Filtered reads were clustered at 90% thresholds for clustering within each sample. Clusters with a minimum depth of coverage less than five were discarded. Error rate and heterozygosity were jointly estimated based on counts of site patterns across clustered reads for each sampled individual and the average parameter values were used for consensus base calling. Consensus loci were then clustered across samples at 90% similarity and aligned. We filtered loci to be shared by at least 4–13 taxa, which hereafter refer to these 10 alignments as min4-min13, and then wrote output files.

We assembled the genome skimming data using the GetOrganelle v1.9.77 (https://github.com/Kinggerm/GetOrganelle) with a range of k-mers of 65, 75, 85, 95 and 105. The published plastome, *F. rimosivaginus* (NC_015831.1) was used as the reference genome for assembly of all other accessions. Contigs were visualized and extracted using Bandage v0.8.0 (Wick et al., 2015). We filled the gaps produced by non-overlapping contigs using GapCloser as a package for SOAPdenovo2 (https://sourceforge.net/projects/soapdenovo2/files/GapCloser). Chloroplast sequences were checked by aligning the contigs to the reference with pairwise align option and scanned by eye to confirm appropriate mapping using Geneious v.9.1.4 (Kearse et al., 2012). Finally, whole plastome sequences from all 13 species were aligned with MAFFT v.7.222 (Katoh and Standley, 2013) using the default settings. A few poorly aligned regions were manually adjusted in Geneious.

2.4. Phylogenomic analyses

For each assembled RAD data set, all the loci were concatenated and maximum likelihood (ML) trees were inferred in RAxML v8.2.10 (Stamatakis, 2014). Bootstrap supports were estimated from 100 replicates and the best-scoring ML tree was searched for using the GTR + Γ nucleotide substitution model.

To account for incomplete lineage sorting (ILS), we also inferred species trees based on quartet trees inferred from phylogenetic invariants using the program *tetrad* implemented in *ipyrad* (https://github.com/dereneaton/ipyrad). To avoid issues with linkage, we opted to use only one SNP per locus for a total of 15,837 (min6), 2221 (min9) and 155 SNPs (min12). We inferred all 715 possible quartets for the 13 taxa, performed 100 bootstraps replicates and constructed a 50% majority-rule consensus tree.

The plastome dataset was unpartitioned, and subsequent phylogenetic analyses were performed with GTR + Γ nucleotide substitution model. The ML trees with bootstrap support were inferred from 1000 bootstrap replicates and the best-scoring ML tree was searched.

2.5. Network analysis

We used network analysis on the 13 taxa of the min9 dataset to visualize the signal in the dataset and to investigate possible phylogenetic signal conflicts. SplitsTree4 (Huson and Bryant, 2006) was used with the Neighbor-Net algorithm and uncorrected *P*-distances. The position of *S. kumasasa* in the ML trees was inconsistent (see Results for more details); to further compare the effect of *S. kumasasa* on the conflict, we excluded it from network analyses.

3. Results

3.1. Characteristics of the ddRAD-seq and genome skimming data sets

After quality filtering, we obtained 2,577,856 to 21,573,017 paired-end reads for 13 samples with an average of 7.6 million (Appendix: Table S2). *De novo* assembly using *ipyrad* produced 102 K ± 55.7 K (mean ± SD) consensus loci per sample under clustering (90% similarity). Datasets that were assembled with different minimums for sample coverage had different proportions of missing data: The largest but most incomplete assembled data matrix that includes all loci shared across at least four samples (min4) has 57.55% missing data for 13 individuals across 47,217 loci, whereas all other matrices have fewer missing data (0.49–50.65%; Appendix: Table S3).

Nine complete plastomes were newly determined. All these plastomes were conservative and showed high similarity in genome structure and gene content with published plastomes of bamboos (Burke et al., 2012; Wu and Ge, 2012; Ma et al., 2017). Their genome size ranged narrowly from 139,467 to 139,786 bp with an alignment of 140,851 bp across 13 species, characterized by extremely low sequence divergence with only 775 variable sites (0.55%) and 354 parsimony-informative sites (PICs, 0.25%).

3.2. Well-resolved phylogeny with ddRAD-seq data

The phylogenies inferred using ML for each of the ten concatenated data sets were highly supported and generally congruent despite variation in the proportion of missing data. The larger and more incomplete data sets yielded similar or identical topologies to the smaller and more complete data sets, although topologies of the latter data sets often had lower bootstrap supports. All phylogenetic analyses recovered strong support for *Shibataea* as monophyletic (BS = 100%), and *Phyllostachys* as its sister group, which is consistent with recent phylogenetic results that used RAD-seq data sets (Wang et al., 2017). Within *Shibataea*, the order of relationships was generally congruent among different data sets except for the position of *S. kumasasa*. Conflicts among data sets with regard to these relationships are summarized into three alternative topologies. Topologies 1–3 are presented using ML trees inferred from dataset min6, min9 and min12, respectively (Fig. 1a–c). Except for *S. kumasasa*, all of the concatenated ML topologies resolved *S. hispida* as the earliest diverged species (BS = 100%, 100%, 100%), followed by *S. chinensis* (BS = 100%, 100%, 95%), while the remaining species of *Shibataea* can be further divided into two clades (BS = 100%, 100%, 61%).

The tetrad species trees had lower bootstrap support values across some nodes, with three, two, and four nodes receiving less than 80% support in the min6, min9, and min12 data sets (Fig. 1d–f). Interestingly, position of *S. kumasasa* was resolved differently between the tetrad species tree topologies and the concatenated ML tree topologies from min6 and min12 data sets. However, the topologies of all the tetrad species trees were identical to topology 2. Taking into consideration statistical support, the species trees, and the missing data used in building trees, we focused our remaining analyses on topology 2 (assembled from data set min9).

3.3. Network analysis

The neighbor-net network constructed from the min9 data set revealed *Shibataea* as monophyletic, while also revealing conflicting signals that were evident in phylogenetic analyses. In particular, clusters connected by many parallel short edges indicate character conflicts among *S. kumasasa*, *S. changshanensis*, and *S. nanpingensis* (Fig. 2a). After discarding *S. kumasasa*, the remaining relationships were more tree-like and fewer conflicts were contained (Fig. 2b).

3.4. Plastid phylogenomic analyses

Phylogenetic analyses of plastid genomes recovered *Shibataea* as monophyletic, with relationships among them generally strongly supported (Fig. 3). Two species belonging to clade IV, *F. rimosivaginus* and *G. tessellatus*, were strongly supported as a sister group of *Shibataea*, which is congruent with previous studies based on plastid genes (Zeng et al., 2010; Zhang et al., 2012; Ma et al., 2017). Within *Shibataea*, two monophyletic clades were strongly supported (BS = 99%, 89%).

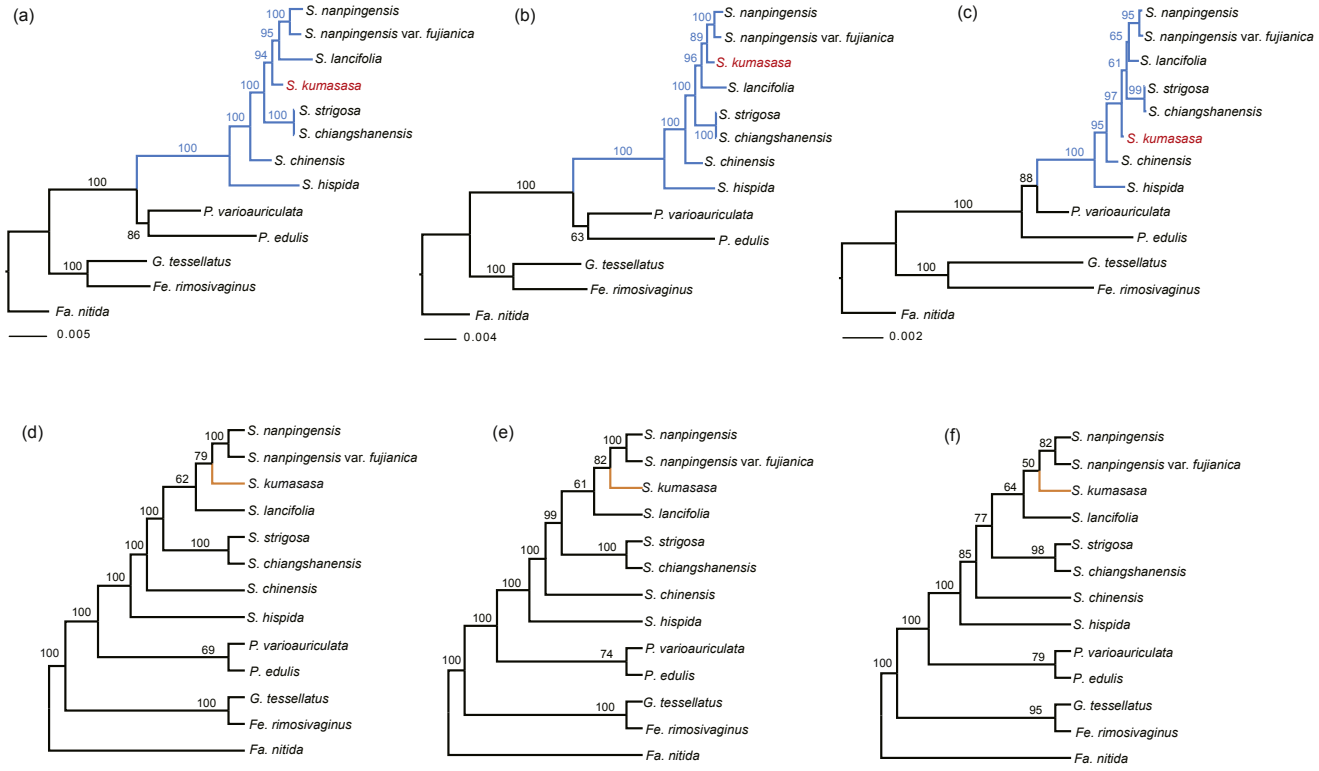

Fig. 1. A summary of three alternative maximum likelihood (ML) topologies and species trees of *Shibataea*. (a)–(c) represent ML trees reconstructed from min6, min9, and min12 data sets, respectively. (d)–(f) represent consensus species tree inferred from *tetrad* analysis based on min6, min9, and min12 data sets, respectively. Numbers associated with nodes indicate bootstrap values (BS).

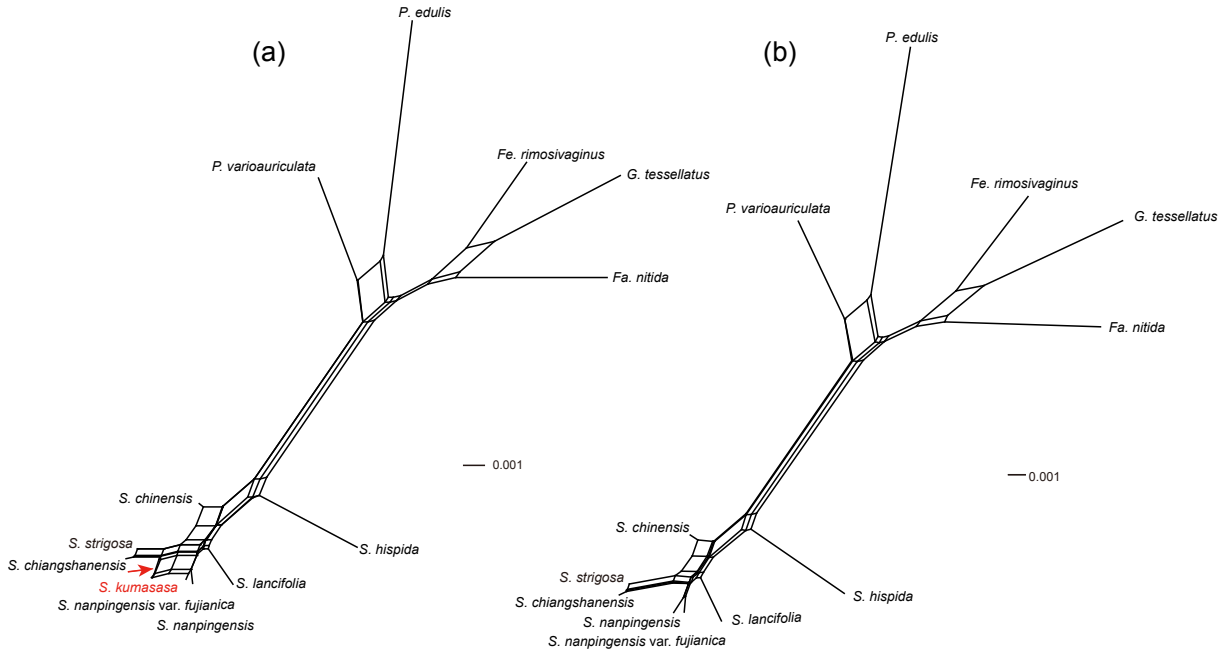

Fig. 2. Split network of *Shibataea* based on min9 data set. (a) contains all 13 species, while (b) contains 12 species with *S. kumasasa* discarded.

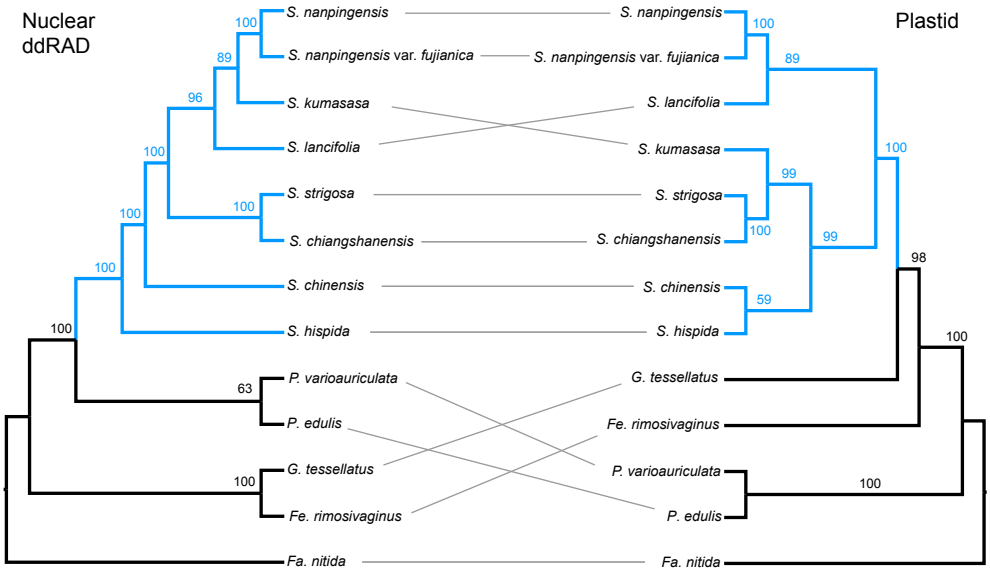

Fig. 3. Tanglegram of the nuclear ddRAD (left) and plastid (right) phylogenies of *Shibataea*. Gray lines connect taxa between the phylogenies. Maximum likelihood bootstrap support values are shown above branches.

3.5. Incongruence among the different genomic data sets

The plastid topologies conflict significantly with the nuclear ddRAD results (Fig. 3). Our plastid tree was consistent with trees from previous studies that were based on chloroplast sequences (Zeng et al., 2010; Zhang et al., 2012; Ma et al., 2014, 2017). Specifically, all *Shibataea* species formed clade IV (*Shibataea* clade) together with *Fe. rimosivaginus* and *G. tessellatus* (Fig. 3). In our ddRAD tree, by contrast, genus *Shibataea* grouped with *Phyllostachys*, and the remaining clade IV genera formed a new clade. Moreover, the plastid and ddRAD trees were in conflict at the genus level. The plastid tree shows *S. hispida* and *S. chinensis* clustered with the clade that contains *S. chiangshanensis*, *S. strigosa* and *S. kumasasa*. The ddRAD tree, however, shows *S. hispida* and *S. chinensis* are early diverged species. The relationships between *S. nanpingensis*, *S. nanpingensis* var. *fujianica* and *S. lancifolia* in the ddRAD tree are generally consistent with those in the plastid tree, except that in the ddRAD tree, *S. kumasasa* is nested with *S. nanpingensis* and *S. nanpingensis* var. *fujianica*.

4. Discussion

4.1. Well-supported relationships of Shibataea using ddRAD-seq data

Our phylogenomic analysis based on ddRAD-seq data congruently recovered *Shibataea* as monophyletic. Trees inferred from ten ddRAD data sets recovered three topologies that all support

identical orders of species in *Shibataea* except for *S. kumasasa*, as summarized in Fig. 1. Species trees reconstructed from different data sets generated congruent topologies. These results illustrate the great promise of ddRAD-seq data for the resolution of an intractable bamboo genus and other non-model plant groups. As we presented here, the potential power of genomic data is not limited to resolving phylogenetic relationships, but in revealing genealogical discordance that would be otherwise undetected. We propose that incongruence in the placement of *S. kumasasa* among trees estimated from different ddRAD data sets is likely due to the conflicting signals, as revealed by network analysis (Fig. 2).

4.2. Conflicting signals and overlapped distribution imply past hybridization events

As revealed by previous empirical studies in bamboos (Zhang et al., 2012; Wysocki et al., 2015, 2016), incongruence between the topologies obtained from nuclear and plastid genomic datasets is also found in *Shibataea*. According to Maddison (1997), several factors can contribute to such conflicts, including phylogenetic uncertainty and/or biological factors such as ILS and interspecies gene flow. The conflicting nodes in our trees are well supported (BS > 89%, Fig. 3); therefore, the incongruence observed in these trees is unlikely to be due to errors in phylogenetic reconstruction.

Phylogenetic data sets may show a high degree of gene tree incongruence due to incomplete lineage sorting (ILS) of genes (e.g. Pollard et al., 2006; García et al., 2017). In our study, data sets from concatenating loci shared across different taxa disclose three alternative topologies. Moreover, Zhang et al. (2012) found more than half of the species sampled in their study possessed two alleles of the *GBSSI* gene, which supports the ancestral polymorphism of these species and demonstrates that ILS may be common in Arundinarieae.

Despite long reproductive cycles — from a few up to 120 years— in temperate bamboos (Janze, 1976; Ma et al., 2017), hybridization is still proposed to have played a significant and recurrent role in bamboo evolution (Triplett, 2008; Triplett and Clark, 2010; Yang et al., 2013; Triplett et al., 2014). In our study, phylogenetic discordances between the ddRAD tree and the plastid tree are focused on *S. kumasasa*. This species formed a well-supported clade with *S. nanpingensis*, *S. nanpingensis* var. *fujianica* and *S. lancifolia* in the ddRAD tree, but clustered closely with *S. strigosa* and *S. chiangshanensis* in the plastid tree. *S. kumasasa* is very likely hybridized with either *S. strigosa*, *S. chiangshanensis* or their ancestor, and captured their plastome during the introgression. And *S. nanpingensis* or *S. lancifolia* may be the paternal donor when they hybridized with *S. kumasasa*.

Consistent chromosome number among temperate bamboos (2n = 48) (Zhou et al., 2017) provides adequate opportunity for plants to overcome hybridization barriers. One of the most important conditions for hybridization is overlapping geographic distributions. Of the species examined in our study, *S. strigosa*, *S. chiangshanensis* and *S. nanpingensis* are endemic species with narrow distributions. Specifically, *S. strigosa* is narrowly endemic to Longquan, Zhejiang province in Eastern China; *S. chiangshanensis* only occurs in Jiangshan, Zhejiang; and *S. nanpingensis* is endemic to Nanping, Fujian province in Eastern China (Fig. 4). Moreover, *S. kumasasa* and *S. lancifolia* are widely distributed in Zhejiang and Fujian provinces, thus, their overlapping distribution with the other three species may largely promote gene exchange. This pattern is

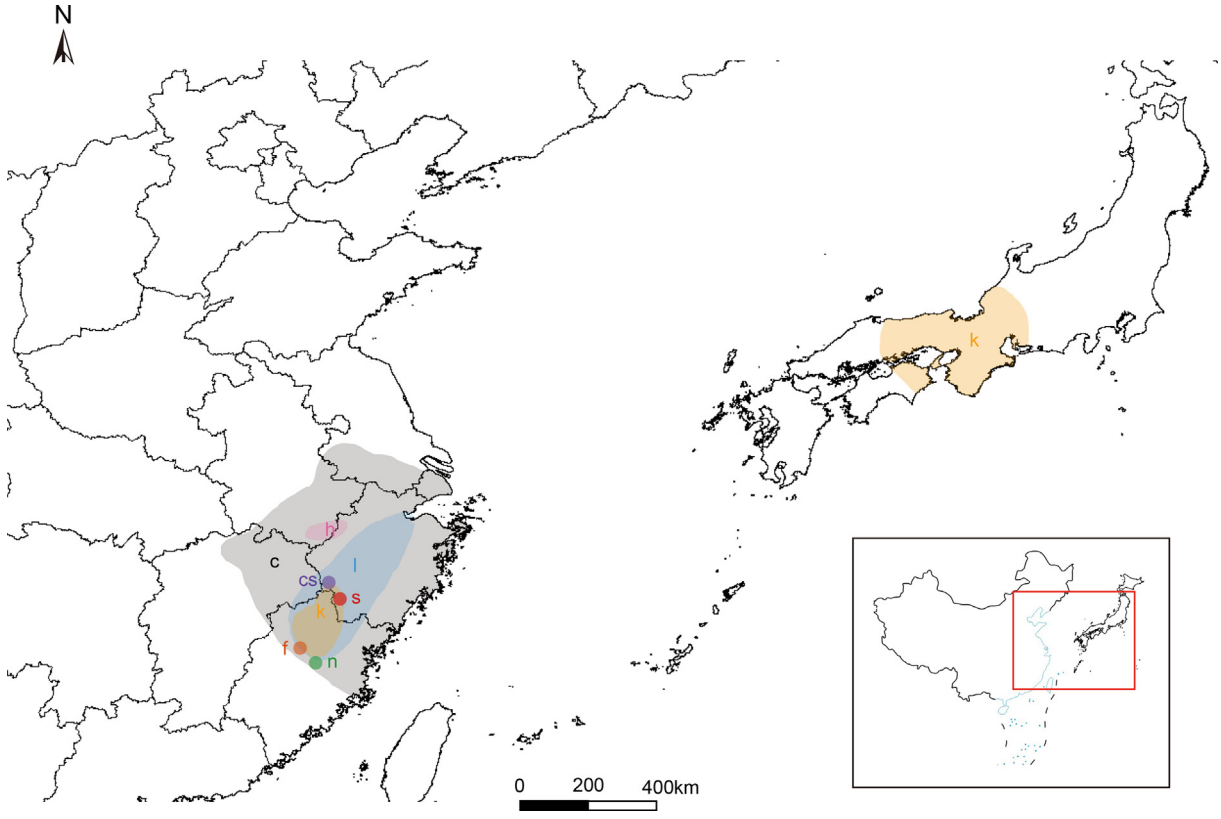

Fig. 4. Geographic distribution of species of *Shibataea* (after Hu et al., 1989). c, cs, f, h, k, l, n, s represents *S. chinensis*, *S. chiangshanensis*, *S. nanpingensis* var. *fujianica*, *S. hispida*, *S. kumasasa*, *S. lancifolia*, *S. nanpingensis*, *S. strigosa*, respectively.

quite common in plants, even in the temperate bamboos. In North American *Arundinaria*, Triplett and Clark, 2010 have identified hybrids among *Arundinaria gigantea*, *A. tecta*, and *A. appalachiana* in regions of geographic range overlap. In addition, in the American live oaks, Eaton et al. (2015) found that species occurring in close geographic proximity show some degree of gene flow, and provide evidence of introgression that is concordant with the present-day geographic distributions of species. Further analyses at the population level should be carried out to provide more evidence of admixture between *S. kumasasa* and the other four species.

5. Conclusion

In this study, we reconstructed the first highly supported phylogenies of *Shibataea* using genome-wide markers both from ddRAD-seq sequences and plastid genomes. We compared these genealogies and revealed conflicting relationships. Together with the result of network analysis, *S. kumasasa* was identified as the most admixed species. We then elucidated the potential biological mechanism which may have contributed to the incongruence observed here.

Conflicts of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2019.05.003.

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