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Reappraisal of *Tashiroea* as a genus independent of *Bredia* (Melastomataceae) based on molecular data

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Abstract

Molecular phylogenetic analyses using ITS sequences were used to reconsider the taxonomic validity of *Bredia okinawensis*, *B. yaeyamensis*, and *B. sinensis* as an independent genus *Tashiroea*. The result showed that the three species formed a different phylogenetic lineage from other species of *Bredia* including its type species. We suggest that *B. okinawensis*, *B. yaeyamensis*, and *B. sinensis* should be treated as *T. okinawensis*, *T. yaeyamensis*, and *T. sinensis*, respectively, following Ito and Matsumura (1899) and Diels (1924).

Keywords: Biogeography, East Asia, ITS, Taxonomic revision

Introduction

Ito & Matsumura (1899) described the genus *Tashiroea* Matsumura (Melastomataceae) with two new species: *T. yaeyamensis* Matsumura (Ito & Matsumura 1899: 489) (Fig. 1A) based on a type specimen from Iriomote Island in the Ryukyu Archipelago (hereafter called the Ryukyus) of Japan, situated between Japan proper and Taiwan (Fig. 2); and *T. okinawensis* Matsumura (Ito &Matsumura 1899: 490) (Fig. 1B) based on a type specimen from Okinawa Island in the Ryukyus. Subsequently, a third *Tashiroea* species, namely *T. sinensis* Diels (1924: 198) (Fig. 1C), was described based on a specimen from Fujian, China. Li (1944), however, transferred the three *Tashiroea* species to *Bredia* Blume (1849: 24), and published the names *B. okinawensis* (Matsumura) H.L. Li (1944: 21), *B. yaeyamensis* (Matsumura) H.L. Li (1944: 21), and *B. sinensis* (Diels) H.L. Li (1944: 22); because, in morphology, *T. okinawensis* and *T. yaeyamensis* were closely related to *B. oldhamii* J.D. Hooker (1871: 68) described based on a type specimen collected from Taiwan; and *T. sinensis* was closely related to *B. grabra* Merrill (1927; 12) described a type specimen collected from Zhejiang of China.

The genus *Bredia* contains about 20 species, primarily distributed in warm-temperate and subtropical areas of East Asia, including the Ryukyus, and was established based on the type species *B. hirsuta* Blume (1849: 25) (Fig. 1D), which is endemic to the Ryukyus (Ohashi 2016). Li's (1944) taxonomic concept is supported by most of the flora of East Asian and major references (Li 1950; Hatusima 1975; Walker 1976; Renner 1993; Huang & Huang 1993; Iwatsuki 1999; Chen & Renner 2007; Yeh *et al*. 2008a; Ohashi 2016).

Recently, Zeng *et al*. (2016) revealed that *Bredia* and *Phyllagathis* (Blume 1831: 507) were each polyphyletic; *B. fordii* was included in a lineage that was distinct from the clade of *B. quadrangularis*, *B. sinensis*, and *P. nudipes*. However, taxonomic relationship between the type species *B. hirsuta* and the three species of *B. okinawensis*, *B. yaeyamensis*, and *B. sinensis* (or *T. okinawensis*, *T. yaeyamensis*, and *T. sinensis*) has never been tested using molecular techniques.

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FIGURE 1. Plants of six *Bredia* species. (**A**) *B. yaeyamensis*, (**B**) *B. okinawensis*, (**C**) *B. sinensis*, (**D**) *B. hirsuta*, (**E**) *B. oldhamii*, and (**F**) *B. rotundifolia*.

In this study, we propose the transfer of *B. okinawensis*, *B. yaeyamensis*, and *B. sinensis* to *Tashiroea* based on morphological and molecular data.

Materials and Methods

Taxon sampling and sequence data from DNA database

We followed Li's (1944) taxonomic concept and did not separate *Tashiroea* from *Bredia* (Table 1) before discussion of the present paper. Based on results of the present phylogenetic analysis, we will discuss which treatment (*Bredia* vs *Tashiroea*) is more appropriate for the three species in discussion below.

We collected seven species of *Bredia* in total: six species recognized by Li (1944) and a species described thereafter, namely *B. dulanica* C.L.Yeh, S.W.Chung & T.C.Hsu (2008b: 395), from 19 localities (in total 19 plants) in East Asia for the present molecular phylogenetic analysis (Table 1, Fig. 2). Voucher specimens for the 19 plants were deposited in the herbarium of National Museum of Nature and Science, Japan (TNS) (Thiers 2018).

To elucidate the phylogenetic status of *B. okinawensis*, *B. yaeyamensis* and *B. sinensis*, sequence data of other three species of *Bredia* and anther accession of *B. sinensis*, and 14 species of related genera provided by previous studies (Michelangeli *et al.* 2013; Penneys & Judd 2013; Liu *et al.* 2015; Zeng *et al.*; 2016) were used in the present analysis. For the outgroup, we employed *Miconia calycina* Cogniaux (1912:312) referring to Zeng *et al.* (2016) (Table 2).

In total, ITS sequences of 36 accessions of 23 species as ingroup member, and that of an accession of a species as outgroup member were obtained in the present analyses.

TABLE 1. Voucher specimens of *Bredia* and accession numbers for ITS sequenced in the present study

Species	Voucher*	Accession no.
B. dulanica	*GK19975*	LC458450
B. hirsuta	*GK18189*	LC458451
	GK18863	LC458452
B. okinawensis	*GK19544*	LC458453
	GK20041	LC458454
	GK20032	LC458455
	GK19074	LC458456
B. oldhamii	*GK14943*	LC458457
	GK20009	LC458458
	GK19983	LC458459
	GK19957	LC458460
B. rotundifolia	*GK19937*	LC458461
B. sinensis	*GK19241*	LC458462
	GK19248	LC458463
B. yaeyamensis	*GK18904*	LC458464
	GK18900	LC458465
	GK19029	LC458466
	GK18713	LC458467

*GK: Goro Kokubugata

DNA extraction, polymerase chain reaction, and sequencing

Total DNA was extracted from dried leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. The total DNA samples were deposited in the Center for Molecular Biodiversity Research of the National Museum of Nature and Science, Japan.

The entire Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA, including ITS1, 5.8S, and ITS2, was amplified by polymerase chain reaction (PCR). The forward primer 17SE and reverse primer 26SE (Table 3) were used for PCR amplification. The PCR profile comprised 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1.5 min at 72°C, after an initial denaturing for 3 min at 94°C. PCR products were checked by electrophoresis before purification with the ExoStar clean-up kit (USB, Cleveland, OH, USA). Cycle sequencing was performed with a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) using PCR primers listed above with an additional internal reverse primer N2 and the forward primer N3 (Table 3). Cycle sequencing products were then

purified by ethanol precipitation. Automated sequencing was performed with an Applied Biosystems 3130xl Genetic Analyzer. The electropherograms were assembled using the ATGC ver. 4.01 software (Genetyx Co., Tokyo, Japan). Sequence data from this study were deposited in the DNA Data Bank of Japan (DDBJ) database (http://www.ddbj.nig.ac.jp/).

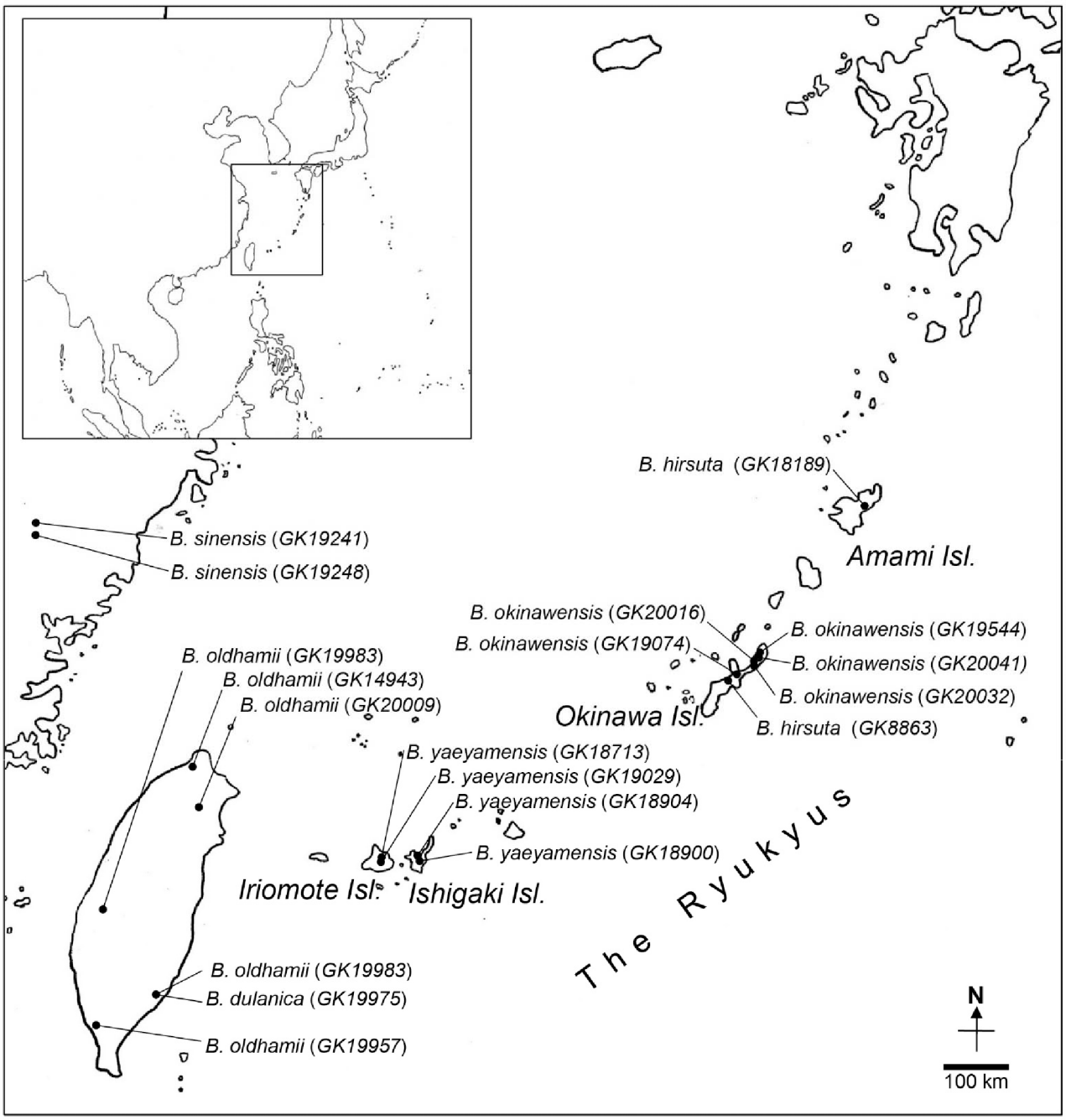

FIGURE 2. Distribution map of 19 collection sites of seven *Bredia* species. For voucher specimen of collection sites refer Table 1.

Phylogenetic analyses

DNA sequences were aligned using the ClustalW 1.8 software (Thompson *et al.* 1994) and then manually adjusted. Phylogenetic analyses were constructed using both maximum parsimony (MP) and maximum likelihood (ML) criteria.

In the MP phylogenetic analysis, using PAUP* version 4.0b10 (Swofford 2002), indels were treated as missing data. Characters were treated as unordered, and character transformations were weighted equally. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed with 200 replicates of random additions of sequences with ACCTRAN character optimization, tree bisection-reconnection (TBR) branch

swapping, and MULTREES and STEEPEST DESCENT options on. Statistical support for each clade was assessed using bootstrap analysis (Felsenstein 1985). Ten thousand replicates of heuristic searches, with TBR branch swapping option on and MULTREES option off, were performed to calculate bootstrap values.

TABLE 2. GenBank accession numbers of ITS sequences reported in previous studies

Species	Voucher	Accession no.*
Ingroup		
Blakea gracilis Hemsley	Boyle 6631	JQ730066[c]
Blastus cohinchinensis Loureiro	S. Zeng B0615	KM521838[a]
Blastus pauciflorus (Bentham) Guillaumin	S. Zeng B0667	KM521839[a]
Bredia fordii (Hance) Diels	S. Zeng X011	KM521840[a]
Bredia quadrangularis Congniaux	Wu et al. FJ05	KT354878[a]
Bredia sinensis (Diels) H. L. Li	Zeng MZ003	KT354879[a]
Bredia sessilifolia H.L.Li	SCBGP374-1	KP093022[b]
Centradenia inaequilateralis G. Don	F.A. Michelangeli 838a	JQ730066[c]
Fordiophyton brevicaule C. Chen	S. Zeng et al. 43858	KM521841[a]
Fordiophyton chenii S. Jin Zeng & X.Y. Zhuang	S. Zeng Q008	KM521843[a]
Fordiophyton cordifolium C.Y. Wu ex C. Chen	S. Zeng X001	KM521842[a]
Fordiophyton faberi Stapf	S. Zeng et al. Y028	KM521844[a]
Fordiophyton huizhouense S. Jin Zeng & X. Y. Zhuang	S. Zeng et al. B536	KM521845[a]
Fordiophyton peperomiifolium (Oliver) Hansen	S. Zeng Q004	KM521846[a]
Melastoma malabathricum L.	Penneys 1998	KY782407[d]
Phyllagathis fengii C. Hansen	*Song et al. YN01*	KT354881[a]
Phyllagathis hispidissima (C. Chen) C. Chen	S. Zeng X009	KM521847[a]
Phyllagathis nudipes C.Chen	Tang et al. T0013	KT354880[a]
Outgroup		
Miconia calycina Cogniaux		

*Reported by [a]Zeng et al. (2016), [b]Liu *et al.* (2015), [c]Michelangeli *et al.* (2013) and [d]Penneys & Judd (2013).

TABLE 3. Primers for PCR and Cycle sequencing in the present study

Primer *	Sequence
17SE[a]	5'-ACG AAT TCA AGG TCC GGT GAA GTG TTC G-3'
26SE[a]	5'-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3'
N2[b]	5'-GGC GCA ACT TGC GTT CAA-3'
N3[b]	5'-GCT CTC GCA GCA TCG ATG AAG-3'

*Reported by [a] Hidayat *et al.* (2005), [b] Yukawa T, TNS, personal communication.

ML phylogenetic analysis was conducted using RAxML-HPC v.8 on XSEDE (8.2.10) (Stamatakis 2014) implemented on the CIPRES Science Gateway V. 3.3 (http://www.phylo.org/) (Miller *et al.* 2010). GTR+G nucleotide substitution model was used, as selected with MrModeltest (Nylander 2004) using Akaike information criterion. Branch support was estimated using rapid bootstrap analysis with 1000 replicates.

Results

In the MP analyses, 197 of 317 variable characters were parsimony informative, and 4 equally most parsimonious trees of 606 steps were obtained with a consistency index of 0.729, a retention index of 0.860, and a rescaled consistency index of 0.627. Differences in topology among the four equally most parsimonious trees were only found within a clade composed of six species of *Fordiophyton* Stapf (1892: 314) (Clade B2 in Fig. 3). The ML phylogenetic analysis yielded a tree with the same topology with this MP tree (data not shown). Therefore, bootstrap values in the ML analysis are presented on the MP tree (Fig. 3).

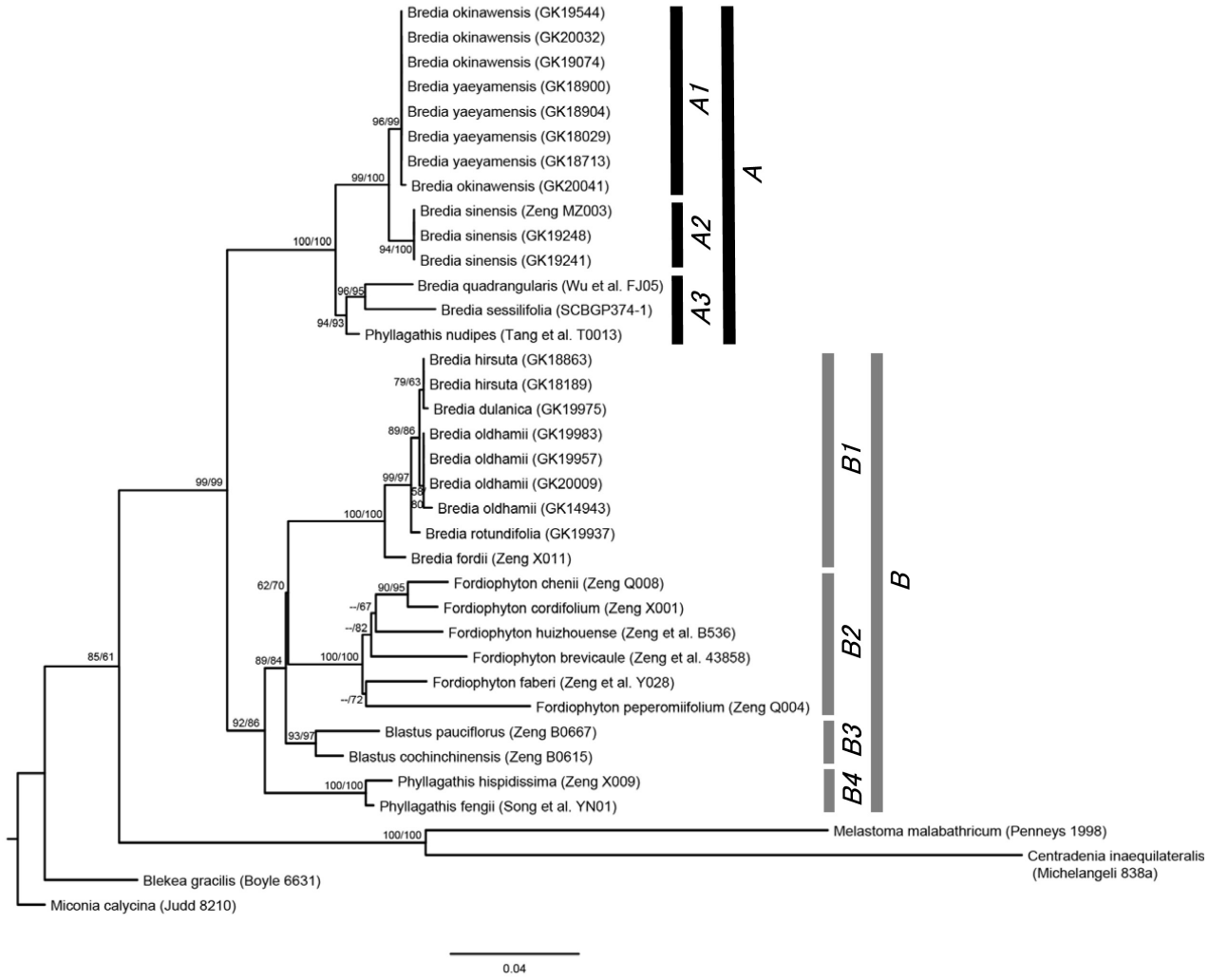

FIGURE 3. One of the four equally most parsimonious trees of *Bredia* and its related genera based on ITS sequences. Bootstrap percentages in the MP/ML analysis are shown above branches.

In four *Bredia okinawensis* plants (from four populations), only one nucleotide substitution was found between *GK20041* and the other three plants of *GK19074*, *19544*, and *20032*. Within the three *B. yaeyamensis* plants (three populations), no intraspecific variation was found and the ITS sequence was identical to that of the three *B okinawensis* plants. In *B. sinensis*, the ITS sequence of *GK19248* was identical to that of *ZengMZ003* reported by Zeng *et al*. (2016), and the sequence differed from that of *GK19241* at two sites: the former accessions had G and G, whereas the latter accession had A/G (double peaks in the electropherogram) and A/G. After aligning the 37 sequences from 24 taxa (including an outgroup species), we obtained a matrix of 832 base pairs.

In the MP tree (Fig. 3), *B. okinawensis* + *B. yaeyamensis* formed a well-supported clade [bootstrap values (BS) in MP / ML = 96% / 99%; Clade *A1*). *Bredia okinawensis* + *B. yaeyamensis* (Clade *A1*) and *B. sinensis* formed a well-supported clade (99 / 100; Clade *A2*), *B. okinawensis* + *B. yaeyamensis* + *B. sinensis* (Clade *A2*) was sister to Clade *A3* (94 / 93) comprising *B. quadrangularis* Cogniaux (1891:473), *B. sessilifolia* H.L. Li (1944:22), and *Phyllagathis nudipes* C. Chen (1984:47) with high bootstrap value (100 / 100; Clade *A*).

In comparison, *B*. *hirsuta* from Japan, the three Taiwanese *Bredia* species (*B*. *dulanica*, *B*. *oldhamii* J.D. Hooker [1871: 68], and *B*. *rotundifolia* [Liu & Ou (1976: 118], S.F. Huang & T.C. Huang [1991:123]) and *B*. *fordii* (Hance [1881: 46]) Diels [1932: 110]) from China formed a clade (100 / 100; Clade *B1*). Clade *B1* was connected to a clade of six *Fordiophyton* species (100 / 100; Clade *B2*). Clades *B1* and *B2* formed a weakly supported clade (62 / 70) that was connected with a clade of two *Blastus* species (93 / 97; *B3*). Clades *B1*, *B2*, and *B3* formed a clade (89 / 84) that was connected to clade *B4* (100 / 100) comprising *Phyllagathis fengii* C. Hansen (1990: 23) and *P*. *hispidissima* (C. Chen) C. Chen (1984: 46) from China, forming clade *B* (= *B1* + *B2* + *B3* + *B4*; 92 / 86).

Discussion

Verification of the genus Tashiroea

Our results revealed that *Bredia* and *Phyllagathis* (Blume 1831: 507) were each polyphyletic; *B*. *fordii* was included in a lineage that was distinct from the clade of *B*. *quadrangularis*, *B*. *sinensis*, and *P*. *nudipes* in accordance with those of Zeng *et al*. (2016). Our results also indicated that five *Bredia* species (Clade *B1*), including the type species *B*. *hirsuta*, are more closely related to *Fordiophyton* (Clade *B2*), two species of *Blastus* Loureiro (1790: 517) (Clade *B3*), and the two *Phyllagathis* species (Clade *B4*) than to *B*. *okinawensis*, *B*. *yaeyamensis*, and *B*. *sinensis* (clades *A1* and *A2*).

Zeng *et al*. (2016) argued that *Fordiophyton* is a monophyletic taxon that is morphologically distinguishable from the *Bredia* species that are included in clade *B1* in our analyses; *Fordiophyton* species have stamens that are distinctly unequal in shape and length and no spur stamen connectives; *Bredia* species have subequal or distinctly unequal stamens and spur stamen connectives. Chen & Renner (2007) reported that the genera *Blastus* and *Bredia* are distinguishable morphologically: the former has a conic or truncate ovary apex and lacks a membranous crown, hypanthium often as long as fruit and usually contracted at or near the apex; whereas the latter has an ovary apex usually with a membranous crown, crown margin often setose and exserted from calyx, and hypanthium not contracted at or near the apex. Therefore, the merger of these genera is not supported and we conclude that *B*. *okinawensis*, *B*. *yaeyamensis*, and *B*. *sinensis* should be treated separately as *Tashiroea* species, following Ito & Masamune (1899) and Diels (1924). Although nobody treated *Bredia quadrangularis* and *B*. *sessilifolia* as *Tashiroea* member, the present molecular analyses indicates the two species could be also included in *Tashiroea*.

In the description of *Tashiroea*, Ito & Matsumura (1899) only mentioned morphological differences from *Barthea* J.D. Hooker in Bentham & Hooker (1867: 731) and *Phyllagathis*, but did not mention morphological differences from *Bredia*.

Also Delis (1924) did not mention morphological differences between *Bredia* and *Tashiroea*. Anatomically, van Viet (1981) reported a difference in vessel morphology between *B*. *hirsuta* plus *B*. *oldhamii* and *B*. *okinawensis* plus *B*. *yaeyamensis*; the former two species have a scalariform inter-vessel pit, whereas the latter two species have alternate/opposite inter-vessel pits. These vessel morphologies are a key character that distinguishes *Tashiroea* from *Bredia*.

Li (1950) argued that *T*. *okinawensis*, *T*. *yaeyamensis*, and *T*. *sinensis* are closely related to *B*. *oldhamii*, *B*. *quadrangularis* and *B*. *sessilifolia*. The author classified the six species into section *Tashiroea* of genus *Bredia* based on their geographical proximity. Our study partly supported Li's concept (1950) that *B*. *quadrangularis* and *B*. *sessilifolia* are closely related to the three *Tashiroea* species; however, it did not support his idea that *B*. *oldhamii* is allied to the three *Tashiroea* species.

Bredia quadrangularis and *B*. *sessilifolia* were phylogenetically clearly separated from the lineage including the type species of the genus *B*. *hirsuta*, and they may also be treated as *Tashiroea* species. Another taxonomic problem remains regarding *Phyllagathis nudipes*, because the genus was revealed to be polyphyletic, as suggested by Zeng *et al*. (2016). However, we do not have enough molecular or morphological data on the genus *Phyllagathis*, which has more than 50 species, to discuss its taxonomic status. Further morphological (particularly anatomical) and molecular investigations are required to solve the taxonomic problems.

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