Botanical Studies (2009) 50: 343-352.

Molecular phylogeny of *Myricaria* (Tamaricaceae): implications for taxonomy and conservation in China

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(Received September 23, 2008; Accepted March 4, 2009)

ABSTRACT. The genus *Myricaria* belongs to the family Tamaricaceae, which consists of thirteen species, ten of which are distributed in China. They are riparian or lake-side shrubs and naturally occur in eastern Asia, extending to central Asia and Europe, with a suggested center of origin and diversity in the Himalayan region. Most of the species are threatened by increasing habitat fragmentation and anthropogenic disturbances like dam and highway construction and over-grazing. Information on molecular phylogenetic relationships is critical for understanding the taxonomy and developing conservation strategies for *Myricaria* species in China. In the present study, DNA sequence data from the nuclear ribosomal internal transcribed spacer region and the plastid *psb*A-*trn*H intergenic spacer were used to infer the phylogeny of the genus. Thirteen morphological traits were also used in conjunction with the molecular phylogenetic relationships. The phylogenetic analysis revealed a basal clade of *M. elegans* to other *Myricaria* species. Molecular evidence resolved one suspicious specimen *Myricaria* sp. that was closely related to *M. wardii*. Furthermore, the results revealed that three widespread species—*M. paniculata*, *M. bracteata* and *M. squamosa*—with little morphological difference have distinct DNA sequences. On the other hand, *M. pulcherrima* and *M. platyphylla* were found to be grouped with the above three widespread species despite their morphological characters being similar to that of *M. elegans*. Also, *M. laxiflora* was found in a more basal phylogenetic position than *M. paniculata* although the two species are morphologically similar. Our phylogenetic analyses provided molecular evidence in supporting the hypothesis that the center of origin and evolution for *Myricaria* species is the Himalayan region. The present study provides useful baseline data for formulating conservation priorities and further taxonomic delineation.

Keywords: Conservation; Morphology; *Myricaria*; Phylogeny; Tamariacaceae; Taxonomy.

INTRODUCTION

The genus *Myricaria* Desv. belongs to the family Tamaricaceae, which comprises three genera, including *Tamarix* L., *Reaumuria* L., and *Myricaria* Desv., and about 110 species widely distributed in Europe, Africa, and Asia (Zhang and Zhang, 1984; Zhang, 2005). Although the equivocal taxonomy, which includes from three to five genera within Tamaricaceae, has remained controversial, *Myricaria* is most closely related to *Tamarix* since it was erected as a genus from *Tamarix* and the first species of *M. squamosa* Desv. was described in 1825 by Desvaux based on the distinct morphological difference in stamens, stigma, and leaflet (Zhang and Zhang, 1984). The most recent revision by Zhang and Zhang (1984) principally considered the taxa native to China, but it included 13 *Myricaria* species. They re-treated a specimen under *M. germanica* (L.) Desv. collected in China as a new species *M. paniculata* P. Y. Zhang et Y. J. Zhang and uplifted *M. germanica* var. *laxiflora* Franch. as a species *M. laxiflora* P. Y. Zhang et. Y. J. Zhang. In this extensive revision, Zhang and Zhang (1984) also combined *M. alopecuroides* Schrank into *M. bracteata* Royle based on the morphological similarity. Otherwise, two species *M. hedinii* O. Paulsen and *M. laxa* W. W. Sm. previously described based on specimens collected from Tibet were discarded because of confounding synonymy with *M. prostrata* Hook. f. et Thoms ex Benth. et Hook. f. and *M. squamosa*. However, differences in morphological characteristics between species or infraspecific taxa are not always clear cut and consistent, and there has been debate as to what degree of morphological differences observed justifies the delineation of taxa. For example, the taxonomic status of the species *M. elegans* Royle has remained debatable and controversial (Zhang et al., 2000; Hua et al., 2004; Zhang et al., 2005).

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Myricaria has a wide geographic distribution extending from eastern Asia to Europe, its original native center and diversity being the Qinhai-Tibet Plateau (QTP), China and adjacent regions (Zhang and Zhang, 1984). It was generally accepted that the common ancestor of *Myricaria* originated in the Paleo-Mediterranean sea region; like other genera in Tamariacaceae, the Himalayas was suggested as the center of origin. The phylogenetic divergence and speciation of *Myricaria* occurred with the rise of the QTP during the Tertiary (Zhang and Zhang, 1984; Zhang et al., 2001). Of the 13 species included in the genus *Myricaria*, ten species and one variety are naturally distributed in China and only three species—*M. germanica* (Linn.) Desv., *M. dahurica* (Willd.) Ehrenb. and *M. longifolia* (Willd.) Ehrenb.—are distributed in Eurasia extending from central Asia to western Europe (Zhang and Zhang, 1984; Wang et al., 2006). *Myricaria* species are all deciduous shrubs of upstanding, recumbent or creeping forms, depending on the habitat, and are distributed in over 14 provinces within and around the QTP and adjacent regions in China (Zhang and Zhang, 1984; Wang et al., 2006). Except for the three widespread species *M. bracteata*, *M. paniculata* and *M. squamosa*, most *Myricaria* species in China have narrow distributions in montane areas close to rivers or lakes. Their vertical distributions in altitudes are variable, ranging from 70 to 5200 meters above sea level (a.s.l.) (Wang et al., 2006). An attempt to document morphological variation and taxonomic revision of *Myricaria* species (Zhang and Zhang, 1984) based on only morphological data failed to produce satisfactory results, but called interspecific relationships among some species into question. Moreover, recent extensive field surveys found that taxonomic status and/or classification of species such as *M. paniculata*, *M. squamosa* and *M. bracteata* appeared to be vague and problematic (Wang et al., 2006). On the other hand, previous studies in phylogenetic relationships and phytochemistry sampled only a limited numbers of species in *Myricaria* and focused on some specific questions (Cheng et al., 2000; Jetter, 2000; Gaskin et al., 2004; Hua et al., 2004). The phylogeny and taxonomy of *Myricaria* is still debatable. More detailed information is needed concerning the origin and phylogeny of *Myricaria* species.

Myricaria species are predominant taxa in the riparian plant communities in western China and play important roles in ecosystem services to local harsh environments and sustainable development in the western region (Wang et al., 2003). However, recent field surveys and studies have indicated that the majority of *Myricaria* species in their native habitats of western China are threatened due to increasing anthropogenic disturbance and habitat fragmentation (Wang et al., 2006). The riparian habitats are very fragile and severely degraded by anthropogenic activities in western China. An increasing number of dams have been constructed for hydroelectricity and agricultural irrigation in the past two decades. A well-known example is *M. laxiflora*, a species distributed exclusively on the riverbanks of the Yangtze River Valley within the Three Gorges Region. Construction of the Three Gorges Dam raised water levels and submerged its most distribution range (Wang et al., 2003; Liu et al., 2006). Several populations of *M. paniculata* are also severely disturbed by dam and highway construction (Wang et al., 2006). In addition, desertification and soil erosion in the range of *Myricaria* has severely damaged natural habitats of many species and impacted survival of local populations. The population sizes of *M. pulcherrima* have been sharply declining due to the disappearance of lakes/wetlands in the Tengger Desert. *Myricaria platyphylla* is also endangered because of overgrazing and habitat degradation (Wang et al., 2006). A well-resolved phylogeny is needed to develop and prioritize the conservation and management strategies in consensus with defining evolutionary significant species and/or taxa (Russello and Amato, 2004; Dávalos et al., 2005; Ewen et al., 2006).

The primary purposes of this study are : 1) to reconstruct a molecular phylogeny of *Myricaria* including all ten species collected from 17 different geographic regions in China and two species obtained by other sources using sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA and the chloroplast *psb*A-*trn*H intergenic spacer; 2) to assess the taxonomic distinctness and rank of the species in comparison to the reported phylogenetic relationships previously established based on morphological and molecular analysis of a number of Chinese endemic *Myricaria* species available; and 3) to address conservation concerns about *Myricaria* species endemic to China in consensus with phylogenetic pattern of the genus.

MATERIALS AND METHODS

Plant material

Several extensive field surveys were conducted during the summers from 2002 to 2005 in western, northwestern, southwestern, and central China. Sources of plant material used in this study are listed in Table 1, including 11 taxa and one suspicious new taxon which were collected at 17 different geographical locations from nine provinces or autonomous regions. The data of two other nonnative taxa obtained from GenBank (ITS sequence nos: AF484746 and AY572413) were also included. Two species, *Tamarix androssowii* Litv. and *Reaumuria songarica* (Pall.) Maxim, from related genera in Tamaricaceae were included in the analyses as outgroups. One unclassified *Myricaria* specimen, tentatively named *Myricaria* sp., was found in the mountain area of Yunnan Province in southwest China and was obviously morphologically different from all documented *Myricaria* species. It was also included in the phylogenetic analyses. All voucher specimens were deposited in Wuhan Botanical Garden Herbarium (HIB). Leaf materials were collected and immediately dried in silica gel for DNA extraction, and sampling sites were recorded using a global positioning system (GPS, Garmin, eTrex).

Table 1. List of *Myricaria* species and outgroups used in this study and accession numbers for sequences deposited in GenBank.

Taxon	Natural distribution	Collection locality	Habitats of sampling locality	Altitude (m)	Longitude /Latitude	Collector and specimen voucher	GenBank accession number	
							ITS	*psb*A–*trn*H[a]
Myricaria alopecuroides Schrank	-	Obtained from NCBI GeneBank	-	-	-	Provided by J. F. Gaskin and B. A. Schaal	AF484746	-
Myricaria bracteata Royle	Tibet, Xinjiang, Qinghai, Gansu, Sichuan, Shaanxi, Inner Mongolia, Shanxi	Sunan, Gansu, China (specimen 1)	Riparian sand	2140	99°44′52″ /38°54′29″	Yong Wang and Yifei Liu, HIB-WY362	EU240596	EU240615
		Zhangye, Gansu, China (specimen 2)	Riparian sand	1430	100°26′54″ /39°03′14″	Yong Wang and Yifei Liu, HIB-WY369	EU240597	-
		Lixian, Sichuan, China (specimen 3)	Riparian sand	2565	102°49″03″ /31°35′08″	Yong Wang and Yifei Liu, HIB-WY264	EU240598	-
Myricaria elegans Royle	Tibet, Xinjiang	Agazi, Yecheng, Xinjiang, China	Riparian sand	2500	76°53′42″ /37°03′43″	Yong Wang and Yifei Liu, HIB-WY379	EU240594	EU240613
Myricaria elegans var. *tsetangensis* P. Y. Zhang et Y. J. Zhang	Tibet	Zedang, Shannan, Tibet, China	Riparian sand	3564	91°49′07″ /29°16′22″	Yong Wang and Yifei Liu, HIB-WY389	EU240595	EU240614
Myricaria germanica (L.) Desv.	Central Asia and Europe	Obtained from NCBI GeneBank	-	-	-	Provided by D. Zhang, Y. Zhang and Z. Chen	AY572413	-
Myricaria laxiflora (Franch.) P. Y. Zhang et Y. J. Zhang	Hubei, Chongqing	Zitong, Banan, Chongqig, China (specimen 1)	Sand island within river	150	106°58′38″ /29°46′11″	Yong Wang and Yifei Liu, HIB-WY105	EU240610	-
		Wangjiang, Zigui, Hubei, China (specimen 2)	Riparian sand	78	110°55′55″ /30°52′24″	Yong Wang and Yifei Liu, HIB-WY108	EU240609	EU240620
Myricaria paniculata P. Y. Zhang et Y. J. Zhang	Tibet, Qinghai, Gansu, Sichuan, Yunnan, Shaanxi, Henan	Helong Bridge, Deqin, Yunnan, China (specimen 1)	Riparian sand	2100	99°25′06″ /28°09′05″	Yong Wang and Yifei Liu, HIB-WY406	EU240599	-
		Xichang, Sichuan, China (specimen 2)	Riparian sand	1616	102°10′27″ /28°09′36″	Yong Wang and Yifei Liu, HIB-WY411	EU240600	EU240616
Myricaria platyphylla Maxim.	Shanxi, Inner Mongolia, Ningxia	Shapotou, Zhongwei, Ningxia, China	Dune in desert	1248	105°02′56″ /37°30′23″	Yong Wang and Yifei Liu, HIB-WY401	EU240606	EU240618
Myricaria prostrate Hook. f. et Thoms. ex Benth. et Hook. f.	Tibet, Xinjiang, Qinghai, Gansu	Yemadaquan, Subei, Gansu, China	Billabong of hillside	3824	96°06′35″ /39°27′52″	Yong Wang and Yifei Liu, HIB-WY357	EU240607	EU240624
Myricaria pulcherrima Batal.	Xinjiang	Keriya River, Yutian, Xinjiang, China (specimen 1)	Riparian sand	1390	81°42′26″ /36°52′05″	Yong Wang and Yifei Liu, HIB-WY371	EU240601	-
		Sangzhu River, Pishan, Xinjiang, China (specimen 2)	Riparian sand	2257	78°19′16″ /37°03′17″	Yong Wang and Yifei Liu, HIB-WY374	EU240602	EU240619
Myricaria rosea W. W. Sm.	Tibet, Yunan	Galong Mountain, Bomi, Tibet, China	Stonily beside brook	3528	95°41′56″ /29°48′20″	Yong Wang and Yifei Liu, HIB-WY399	EU240608	EU240621
Myricaria sp.	Yunnan	Baima Mountain, Deqin, Yunnan, China	Droughty hillside	3920	98°59′09″ /28°24′07″	Yong Wang and Yifei Liu, HIB-WY405	EU240603	EU240622
Myricaria squamosa Desv.	Tibet, Xinjiang, Qinghai, Gansu, Sichuan	Laoyushu, Kangding, Sichuan, China	Riparian sand	3022	101°57′23″ /29°57′14″	Yong Wang and Yifei Liu, HIB-WY410	EU240604	EU240617
Myricaria wardii Marq.	Tibet	Apei, Gongbujiangda, Tibet, China	Riparian sand	3374	93°19′15″ /29°54′05″	Yong Wang and Yifei Liu, HIB-WY385	EU240605	EU240623
Tamarix androssowii Litv.	Xinjiang, Gansu, Inner Mongolia, Ningxia	Shapotou, Zhongwei, Ningxia, China	Desert	1240	105°02′56″ /37°30′24″	Yong Wang and Yifei Liu, HIB-WY002	EU240612	EU240625
Reaumuria songarica (Pall.) Maxim	Xinjiang, Inner Mongolia, Qinghai, Ningxia, Gansu	Gonghe, Delingha, Qinghai, China	Hungriness	3154	97°27′36″ /37°22′26″	Yong Wang and Yifei Liu, HIB-WY345	EU240611	EU240626

[a]Only one sample of each species has been used in *psb*A-*trn*H intergenic spacer phylogenetic analysis because no intraspecific sequence polymorphism has been detected.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from dried leaves using a modified CTAB protocol (Doyle and Doyle, 1987). An informative nuclear ITS region (partial sequences of ITS1 and ITS2, complete sequence of 5.8S) confirmed in previously molecular phylogenetic analysis of Tamaricaceae species (Zhang et al., 2000; Gaskin et al., 2004) was amplified with the primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC) (White et al., 1990) and ITS5p (5′-GGA AGG AGA AGT CGT AAC AAG G-3′) (Swensen et al., 1998). The primers *psb*A (5′-GTTA TGCA TGAA CGTA ATGC TC-3′) and *trn*H (5′-CGCG CATG GTGG ATTC ACAA ATC-3′) developed by Sang et al. (1997), which are widely used for plant phylogenetic analysis (e.g. Small et al., 1998; Shaw et al., 2005), were utilized to amplify the *psb*A-*trn*H intergenic spacer in the chloroplast genome. Reaction volumes were 25 μL and contained 1.5U Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, USA), Replitherm buffer, 2.0 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} dNTP, 0.2 μmol L^{-1} primer, and 25-60 ng sample DNA. PCR was performed in a PTC-200 thermocycler (Bio-Rad Life Science, Hercules, USA). PCR amplifications for the *psb*A-*trn*H intergentic spacer were carried out at an initial denaturation of 94°C for 4 min, followed by 35 cycles at 94°C for 45 s, 50°C for 1 min, and 72°C for 1 min, finally followed by an extension of 7 min at 72°C. Amplification of the ITS region was conducted with 35 cycles at 94°C for 45 s, 50°C for 1 min, and 72°C for 1 min 30 s, followed by a final extension of 7 min at 72°C. PCR products were purified using the E.Z.N.A® Gel Extraction Kit (Omega Bio-Tek, Norcross, USA). Purified PCR products were then sequenced by a commercial laboratory (Sunbiotech Co., Ltd, Beijing, China) in an ABI 3730XL automated sequencer. All sequence data have been deposited in GenBank (Table 1, Accession Nos. EU240594-EU240612 for ITS sequences and EU240613-EU240626 for *psb*A-*trn*H intergenic spacer sequences).

Molecular phylogenetic analysis

Sequences were aligned using Clustal X (Thompson et al., 1997) and then edited manually. For all analyses, characters were equally weighted, gap areas were treated as missing data, and their phylogenetically informative insertion/deletion occurrences were included as unweighted binary characters (presence/absence) according to the method of Simmons and Ochoterena (2000). Sequence divergence was measured using the Kimura two-parameter model implemented in PAUP* version 4.0b10 (Swofford, 2003).

Optimal trees were inferred using maximum parsimony (MP) as implemented in PAUP* based on the two different data sets of nuclear divergence (ITS sequence) and cytoplasm divergence (*psb*A-*trn*H intergentic spacer). Parsimony analyses were conducted using a heuristic search mode with 1000 random-addition sequence replicates, tree bisection-reconnection (TBR) branch-swapping and MULTrees option on. The consistency index (CI) and retention index (RI) (Kluge and Farris, 1969; Farris, 1989) were calculated. Non-parametric bootstrap analyses (Felsenstein, 1985) were carried out for 1000 replicates of full heuristic search with tree TBR branch swapping to infer node stability. The incongruence length difference (ILD) was tested to determine whether the ITS and *psb*A-*trn*H intergentic spacer data partitions differed significantly from random partitions of the combined data. This was implemented as the partition homogeneity test in PAUP* by using 100 replicates and 1,000 random-addition starting sequences.

Bayesian analyses were conducted using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) based on the same data sets and also the combined data set to provide an additional confirmation consistent with the trees obtained from the MP method. The best-fit model of nucleotide sequence evolution was determined by the program Modeltest 3.7 (Posada and Crandall, 1998). This was conducted by using a more general GTR + I + G model (General Time-Reversible model, with gamma distribution (Γ) and proportion of invariable sites (I), Rodrígues et al., 1990) for Bayesian simulation. Values for model parameters were allowed to vary in different partitions but were not specified a priori (Silva-Brandão et al., 2005). Each search was started from random trees with four MCMCs (Markov's chain Monte Carlo simulations) and was run for 6,000,000 generations. We sampled trees every 100 generations and, after excluding the burn-in values, a majority-rule consensus tree was constructed with posterior probabilities for all nodes in the tree. *Tamarix androssowii* and *Reaumuria songarica* were used as the outgroups in all analyses.

Morphological analysis

Thirteen morphological characters (Appendix) were determined based on the key for species descriptions in Yang and Gaskin (2007) to examine morphological variation. The 13 characters of all *Myricaria* plants used in the present study were morphologically verified and transformed into a digitalized matrix that was then subject to a parsimony analysis using the PAUP program. The parameters set for the morphological analysis is consistent with those for MP analysis of molecular data.

RESULTS

Sequence characteristics and comparisons

The sequence variability of all analysed *Myricaria* species and two outgroups is summarized in Table 2. Within *Myricaria*, the aligned length of the ITS region was 687 bp and comprised 65 variable characters, of which 47 (72.3% of the variable characters) were potentially informative. The G+C content ranged from 50.8% to 52.4% with a mean value of 51.1%. In comparison, the

Table 2. Summary of the sequence variability in all analysed *Myricaria* species and 2 outgroups.

Species group	Gene/Intergenic spacer	Aligned length	Sequence length	Variable sites	Informative sites	Mean G + C content
Within *Myricaria*	ITS	687	643 - 685	65 (9.5%)	47 (6.8%)	51.1%
	*psb*A-*trn*H	416	390 - 400	30 (7.2%)	12 (2.9%)	27.2%
	Total	1103	1066 - 1086	92 (8.3%)	54 (4.9%)	42.5%
With outgroups	ITS	689	643 - 686	220 (31.9%)	96 (13.9%)	52.1%
	*psb*A-*trn*H	439	362 - 413	98 (22.3%)	27 (6.2%)	27.8%
	Total	1128	1005 - 1086	318 (28.2%)	123 (10.9%)	43.5%

*psb*A-*trn*H sequences, with an aligned length 416 bp, revealed a less informative resolution in that only 12 (40.0% of the variable characters) parsimony informative sites were found in a total of 30 variable nucleotide sites. The G+C content of *psb*A-*trn*H ranged from 26.6% to 29.8% with a mean value of 27.2%. However, the variable sites and the G + C content became high when analyses were conducted on combing *Myricaria* species and two outgroups (Table 2). The ITS sequence divergence ranged from 0% to 7.89% among *Myricaria* species and from 0% to 30.14% when outgroups were included while the *psb*A-*trn*H sequence divergence ranged from 0.25% to 4.82% and from 0.26% to 18.03% without and with outgroups, respectively.

Molecular Phylogenetic relationships

Parsimony analysis based on ITS sequences yielded twelve most parsimonious trees of 279 steps (CI = 0.957; RI = 0.929). The strict consensus tree is shown in Figure 1A, revealing consistency with taxonomic treatment based on morphology for most species (Zhang and Zhang, 1984). No intraspecific polymorphisms were detected in the specimens collected from different regions and a preferential clustering appeared in accordance with the taxonomic relationships of *M. pulcherrima*, *M. paniculata*, *M. laxiflora* and *M. bracteata*. However, it was interesting to note that three specimens of *M. bracteata* were parallel clustered with the *M. alopecuroides* that was previously treated as *M. bracteata* by Zhang and Zhang (1984). Also, this largest parallel cluster in the ITS tree included two specimens of *M. paniculata* which are morphologically similar to *M. bracteata* and *M. alopecuroides*. The suspicious specimen *Myricaria* sp. was presented in a paraphyletic relationship with four other species: *M. squamosa*, *M. wardii*, and *M. platyphylla* (native in China) and the species *M. germanica* (native in Europe). Two

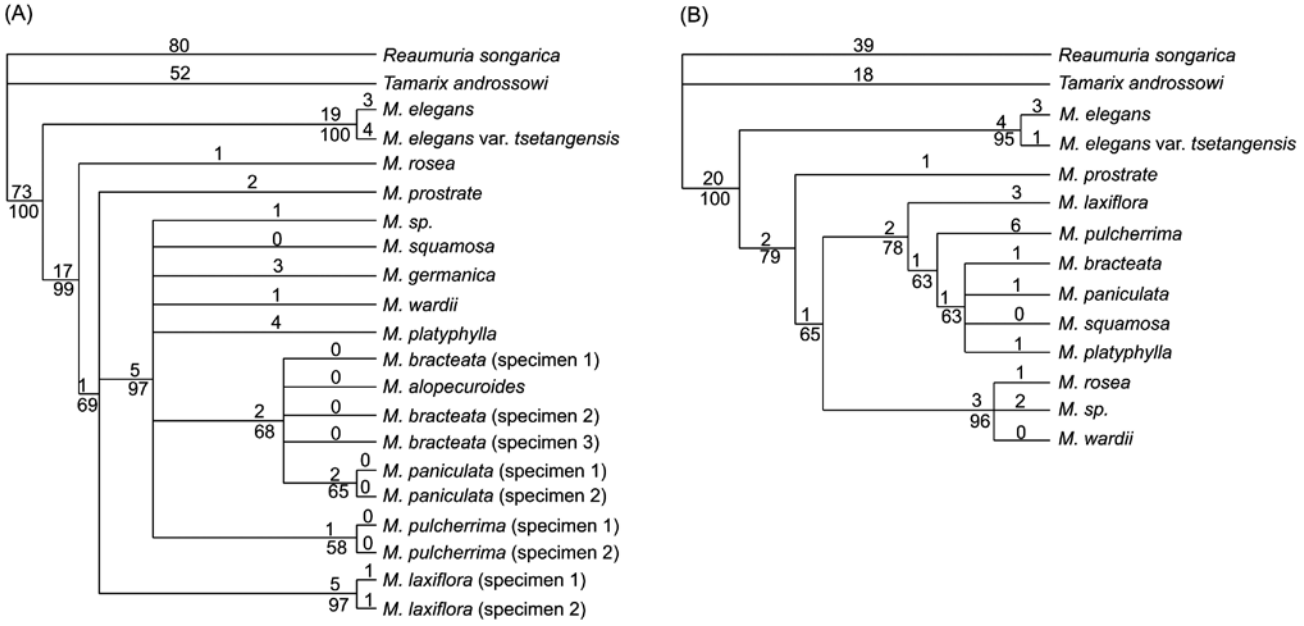

Figure 1. Phylogenetic analysis based on Maximum Parsimony method for *Myricaria*. (A) Strict consensus tree of 12 most-parsimonious trees of 279 steps (CI: 0.957; RI: 0.929) based on ITS sequences; (B) Strict consensus tree of 13 most-parsimonious trees of 111 steps (CI: 0.946; RI: 0.882) based on *psb*A-*trn*H sequences. Numerals above each branch are branch lengths and Bootstrap percentages (60%) are given below branches.

varieties of *M. elegans* (*M. elegans* var. *elegans* and *M. elegans* var. *tsetangensis*) grouped together with a high bootstrap value (100%) and formed the basal node in the tree.

In comparison to the phylogenetic tree topology based ITS sequences, a slight difference from the *psb*A-*trn*H tree was revealed. Parsimony analysis of *psb*A-*trn*H sequences resulted in 13 most parsimonious trees of 111 steps (CI = 0.946; RI = 0.882). The strict consensus tree is shown in Figure 1B. The most significant difference in the *psb*A-*trn*H tree is the appearance of a single clade including *M. rosea*, *M. wardii*, and the suspicious specimen *Myricaria* sp. with a high bootstrap value (96%), and a clade paraphyletically including *M. paniculata*, *M. bracteata*, *M. squamosa*, and *M. platyphylla*.

The ILD test identified incongruence between the strict consensus tree derived from the ITS and the one derived from the *psb*A-*trn*H sequences (P = 0.0100) by parsimony analyses. However, the model-based Bayesian analysis, which uses the best-fit model of nucleotide sequence evolution to obtain a posterior phylogenetic tree (Liu and Pearl, 2007), can partially reduce the impact from sequence heterogeneity. Moreover, the Bayesian tree, derived from a combined data set, revealed a resolved node-construction and phylogenetic relationships among *Myricaria* species with higher bootstrap values. The Bayesian tree was thus shown in Figure 2 to depict the overall species relationship. Within this Bayesian tree, *M. pulcherrima* and *M. platyphylla* were more closely related to *M. paniculata*, *M. bracteata*, and *M. squamosa* and formed the largest phylogenetic group while a sister lineage to it was made up of the species *M. wardii* clustered together with the suspicious specimen *Myricaria* sp. *M. laxiflora* took up a relatively inner phylogenetic position in the tree together with the species *M. rosea* while *M. prostrate* was found phylogeneticly more basal than the other species, with the exception of the most basal clade *M. elegans* and its variety *M. enegans* var. *tsetangensis*.

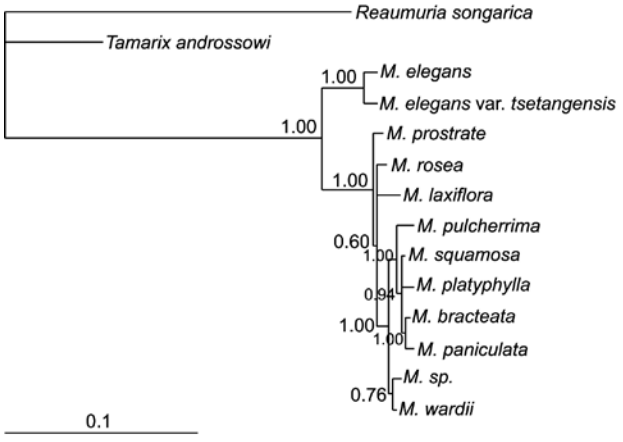

Figure 2. Phylogenetic tree inferred by Bayesian analysis from the combined ITS and *psb*A-*trn*H data set for *Myricaria* species in China. Values at nodes indicate Bayesian posterior probability.

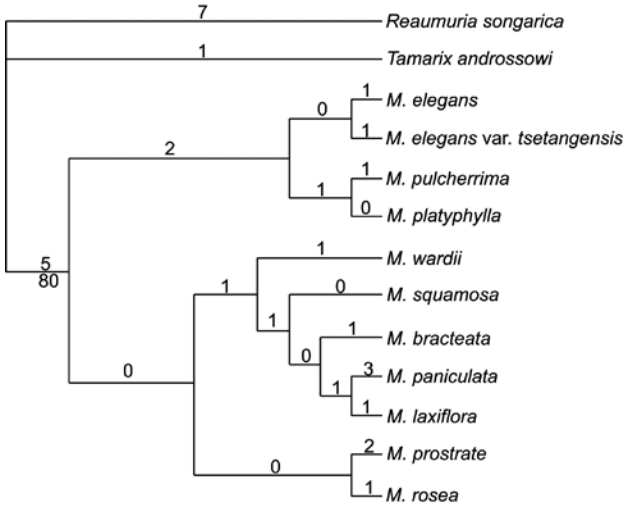

Figure 3. Morphologically parsimony relationships resulting from 13 morphological characters of *Myricaria* species naturally distributed in China. Strict consensus tree was produced from 32 most-parsimonious trees of 184 steps (CI: 0.656; RI: 0.607). Numerals above each branch are branch lengths and Bootstrap percentages are given below branches.

Morphological relationships

The morphological data set of 13 characters produced 184 minimum-length trees of 32 steps with a CI = 0.656 and RI = 0.607. Three main groups were identified in the strict consensus tree despite most clade nodes supported with lower bootstrap values (<60%) (Figure 3). The first group contained *M. prostrata* and *M. rosea* which both feature a spreading growth form. The third group contained *M. platyphylla*, *M. pulcherrima*, *M. elegans*, and *M. elegans* var. *tsetangensis*, all species with large leaves. The second group—including *M. laxiflora*, *M. bracteata*, *M. squamosa*, *M. paniculata*, and *M. wardii*—was the largest morphological group in the parsimony tree, and all species were characterized by upright growth and small leaves, but differed in geographic distribution and altitude range.

DISCUSSION

Incongruence between phylogenetic trees

Although the general topologies of the two MP trees from the nuclear ITS and chloroplast *psb*A-*trn*H sequences are very similar, some incongruent clade arrays have been observed between the trees (Figure 1). The phylogenetic incongruence might involve some evolutionary mechanisms, including incomplete lineage sorting, hidden paralogy and horizontal gene transfer (Galtier and Daubin, 2008). Nevertheless, the chloroplast DNA genome is believed to evolve more slowly than the nuclear DNA genome (Palmer, 1987; Wolfe et al., 1987). In the present study, the nuclear ITS region showed considerably more informative sites than did the chloroplast *psb*A-*trn*H region (Table 2). Thus, the observed incongruence may

possibly have resulted from the variable evolutional rates of different genomes. The ancestral polymorphism of chloroplast genome is possibly not fully resolved into the related monophyletic lineages when the second speciation occurs. More analyses on different gene sequences should help confirm a true discordance between nuclear and chloroplast gene trees.

Phylogenetic relationships

The *M. elegans* and its variants *M. elegans* var. *tsetangensis* formed a basal clade to the rest of the species. This is in agreement with the notion that *M. elegans* may be the evolutionary bridge between *Tamarix* and *Myricaria* (Zhang et al., 2000; Hua et al., 2004; Zhang, 2005) or an interspecific hybrid between them. Deciding which was the case was considered to be a difficulty of taxonomic delineation in previous studies (Zhang et al., 2000, 2001; Zhang, 2005). However, the phylogenetic analysis in the present study demonstrates that the placement of *M. elegans* into *Myricaria* is highly supported by parsimony trees with bootstrap values of 100% and by the Bayesian tree with the posterior probability of 1.00, which was well in accordance with the evidence reported by Hua et al. (2004) and our morphological analysis. Thus, it is reasonable to believe that *M. elegans* is an evolutionary bridge rather than an interspecific hybrid origination.

The ITS tree showed no resolution between *M. alopecuroides* (ITS sequence from GeneBank) and *M. bracteata*. This is consistent with the recent taxonomic re-treatment by Zhang and Zhang (1984), who merged *M. alopecuroides* into *M. bracteata*. In contrast, the ITS tree revealed molecular divergence among *M. paniculata*, *M. squamosa*, and *M. bracteata* although they were all widespread species with little in the way of interspecific morphological difference, i.e. differences in inflorescence, bractlet size, and shoot involucra (Wang et al., 2006). Molecular phylogenetic analysis of the nuclear genome was thus of value in discriminating among these three species.

Interestingly, all phylogenetic trees (Figures 1-3) pointed to a close relationship of the suspicious specimen *Myricaria* sp. to *M. wardii*. In morphological and taxonomic examination, the most distinct difference of *Myricaria* sp. from other described *Myricaria* species was its bigger, tree-like branches. Based on the key by Zhang and Zhang (1990), the suspicious specimen could not be unambiguously classified in the context of current taxonomic treatment of *Myricaria*. The habitat of the suspicious taxon was also unique, occurring in a drought-ridden mountain slope far away from a brook (Table 1). However, the natural range of both *M. wardii* and the suspicious specimen *Myricaria* sp. either overlap or are adjacent to each other. Considering its geographical distribution and close phylogenetic relationship to *M. wardii*, we propose that the suspicious specimen *Myricaria* sp. be considered a variety or ecological cline of *M. wardii*.

In comparing morphological and molecular trees, a clear difference in specific relationships among the species emerge. On the molecular tree, the large leaf species *M. platyphylla* and *M. pulcherrima* showed close phylogenetic relationships to the small leaf species *M. squamosa*, *M. bracteata*, and *M. paniculata*. In taxonomy, leaf traits (e.g. large vs. small leaves) were the most important attributes in distinguishing *M. platyphylla* and *M. pulcherrima* from other *Myricaria* species (Zhang and Zhang, 1984), and this was well demonstrated on the morphological tree (Figure 3). The phylogenetic relationships as revealed on the molecular tree may reflect some unique evolutionary histories or local adaptive patterns which may or may not be in accordance with morphological changes. The phylogenetic position of *M. laxiflora* revealed in the present study is also quite interesting. On the morphological tree, *M. laxiflora* was most closely related to *M. paniculata* (Figure 3). However, on the Bayesian tree, *M. laxiflora* was adjacent to *M. rosea* at a more basal position than *M. paniculata*. Following the coalescent theory, older evolutionary events tended to have interior phylogenetic positions (Posada and Crandall, 2001). *Myricaria laxiflora* might have an older evolutionary history than *M. paniculata* as suggested by Wu et al. (2003a, b).

Previous studies hypothesized that the center of origin and distribution for *Myricaria* species was the Himalayan region (Zhang and Zhang, 1984; Zhang et al., 2001). Our analysis provided molecular evidence to support this hypothesis. In the Bayesian tree, basal clades including *M. elegans*, *M. prostrata*, and *M. rosea* are mostly distributed in the Himalayas and adjacent regions over the QTP. In contrast, other clades on the phylogenetic tree represented younger evolutionary derivatives that contain widespread species such as *M. bracteata*, *M. paniculata*, and *M. squamosa*. These species mainly occur in the regions outside the QTP, suggesting recent species evolution and expansions. The high altitudes of the mountains in the QTP might also affect the morphological characters of some *Myricaria* species due to long-term adaptive evolution, such as in the recumbent or creeping growth forms of *M. prostrata* and *M. rosea*.

Conservation implication for *Myricaria* species in China

Due to the important roles of *Myricaria* in ecoservice for the vulnerable ecosystem in western China and the threatened and endangered status of most species in the genus *Myricaria* (Wang et al., 2003), conservation strategy and practical action plans are urgently needed to safeguard the endangered and/or quickly disappearing species. The application of phylogenetic analysis to assist determination of conservation priorities has been advocated in many cases in which natural resources are scarce or not all taxa can be equally protected (Hibbett and Donoghue, 1996; Eastwood et al., 2004; Yao et al., 2008). In the genus *Myricaria*, the *M. laxiflora* is a good example of how

the present phylogenetic information can aid ongoing conservation and restoration activities (Wang et al., 2003; Liu et al., 2006). After molecular analysis had revealed its phylogenetic importance and its wild extinct status, this species was given the highest priority (Liu et al., 2006). Similarly, *M. platyphylla* and *M. pulcherrima* should be considered for higher conservation priorities because of their extremely endangered status in the wild due to habitat degradation and fragmentation. Conservation of biodiversity should focus on the significant and broad representation of evolutionary diversity for extant organisms (Russello and Amato, 2004). In the genus, the majority of endemic species, such as *M. prostrata* and *M. elegans*, are genetically divergent based on our phylogenetic examination, suggesting special conservation management should be formulated to preserve these key phylogenetically-based species against the increasing pressure of grazing and other anthropogenic activities in their habitats. Although only two species, *M. laxiflora* and *M. pulcherrima*, are currently on the China Species Red List (http://www.chinabiodiversity.com/redlist/), increased disturbance of riparian habitats (Wu et al., 2003a, b) has raised the threat to most *Myricaria* species. We propose that all *Myricaria* species should be included in the list. Detailed assessments of their threatened status based on more populations and local evolutionary units among and within species are currently under way to conserve these Chinese *Myricaria* species.

Acknowledgements. This research was partially supported by the Chinese Academy of Science (KSCX2-YW-N-061), the Migration Office of Hubei Province (Grant 05053935) and the South China Botanical Garden, Chinese Academy of Sciences (Director Grant-200711). We thank Professor Yaojia Zhang for his valuable suggestions and comments. We also thank Dr. Zhongping Chen, Prof. Borong Pan, Dr. Daoyuan Zhang, Dr. Weikai Bao, and many other local conservation officers and workers for their help with the field survey and sampling; and Dr. Xiaohong Yao, Dr. Ting Wang and the reviewers for their critical comments on an early version of this manuscript.

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Appendix. Character list for morphological relationship analysis of *Myricaria* species (refer to Yang and Gaskin, 2007).

1. Growth forms: upstanding (0), recumbent (1), creepy (2).
2. Leaf base status: amplexicaul (0), free (1).
3. Leaf size: large (5-15mm, 0), small (1.5-5mm, 1).
4. Epidermis: present (0), absent (1).
5. Inflorescence shape: raceme (0), paniculiform (1), raceme in spring but paniculiform in summer (2), single (3).
6. Inflorescence position: terminal (0), axillary (1), mixed (2).
7. Flower number per inflorescence: equal or more than five (0), less than five (1).
8. Flowering times: one times (0), two times (1).
9. Stamen numbers: 6-8 (0), 4-5 (1), 10 (2).
10. Stamen filament base status: free (0), connate (1), connivent (2).
11. Gynoecium: with short gynophore (0), no gynophore (1).
12. Seed pappus: totally covering (0), covering seed top and aristate stem (1), only covering aristate stem (2).
13. Aristate: bearing (0), free (1).

中國水柏枝屬植物的分子系統分類與保育研究

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水柏枝屬隸屬於檉柳科共包含約 13 個物種，其中 10 種分佈於中國。該屬植物多爲河邊或湖邊灌木，以喜馬拉雅地區爲分佈中心並覆蓋從東亞到中亞和歐洲的廣泛地區。隨著不斷增加的人爲干擾例如築壩、修路和過度放牧等導致的生境片斷化加劇，中國大多水柏枝植物的生存狀態受到極大威脅。分析水柏枝屬植物的分子系統關係對於理解它們的分類並制定正確的保育措施具有重要作用。本文利用核基因組 ITS 序列和葉綠體基因組 *psb*A 和 *trn*H 基因間序列資訊分析水柏枝屬植物的分子系統關係，並和形態關係進行比較研究。分子系統樹顯示秀麗水柏枝是最基部的進化支系。同時，分子分析解決了一個形態學分類困難標本的系統分類歸屬問題即作爲小花水柏枝的生態變型，並建立了三個形態近緣種三春水柏枝、寬苞水柏枝和具鱗水柏枝間的分子系統關係。雖然心葉水柏枝和寬葉水柏枝的形態特徵更接近於秀麗水柏枝，但分子分析表明它們同前面三個形態近緣種更加的近緣 ； 類似的，疏花水柏枝也表現了分子和形態關係的差異，即其具有較古老的分子系統發育但形態上類似於三春水柏枝。我們的系統分析支援水柏枝屬植物的喜馬拉雅起源和進化中心假說。基於本分子系統分析，相關物種的保育地位得到了討論。

關鍵詞 ： 保育 ； 形態學 ； 水柏枝屬 ； 系統發育 ； 檉柳科 ； 分類學。