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THE PHYLOGENETIC RELATIONSHIPS AMONG GERMPLASM RESOURCES OF WILD RAMIE (*BOEHMERIA NIVEA* L. GAUD) IN CHINA BASED ON *trn*L-F AND ITS SEQUENCES

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Abstract

Ramie (*Boehmeria nivea* L. Gaud) is an important fiber crop in China, which also possesses many wild species in genus *Boehmeria* Jacq. However, the taxonomic position of these species has not been settled. To determine the evolutionary relationships among the members of the genus *Boehmeria*, the combination of ITS and *trn*L-F sequences were used for molecular phylogenetic analyses of 31 ramie accessions (28 species and three varieties) including multiple materials collected in high-altitude regions that have not been previously reported (*B. clidemioides* var. *diffusa*, *B. bicuspis* and *B. longispica*). The ITS and *trn*L-F trees produced showed that *Boehmeria* was classified into four separate clusters. The Sect. *Duretia*, which has a high evolutionary level, clustered with Sects *Zoilingeriana* and *Phyllostachys*. The grouping pattern of clustering differed from traditional taxonomy and indicated possible interspecific hybridization among *Boehmeria.* We found that *B. malabarica* Wedd. var. *leioclada* of Sect. *Boehmeria* clustered into a clade with Sect. *Tilocnide*, providing solid support for the expansion of wild ramie core germplasm resources. The molecular results did not support the intraspecific geographic migration of *Boehmeria*. This study, therefore, established relationships among wild species which will help in ramie crop improvement programs. The results will be important for the collection and conservation of germplasm resources of Chinese wild ramie.

Key words: *Boehmeria; trn*L-F; ITS; Relationship; Germlplasm resources.

Introduction

Ramie (*Boehmeria nivea* L. Gaud) is a perennial phloem fiber crop of the Urticaceae. It is mainly cultivated in China and other Asian countries, such as the Philippines, India, Korea and Thailand (Li & Hu, 1987; Liu *et al*., 2010). It has become an urgent task to improve ramie fiber yield and quality. This has proven difficult using the existing breeding material and technology. However, the wild resources may have excellent genes and traits not found in cultivated ramie in regard to disease and drought resistance. Therefore, wild varieties may be very valuable germplasm resources (Jiang & Jie, 2005; Yang, 2006). The study of ramie germplasm resources and the evolutionary relationships among species of the genus *Boehmeria* is of great significance for ramie breeding.

Since the establishment of *Boehmeria* by Jacquin, there have been great differences in its classification (Blume & Brill, 1856; Weddell, 1869; Satake, 1936). According to the morphological characteristics, Chinese ramie was classified by Wang into 32 species and 11 varieties, belonging to 5 groups: Sects *Boehmeria*, *Tilocnide*, *Zoilingeriana*, *Phyllostachys* and *Duretia* (Wang & Cheng, 1995). Some researchers have performed karyotyping studies (Yang *et al*., 2000) and isozyme analyses (Hu *et al*., 1991; Zeng & Hong, 2009). However, these studies were based on morphological, cytological, physiological and biochemical characteristics (Chen *et al*., 2011), with little evidence on the molecular level. RAPD, SSR and ISSR have been applied to the classification of wild ramie germplasm resources (Guo *et al*., 2003; Liu *et al*., 2009; Liao *et al*., 2010, 2014). However, many species were not included in these previous studies. The internal transcribed spacer (ITS), located between the 18S and 26S rRNA genes, is a part of the transcription unit of nuclear ribosomal DNA, which is under limited selection pressure. The evolutionary rate of this unit is faster than that in the coding region (Baldwin *et al*., 1995; Varshney *et al*., 2005). ITS has been successfully used in studies of genetic relationships and phylogenetic research of some plant species (Jamil *et al*., 2014), such as *Crotalaria* (Shweta *et al*., 2013), *Dianthus* (Mohammad *et al*., 2013) and Apioideae (Galina *et al*., 2013). The *trn*L-F sequence, located in the chloroplast genome, has a moderate size and suffers little selective pressure from the outside environment, so its evolutionary rate is faster than other coding sequences. The *trn*L-F sequence has been widely used in the study of plant systematics (Taberlet *et al*., 1991; Olmstead & Palmer, 1994; Kajita *et al*., 1998; Bakker *et al*., 1999; Kenneth *et al*., 2005; Ivana *et al*., 2013; Wang *et al*., 2013).

At present, only the ITS of the Sect. *Duretia* of *Boehmeria* has been reported (Kang *et al*., 2008), and there has been no related report on cpDNA labeled by *trn*L-F sequences. In the past, the collection and application of *Boehmeria* resources mainly relied on plant materials collected in low-altitude regions. Few studies on the wild resources of high-altitude regions have been conducted, due to a lack of materials. In this study, to more clearly evaluate the genetic relationships among wild Chinese ramie germplasm resources, materials were collected from many regions, including some newly reported wild materials from high-altitude regions. The phylogenetic tree of 31 accessions of *Boehmeria* from different groups was first constructed using the combination of ITS and *trn*L-F sequences. Then, the genetic relationship was studied to provide a necessary theoretical basis for the introduction, breeding and mining of desired *Boehmeria* genes.

Materials and Methods

Experimental materials: The experimental materials included 28 species and three varieties of *Boehmeria*, which could be classified into five groups (Sects *Boehmeria*, *Tilocnide*, *Zoilingeriana*, *Phyllostachys* and *Duretia*), including several samples collected in high-altitude regions that have not been previously studied, (including *B. clidemioides* var. *diffusa*, *B. bicuspis*, *B. longispica* and *B. macrophylla* var. *rotundifolia*). This is the first report concerning *B. densiglomerata*, *B. macrophylla* and *B. bicuspis*. *Pilea* was considered as an outgroup (Fig. 1, Table 1). The experimental materials were from the *Boehmeria* Wild Germplasm Resources Repository in Huazhong Agricultural University. Additional materials were from the China Bast & Leaf Fiber Crops Research Institute (Jiangxi Academy of Agricultural Sciences, China).

DNA extraction and PCR amplification: Genomic DNA was isolated from leaves using the modified CTAB method of Doyle & Doyle (1987). The integrity and quality of DNA were evaluated by electrophoresis on 1% agarose gels. Gel images were captured on a Gene Genius imaging system (Gene Co. Ltd., Hong Kong, China). The DNA was stored at −20°C.

The primers of ITS were designed according to the general primers reported by White (1990). The primer sequences were 5□-TCC GTA GGT GAA CCT GCGG-3□ for ITS1 and 5□-TCC TCC GCT TAT TGA TAT GC-3□ for ITS4. The primers of *trn*L-F were designed according to the general primers reported by Fernández *et al*. (2001). After comprehensive comparisons, the primers were designed; primer T1: 5□-AAA ATC GTG AGG GTT CAA GTC-3□ and T2: 5□-GAT TTG AAC TGG TGA CAC GAG-3□, synthesized by Shanghai Sunny Biotechnology Co. Ltd. (Shanghai, China).

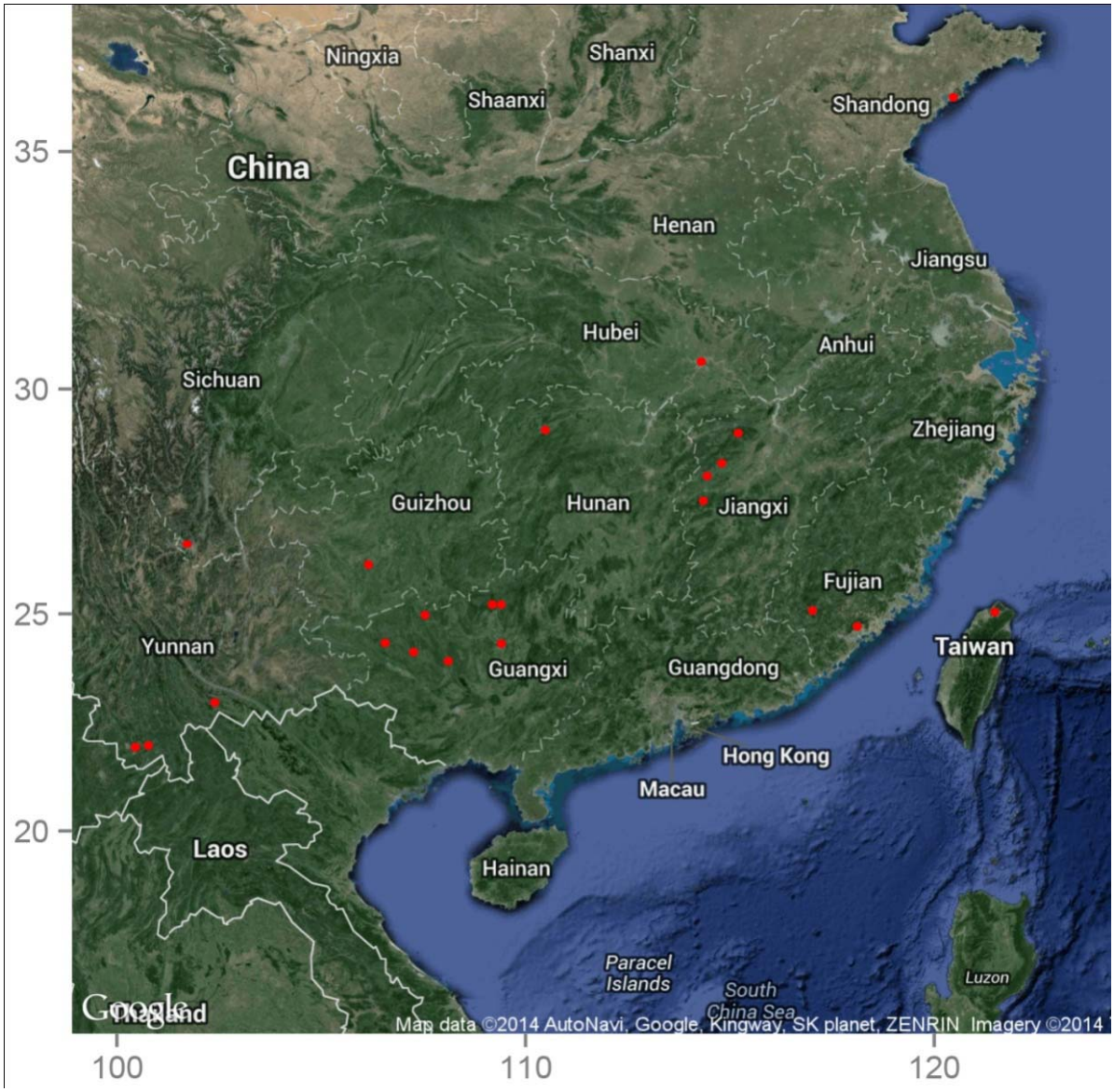

Fig. 1. Map of China with red dots showing locations of collecting areas for samples of *Boehmeria* used in this study (source: Google Earth).

Table 1. List of 32 *Boehmeria* accessions used in the study.

Group	Species	Collection	Coordinate	High	GenBank Number	
					trnL-F	ITS
Sect. *Boehmeira*	*B. glomerulifera*	Menglun Yunnan	N22°14'E100°77'	745	KF782842	KF835855
	B. malabarica Wedd. var. *leioclada*	Menghai Yunnan	N22°26'E100°42'	1230	KF782859	KF835872
Sect. *Tilocnide*	*B. nivea* var. *nipononivea*	Mingyueshan Jiangxi	N27°81'E1114°42'	980	KF782847	KF835860
	B. nivea var. *tenacssima*	Lingyinshan Hangzhou	N25°22' E109°18'	126	KF782848	KF835861
	B. nivea var. *viridula*	Douan Guangxi	N23°93'E108°25'	145	KF782849	KF835862
	B.nivea	Wuhan Hubei	N30°58'E114°30'	30	KF782872	KF835885
Sect. *Zollingerianae*	*B. zollingeriana*	Nanxiang Yunnan	N21°96'E100°45'	1236	KF782843	KF835856
	B. blinii	Lingyun Guangxi	N24°34'E106°56'	455	KF782861	KF835874
Sect. *Phyllostachys*	*B. bicuspis*	Linzhi Tibet	N29°65'E94°36'	2358	KF782845	KF835858
	B. clidemioides var. *diffusa*	Panzhihua Sichuan	N26°39' E101°42'	1931	KF782862	KF835875
	B. pseudotricuspis	Qianlingshan Guizhou	N26°58'E106°68'	1200	KF782867	KF835880
Sect. *Duretia*	*B. spicata*	Nanshan Shandong	N36°108'E120°47'	893	KF782844	KF835857
	B. gracilis	Qianlingshan Gunzhou	N26°60'E106°68'	1065	KF782846	KF835859
	B. tricuspis	Zhangjiajie Hunan	N25°20'E109°27'	800	KF782850	KF835863
	B. formosana Hayata var. *formosana*	Rongan Guangxi	N22°99'E102°39'	183	KF782851	KF835864
	B. macrophylla	Lvchun Yunan	N23°00'E102°13'	1098	KF782852	KF835865
	B. longispica	Panzhihua Sichuan	N26°39'E101°42'	1931	KF782853	KF835866
	B. densiflora	Xinbei Taiwan	N25°05'E121°26'	327	KF782854	KF835867
	B. densiglomerata	Nandan Guangxi	N24°97' E107°11'	567	KF782855	KF835868
	B. pilosiuscula	Puwen Yunnan	N24°72'E118°62'	880	KF782856	KF835870
	B. siamensis	Hongjing Yunnan	N21°58'E101°12'	740	KF782857	KF835871
	B. macrophylla Hornem. var. *canescens*	Hongjing Yunnan	N21°58'E101°12'	570	KF782858	KF835873
	B.macrophylla Hornem. var. *rotundifolia*	Lingzhi Tibet	N29°51'E93°27'	2100	KF782860	KF835874
	B. platyphylla	Panzhihua Sichuan	N26°39'E101°42'	1720	KF782863	KF835876
	B. strigosifolia var. *mollis*	Longyan Fujian	N25°08'E117°01'	1370	KF782864	KF835877
	B. polystachya	Bama Guangxi	N24°08'E107°15'	250	KF782865	KF835878
	B. clidemioides Miq. var. *clidemioides*	Jingan Jiangxi	N29°05'E115°20'	281	KF782866	KF835880
	B. formosana var. *fuzhouensis*	Wanzai Jiangxi	N28°11'E114°44'	500	KF782868	KF835881
	B. platanifolia	Yifeng Jiangxi	N28°39'E114°80'	380	KF782869	KF835882
	B. macrophylla var. *scabrella*	Menglun Yunnan	N21°58'E101°12'	700	KF782870	KF835883
	B. strigosifolia	Liuzhou Guangxi	N24°32'E109°41'	55	KF782871	KF835884
Out group	*Pilea. cadierei*	Menglun Yunnan	N21°58'E101°12'	570	KF835854	KF835853

Phylogenetic analysis: The Align tool using clustalW in MEGA 5.0.5 (Tamericanura *et al*., 2011) was used to perform the multiple sequence alignment. The gaps were treated as missing data. The variation sites and parsimony informative sites of the ITS and *trn*L-F sequences were calculated by DnaSP v.5.10 (Librado & Rozas, 2009). The consistency (CI) and retentivity (RI) were determined by the construction of a matrix using PUAP4.0 (Swofford, 2001). The phylogenetic analyses of ITS and *trn*L-F sequences from all plant materials were conducted by the Neighbor-Joining (NJ) method (Austerlitz *et al*., 2009). The reliability of each clade was estimated by 1000 bootstrap replicates. The partition homogeneity test was performed by PUAP4.0 to analyze the correlation of *trn*L-F and ITS. Finally, a phylogenetic tree with support rates > 50% was formed.

Results

***trn*L-F sequence analysis:** The *trn*L-F sequences of 32 individuals were tested in this study, including 31 accessions of Chinese *Boehmeria* and one species of *Pilea* (GenBank accession numbers are in Table 1). The length was 487 bp after the alignment. There were 31 (6.3%) polymorphic variation sites, including 16 single informative sites and 15 parsimony informative sites (Table 2). Among the single informative sites, there were 16 with two variants and no site with three variants. Among the parsimony informative sites, there were 13 with two variants and two sites with three variants (Table 3).

ITS sequence analysis: The ITS sequences of 32 individuals were amplified, including 31 accessions of Chinese *Boehmeria* and one species of *Pilea* (GenBank accession numbers are in Table 1). After a manual examination and alignment, the length was 717 bp. There were 269 (37.5%) polymorphic variation sites, including 116 single informative sites and 153 parsimony informative sites (Table 2). Among the single informative sites, there were 107 with two variants, eight with three variants and one site with four variants. Among the 153 parsimony informative sites, there are 88 with two variants, 57 with three variants and eight sites with four variants (Table 3).

Analysis of the combination of ITS and *trn*L-F sequences: The result of the partition homogeneity test was p = 0.40, indicating that the two datasets (*trn*L-F and ITS) showed no significant incongruence. We applied the NJ method for the phylogenetic analysis of the *trn*L-F and ITS sequences. The constructed phylogenetic tree (Fig. 2) had a tree length of 855, CI of 0.7532 and RI of 0.7925. The consistency index after readjustment (RC) was 0.5969 (Table 3).

Table 2. Comparison of phylogenetic information for *Boehmeria* species from internal transcribed spacer (ITS), trn*L-F* and combined data sets.

Parameter	ITS	trnL-F	Combines
Number of accession	31	31	31
Range of sequence length (bp)	717	487	1204
Number of variable sites (%)	269 37.5%	31(6.3%)	300(24.9%)
Number of singleton variable sites (%)	116(16.1%)	16(51.6%)	132(11.0%)
Number of informative sitesites (%)	153(21.4%)	15(48.4%)	168(13.9%)
Consistency index (CI)	0.736	0.8475	0.7532
Retention index (RI)	0.7815	0.8421	0.7925
Rescaled consistency index (RC)	0.5752	0.7136	0.5969
Homoplasy index (HI)	0.264	0.1525	0.2468

Table 3. Polymorphism and Indel sites of internal transcribed spacer (ITS) and *trn*L-F

Variable site	Variable type	Site numbers	
		ITS	trnL-F
Singleton variable site	Two variants	107	16
	Three variants	8	0
	Four variants	1	0
Informative site	Two variants	88	13
	Three variants	57	2
	Four variants	8	0

A total of 28 species and three varieties of *Boehmeria* were divided into four clusters. *Pilea*, as the outgroup, was first separated from the wild *Boehmeria* in the clustering process. The species of Sect. *Duretia* were separated from those of other groups, forming a single large clade, with a bootstrap rate of 100%. In clade I were clustered *B. bicuspis*, *B. clidemioides* var. *diffusa*, *B. pseudotricuspis* and *B. zollingeriana* with a bootstrap rate of 99%. The other species of Sect. *Duretia* were clustered into clade II. Clade III was formed by *B. glomerulifera* and *B. blinii* from different groups, with a bootstrap rate of 95%. One species (*B. nivea*) and three *B. nivea* varieties (*nipononiveа*, *tenacssima* and *viridula*) of Sect. *Tilocnide* were separated from the first large clade and clustered into clade IV with *B. malabarica* var. *leioclada*, with a support rate of 88%.

Discussion

China is a center of diversity for *Boehmeria*, with abundant wild ramie germplasm resources. This provides a good basis for the breeding of new varieties of ramie (Lai *et al*., 2000). Many available germplasm resources are a key foundation in any breeding process (Shinwari *et al*., 2014). The collection and study of ramie wild germplasm resources is extremely significant for future genetic breeding (Liu *et al*., 2003). Indeed, dense sampling of wild populations across the geographical range may shed light on the path of ramie domestication and suggest a specific geographical origin.

At present, there is disagreement over the origin and evolution of *Boehmeria* plants due to the lack collection of wild resources. Chinese *Boehmeria* plants include 32 species and 11 varieties (Wang, 1981). Several wild samples, used in the present study, were collected in different regions since 2000, including Guangxi, Yunnan, Guizhou, Sichuan, Hunan, Jiangxi and Tibet. The study materials included 28 species, with *B. densiglomerata*, *B. macrophylla* and *B. bicuspis* reported for the first time. The data was comprehensive and provides a supplement to previous studies.

The combination of ITS and *trn*L-F sequences adequately analyzed the genetic relationships among Chinese wild *Boehmeria*. There were some differences in the clustering results of ITS and *trn*L-F sequences, probably caused by different variation in informative sites contained in different molecular markers (Peng *et al*., 2012). The results clearly showed that there were a greater number of informative sites contained in the combination of ITS and *trn*L-F than in a single sequence. Since the combined diversity estimate based on several molecular markers covers more genetic regions than a single marker alone, the genetic distance estimate based on all the molecular markers most likely gives the most unbiased distance estimate (Lim *et al*., 2007; Abhinandan *et al*., 2013; Sultan *et al*., 2013). In this study, the correlation analysis result ($p = 0.40$) indicated that the combination of ITS and *trn*L-F sequences could provide a greater number of informative sites.

Boehmeria plants are generally distributed in the low altitudes of tropical and subtropical regions (Liu *et al*., 2003), and the acquisition and application of *Boehmeria* samples has mainly concentrated in these regions. Few studies have been conducted on the wild resources in high-altitude regions. In this study, multiple species, such as *B. clidemioides* var. *diffusa*, *B. bicuspis* and *B. longispica*, were collected from high-altitude regions. Their *trn*L-F and ITS sequences were clustered into the same large clade, although their morphology and habitats differ from the wild *Boehmeria* plants from low-altitude regions. Variation indicative of intraspecific geographic migration was not found in the ITS and *trn*L-F sequences. This indicates that these sequences of *Boehmeria* are conserved, conforming to the DNA barcode characteristic of ITS (Kress & Erickson, 2007; Anon., 2011). Additionally, *trn*L-F sequences have been amplified in a number of species (Liu *et al*., 2010). In this study, not only was the amplified *trn*L-F fragment of *Boehmeria* relatively short, but it could be used to distinguish the variation between species. Therefore, it is suggested that *trn*L-F can be used as a DNA barcode in *Boehmeria*.

According to the morphological characteristics, *Boehmeria* was divided into five independent groups by Wang (1981) – a result complemented by the molecular results of the present study. However, unlike the morphological analysis, the Sects *Duretia*, *Zoilingeriana* and *Phyllostachys* clustered into the same clade, which does not fully support the view of Wang.

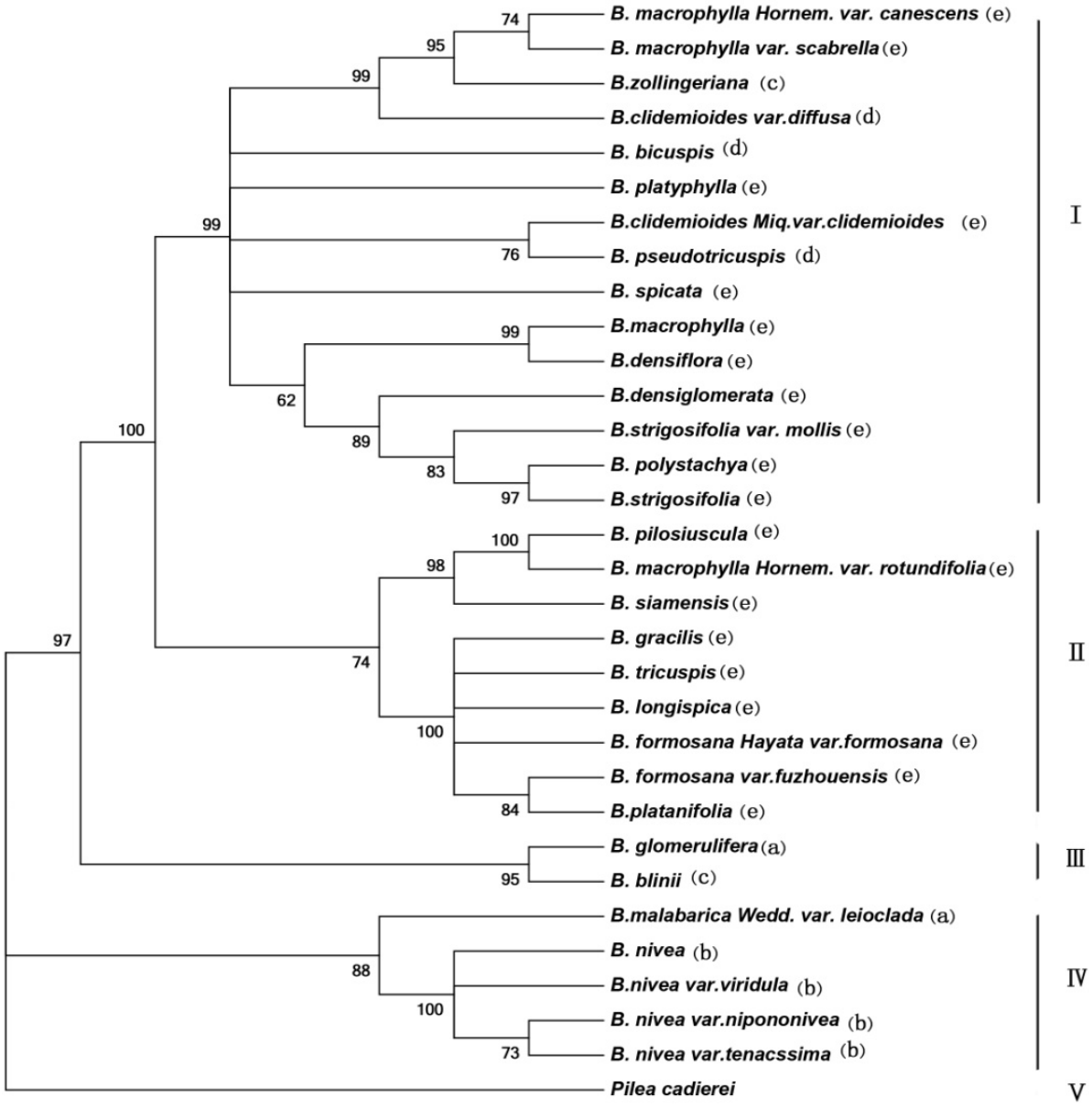

Fig. 2. NJ phylogenetic tree based on the combined ITS and trn*L-F* dataset. The tree has 855 steps, with CI = 0.7532 and RI = 0.7925. Base substitutions are indicated above branches. Bootstrap values are indicated below branches. (a) Sect. *Boehmeria*; (b) Sect. *Tilocnide*; (c) Sect. *Zollingeriana*; (d) Sect. *Phyllostachys*; (e) Sect. *Duretia*

Morphologically, Sect. *Boehmeria* is considered the most primitive group of *Boehmeria* (Wang, 1981, which is supported by the SRAP results (Liao *et al*., 2010). In the present study, *B. glomerulifera* of Sect. *Boehmeria* formed a single clade at the roots, indicating its primitiveness. Additionally, our results showed that *B. malabarica* var. *leioclada* of Sect. *Boehmeria* as well as one species and three varieties of Sect. *Tilocnide* were clustered into a clade. This indicates that the phylogenetic relationship of the two groups is close. These results provide molecular evidence for the view of Wang & Cheng (1995) and provide important information for the expansion of wild core germplasm resources in *Boehmeria* breeding.

As the largest group in *Boehmeria*, Sect. *Duretia* is considered the group at the highest evolutionary level (Wang, 1981). In combination with the morphological study, two types of genetic relationship patterns have been proposed: the Sect. *Phyllostachys*–Sect. *Duretia* pattern and the Sect. *Duretia*–Sect. *Phyllostachys* pattern (Wang, 1981; Zhang, 1998). Sect. *Phyllostachys* was clustered into the first group with Sect. *Duretia*, indicating a close relationship between these two groups. However, the group's evolutionary pattern, based on morphology, is not supported at the DNA level by the analysis of ITS and *trn*L-F sequences in the present study. Our results are not consistent with the RAPD results (Guo *et al*., 2003); however, they are supported by the SARP results (Liao *et al*., 2010).

Sect. *Zoilingeriana* generally has bush morphology.

Unlike other groups, the leaves in this group are alternate or opposite (Wang & Cheng 1995). In this study, the *B. zollingeriana* of Sect. *Zoilingeriana* and Sects *Duretia* and *Phyllostachys* were clustered into the same clade, showing their close relationship (Fig. 2, clade I). Additionally, *B. blinii* of Sect. *Zoilingeriana* was closely related to *B. glomerulifera* of Sect. *Boehmeria* (Fig. 2, clade III). These indicate the possibility of interspecific hybridization among *Boehmeria*. The molecular biological classification provides additional evidence for the traditional morphological classification (Turi *et al*., 2012). The study results are of great significance for the collection and conservation of wild Chinese ramie germplasm resources, and the opening of new fields of study and use of ramie.

Conclusion

There is problematic taxonomy with some species of genus *Boehmeria*. This study aimed at establishing information on the phylogeny and evolution of this group using the combination of *trnL*-F and ITS sequences. This study incorporated the most comprehensive plant materials so far used, making it a good supplement to previous studies. Since little is known about the phylogenetic relationship of wild germplasm resources of ramie in China, this study on the evolutionary relationships in the sections of the genus will improve our understanding of taxonomic demarcations in *Boehmeria* and enable exploring of new fields in the study and use of ramie.

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