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Research Article

Taxonomic status and distribution of *Mirabilis himalaica* (Nyctaginaceae)

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Abstract *Mirabilis himalaica* (Edgew.) Heimerl (Nyctaginaceae) is endemic to the Himalayas where it is used in traditional Tibetan folk medicine and is the only Old World representative of a large New World genus. The systematic position of *M. himalaica* and historical biogeography of *Mirabilis* and related genera was evaluated using two loci (nuclear ribosomal internal transcribed spacer, *rps16*), with divergence times estimated using internal transcribed spacer sequences. All 16 sampled provenances of *M. himalaica* formed a strongly supported terminal clade and at the sectional level formed a clade with sect. *Quamoclidion* sensu stricto, despite their morphology. Section *Oxybaphoides* and sect. *Oxybaphus* were not closely related to *M. himalaica*, suggesting their apparent morphological similarities are convergent. The beast analysis and ancestral area reconstruction indicated that *M. himalaica* separated from related North American species during the late Miocene to early Pleistocene ~5.22 Ma (95% highest posterior density, 2.53–8.18). Both migration by way of the Quaternary Bering land bridge (Beringia) and long-distance dispersal could have contributed to the present-day disjunction between *M. himalaica* and the American species. These results agree with previous studies that suggest *Oxybaphus* should be merged into *Mirabilis*. However, although the infrageneric position of *M. himalaica* is still uncertain, it is not close to sect. *Oxybaphus* as has been suggested previously.

Key words: divergence time, *M. himalaica*, *Oxybaphus*, phylogeny.

1 Introduction

Mirabilis himalaica (Edgew.) Heimerl (Nyctaginaceae) is endemic to the Himalayas, where its roots are important in Tibetan folk medicine for the treatment of nephritis edematous, renal calculus, arthrodynia, and uterine cancer (Yang, 1991; Linghu et al., 2014). The taxonomic position of *M. himalaica* is controversial, principally because of differing opinions on the status of *Mirabilis* L. and *Oxybaphus* L'Her. ex Willd. *Mirabilis* (~60 spp.) is the most speciose genus of Nyctaginaceae, but its generic and specific delimitations have varied historically (Le Duc, 1995; Spellenberg, 2003), due mainly to very few diagnostic reproductive characteristics among morphologically and ecologically variable forms. The problems are exacerbated in section *Oxybaphus* by autogamy, xenogamy, perennial habit, and high chromosome numbers (Spellenberg, 2003).

Standley (1931) merged *Oxybaphus*, *Hesperonia* Standley, *Quamoclidion* Choisy, and *Allioniella* Rydb. into a broadly circumscribed *Mirabilis*, noting that characteristics used to distinguish North American genera allied to *Mirabilis* were not exclusive. This broad generic concept has been adopted widely (e.g., Pilz, 1978; Guan, 1983; Bittrich & Kühn, 1993; Douglas & Manos, 2007; Douglas & Spellenberg, 2010), with *Mirabilis* comprising ~60 species in temperate and tropical North America and South America and one indigenous to southern Asia (Spellenberg, 2003).

Heimerl (1934) recognized six sections within *Mirabilis*: *Mirabilis* Hook., *Mirabilopsis* Heimerl, *Oxybaphus* (L'Her. ex Willd.) Heimerl, *Quamoclidion* (Choisy) A. Gray, *Oxybaphoides* A. Gray, and *Watsoniella* Heimerl. This infrageneric classification has also generally been followed, but often with differing opinions on sectional delimitation (Pilz, 1978; Le Duc, 1995; Levin, 2000; Spellenberg & Tijerina, 2001; Spellenberg, 2003). Ledesma et al. (2011) studied stem anatomy of the five sections (excluding sect. *Watsoniella*), but did not support the current infrageneric classification. Levin (2000) sampled 13 species from sections *Mirabilopsis*, *Mirabilis*, *Oxybaphoides*, and *Quamoclidion*, concluding that sections *Mirabilis* and *Oxybaphoides* were monophyletic, but that sect. *Quamoclidion* was paraphyletic and *Mirabilis coccinea* (Torr.) Benth. & Hook. f. (normally placed in sect. *Mirabilopsis*) was sister to the remainder of the genus.

Some researchers treat sect. *Oxybaphus* as a separate genus with 25 species from warmer regions of the Americas and one species in Asia (e.g., Edgeworth, 1846; Mukherjee, 1984; Brummitt, 1992; Lu, 1993; Tang, 1996; Lu & Gilbert, 2003). *Oxybaphus* differs from *Mirabilis* in possessing much smaller, campanulate or funnelform flowers with long pedicels that open in the morning and a membranous involucre that enlarges in fruit (Lu, 1993; Lu & Gilbert, 2003). As a result, *M. himalaica* has been treated as: (i) part of a broadly defined *Mirabilis* (sensu Spellenberg, 2003) with two varieties: var. *himalaica* from India to China and var. *chinensis* Heimerl endemic to China (Heimerl, 1932; Guan, 1983; Wu & Chen, 1997); or (ii) as *Oxybaphus himalaicus* Edgew. (Edgeworth, 1846), again with two varieties: var. *himalaicus* and var. *chinensis* (Heimerl) D. Q. Lu (Lu, 1993; Tang, 1996; Lu & Gilbert, 2003; Peng et al., 2014).

In addition, abundant morphological variation within *M. himalaica* has created problems for taxon delimitation in the field. For example, *M. himalaica* var. *chinensis* is distinguished from var. *himalaica* in having five rather than four stamens and sparsely hairy to glabrescent rather than densely hairy stems (Heimerl, 1932; Tang, 1996; Lu & Gilbert, 2003). However, examination of multiple *M. himalaica* samples from Xizang, Yunnan, Sichuan, and Gansu found that stamen number varied from two to six between different flowers on the same plant. Cai et al. (2013) similarly found that stamen number varied in cultivated *M. himalaica* plants from six different provenances. This suggests that this character cannot separate these putative varieties, particularly as similar intraspecific variation in stamen numbers is known for other Nyctaginaceae species such as *Tripterocalyx crux-maltae* (Kellogg) Standl. and *T. micranthus* (Torr.) Hook. (Galloway, 1975). The other putatively defining feature (indumentum density) is also difficult to quantify, especially in living plants.

Lin et al. (2016) concluded that there was no significant difference between internal transcribed spacer (ITS) sequences of wild and cultivated *M. himalaica* from five Chinese districts. However, to date, there have been no broader assessments of this species' variability, or its phylogenetic and biogeographic placement in *Mirabilis* relative to *Oxybaphus*.

Mirabilis himalaica is the only Old World representative of this large New World genus (Lu & Gilbert, 2003). The intercontinental disjunction between *M. himalaica* in Asia and the remainder of the genus in the Americas is one of the more remarkable disjunctions in Nyctaginaceae (Douglas & Manos, 2007; Ranjitkar et al., 2014). However, the Asia/North American disjunction is a classical biogeographic pattern in the Northern Hemisphere and many distinctive taxa show this distribution pattern, including *Liriodendron* L. (Magnoliaceae) (Parks & Wendel, 1990), *Kelloggia* Torrey ex Bentham & J. D. Hooker (Rubiaceae) (Nie et al., 2005), *Sassafras* J. Presl (Lauraceae) (Nie et al., 2007), *Astilbe* Buch.-Ham. (Saxifragaceae) (Zhu et al., 2013), and *Osmorhiza* Raf. (Apiaceae) (Yi et al., 2015). Nevertheless, the presence of *M. himalaica* in Asia is considered unusual, with long-distance dispersal proposed, but not tested (Douglas & Manos, 2007).

Mirabilis himalaica is characterized by an annual, herbaceous habit, terminal or axillary, viscid, involucrate, one-flowered inflorescences of 5-partite flowers with a rosy, campanulate perianth contracted above a 2-celled ovary (Mukherjee, 1984; Lu & Gilbert, 2003). Most studies have focused on its medicinal value (He et al., 1996), chemical constituents (Zhang et al., 1997; Linghu et al., 2014), propagation (Xu et al., 2013), extraction technology (Suolang et al., 2012), allelopathy effects (Xin et al., 2012), and predicted responses to climate change (Ranjitkar et al., 2014). However, its systematic position in relation to New World taxa is unresolved.

This study therefore aims to: (i) determine the taxonomic status of *M. himalaica* and its systematic position in relation to the New World taxa using ITS and *rps16* DNA sequences; and (ii) estimate the divergence time and hypothesize possible causes for its disjunct distribution by molecular clock and ancestral area reconstruction methods.

2 Material and Methods

2.1 Sampling, DNA extraction, sequencing, and sequence alignment

All *Mirabilis* species with ITS sequences in GenBank were used to construct a phylogeny (excluding *M. expansa* (Ruiz & Pav.) Standl. cv. Lima). Based on the molecular topology of Douglas & Manos (2007), species from close relatives (*Acleisanthes* A. Gray and *Commicarpus* Standl.) and more distant taxa (*Pisonia* L. and *Colignonia* Endl.) were chosen as outgroups. Details of ITS and some *rps16* sequences obtained from GenBank and included in this study are provided in Levin (2000), Douglas & Manos (2007), Lee et al. (2013), Hayward & Horton (2014), and Xu et al. (2018). The taxa sampled, together with their GenBank accession numbers, are listed in Table S1.

Samples of *M. himalaica* were collected from Yunnan (LGH1S, BZL2S, and LP1S), Gansu (DLD1S and FC1S), Sichuan (XIA2S, GZZ1, MRK1S, and GQC1S), and Xizang (DBC2S, TNC19, YRC1S, NM03, ZDZ1S, LMS1, and LD08) (all in China). All vouchers are deposited in the Research Institute of Xizang Plateau Ecology Herbarium (XZE), Linzhi, Bayi District, Xizang Autonomous Region, China. The sequences of *M. himalaica* from two loci nuclear ribosomal ITS (nrITS) and *rps16* are deposited in the Dryad repository (Data S1).

Total DNA was extracted from silica-gel dried leaves using the 3× CTAB method (Wang & Li, 2007), with ITS sequences amplified and sequenced using the ITS4 primer of White et al. (1990) and ITS5A primer of Stanford et al. (2000). Reactions were carried out in a total volume of 20 μL consisting of 10× buffer 2.0 μL, $MgCl_2$ (25 mmol/L) 2.0 μL, dNTPs (10 mmol/L) 2.0 μL, DMSO (5%) 1.0 μL, each primer (10 μmol/L) 1.0 μL, BSA (1 mg/mL) 2.0 μL, Taq DNA polymerase (5 U/μL) 0.2 μL, and DNA template (5–10 ng/μL) 2.0 μL. Amplification conditions included initial denaturing at 94 °C for 4 min, followed by 35 cycles of 45 s at 94 °C, 1 min at 53 °C, 1 min at 72 °C, and a final extension for 10 min at 72 °C.

The *rps16* sequences were amplified and sequenced with the primers *rps*F and *rps*R2 (Oxelman et al., 1997). Reactions were carried out in a total volume of 25 μL consisting of 10× buffer 2.5 μL, $MgCl_2$ (25 mmol/L) 2.5 μL, dNTPs (10 mmol/L) 2.0 μL, each primer (10 μmol/L) 1.0 μL, Taq DNA polymerase (5 U/μL) 0.3 μL, and DNA template (5–10 ng/μL) 1.0 μL. The amplification conditions included an initial denaturing at 95 °C for 2 min, followed by 33 cycles of 30 s at 95 °C, 1 min at 56 °C, 2 min at 72 °C, and a final extension for 10 min at 72 °C.

All amplified products were then sequenced in both directions using BigDye 3.1 reagents in an ABI 3770 automated sequencer (Applied Biosystems, Carlsbad, CA, USA). The resulting sequences were assembled and edited using Sequencher 4.5 (GeneCodes, Ann Arbor, MI, USA). Sequences were aligned initially with Geneious 6.1.2 (Biomatters), followed by manual adjustment in BioEdit 7.0.9.0 (Hall, 1999).

2.2 Taxonomic status of *M. himalaica*

Phylogenetic analyses used maximum parsimony (MP) in paup 4.0b10 (Swofford, 2003) and Bayesian inference with MrBayes 3.12 (Huelsenbeck & Ronquist, 2001). As ITS sequences were available for more species of *Mirabilis*, we used both ITS + *rps16* and ITS data alone to build phylogenies. Four samples were chosen to represent *M. himalaica*, as there were only three sequence variants for this taxon in our ITS data.

The heuristic search options for MP analysis were: 100 random taxon additions, tree bisection–reconnection branch swapping, collapse of zero-length branches, and character state changes equally weighted. Gaps were treated as missing data and 1000 trees were saved from each random sequence addition. Bootstrap support (BS) values for internal nodes were estimated with 1000 heuristic bootstrap replicates.

Bayesian analyses used the best-fit evolutionary models GTR+I for ITS and TIM for *rps16*, based on the Akaike Information Criterion as determined by Modeltest 3.7 (Posada & Crandall, 1998). The Markov chain Monte Carlo algorithm was run for 5 000 000 generations with one cold and three heated chains, starting from random trees and sampling one out of every 500 generations. After discarding the first 2500 trees (25%) as burn-in, the remaining trees were used to construct a consensus tree, with the proportion of bifurcations given as posterior probabilities (PP).

2.3 Divergence time and disjunct distribution of *M. himalaica*

Internal transcribed spacer sequence divergence times were evaluated using beast version 1.8.1 (Drummond & Rambaut, 2007), with BEAUti analysis using a GTR nucleotide-substitution model with gamma + invariant sites distribution and gamma shape distribution with four categories, based on the results from Modeltest. "Lognormal relaxed clock" clock model and "speciation: Yule Process" tree model options were implemented in the analysis. The Markov chain Monte Carlo program was set as follows: length of chain 40 000 000 and log parameters every 1000 generations. Tracer version 1.6 (Rambaut et al., 2014) was used to detect the effective sample size for which all parameter values exceeded 200. The condensed tree was obtained using TreeAnnotator with a 25% burn-in.

Muller (1981) listed three possible Nyctaginaceae fossils: *Phaeoptilum* Radlk.-type pollen, *Mirabilis*-type pollen, and *Pisonia*-type pollen. *Phaeoptilum*-type pollen was related by Muller (1981) to *Lymingtonia* Erdtman from the Lower Eocene of Europe, but Jaramillo & Dilcher (2001) reported that *Lymingtonia* pollen from the Upper Paleocene of Colombia (58.7–55.8 Ma) could only be assigned reliably to Caryophyllales.

Similarly, *Magnaperiporites spinosus* González Guzmán from the Lower Eocene (55.8–48.6 Ma) of Venezuela (González Guzmán, 1967) was regarded as having *Mirabilis*-type pollen. This palynomorph ranges from the Maastrichtian to Pliocene and Muller (1981) related it to Nyctaginaceae tribe Nyctagininae, as did Salard-Cheboldaeff (1981), who related it to *Commicarpus* and Beucher (1975), where it was described as "*Boerhavia* L. pollen". However, more recent studies place this palynomorph in Malvaceae (Biagolini et al., 2013). Because of these conflicting affinities, these *Lymingtonia* and *Magnaperiporites* palynomorphs were excluded from the current study.

Accordingly, *Pisonia*-type pollen (Nyctaginaceae: Pisonieae) from the Lower Miocene (23.0–16.0 Ma) of the Marshall Islands (Leopold, 1969; Muller, 1981; Távora et al., 2010) was used for the internal fossil calibration point in Nyctaginaceae. The crown age of *Pisonia* was therefore set applying a normal distribution of 19.5 ± 2.1 Ma, approximating the mean fossil age. Although several Late Cretaceous and Cenozoic macrofossils have been assigned to *Pisonia* (see Knowlton, 1919), they are unverified and cannot be used for phylogenetic dating with certainty.

The earliest reliable fossil occurrence for Phytolaccaceae (the sister family to Nyctaginaceae in Brockington et al., 2009) is *Coahuilacarpon phytolaccoides* Cevallos-Ferriz, Estrada-Ruiz & Pérez-Hernández from the Late Campanian (84.9–70.6 Ma) of Mexico (Cevallos-Ferriz et al., 2008). As this fossil shares infructescence characters with extant *Phytolacca* L., we used *Phytolacca* and related Phytolaccaceae (nine species from six genera) as an external calibration point, setting the crown age of the family with a normal distribution centered at 77.8 Ma and standard deviation of 4.4 Ma.

Ancestral area reconstruction was carried out using the reduced dataset of the condensed tree from beast analysis, by the Lagrange (dispersal–extinction–cladogenesis) method using the default settings (Ree & Smith, 2008). Three biogeographic regions were delimited for *Mirabilis* based on extant species distributions: A, North America; B, South America; and C, the Himalayas.

3 Results

3.1 Taxonomic status of *Mirabilis himalaica*

Both MP and Bayesian analyses were carried out with combined ITS + *rps16* and ITS-only analyses. As the same major clades were recognized in both analyses, we only present the Bayesian topologies here, with both PP values and BS values for the two matrices (Figs. 1, 2). Alignments of the ITS + *rps16* and ITS-only dataset and tree files of phylogenetic analyses are deposited in the Dryad repository (Data S2).

All the *M. himalaica* samples formed a terminal clade with strong support in both the ITS + *rps16* (PP = 100; BS = 100) and ITS-only (PP = 100; BS = 99) analyses (Figs. 1, 2). Within the *M. himalaica* accessions, LD08 was sister to the remainder with moderate support (PP = 100; BS = 78) (Fig. 1).

Mirabilis was also supported strongly as monophyletic (PP = 100; BS = 100) in the ITS + *rps16* and ITS-only analyses (Figs. 1, 2). Furthermore, it was divided into three clades in the ITS topology (Fig. 2): (i) *M. coccinea* (sect. *Mirabilopsis*) was paired with *M. nyctaginea* (Michaux) MacMillan above *M. albida* (Walter) Heimerl (both sect. *Oxybaphus*), these three forming a strongly supported Clade 1 (PP = 100; BS = 100) as sister to the remainder of *Mirabilis*; (ii) the taxa from sect. *Mirabilis* formed a strongly supported Clade 2 (PP = 100;

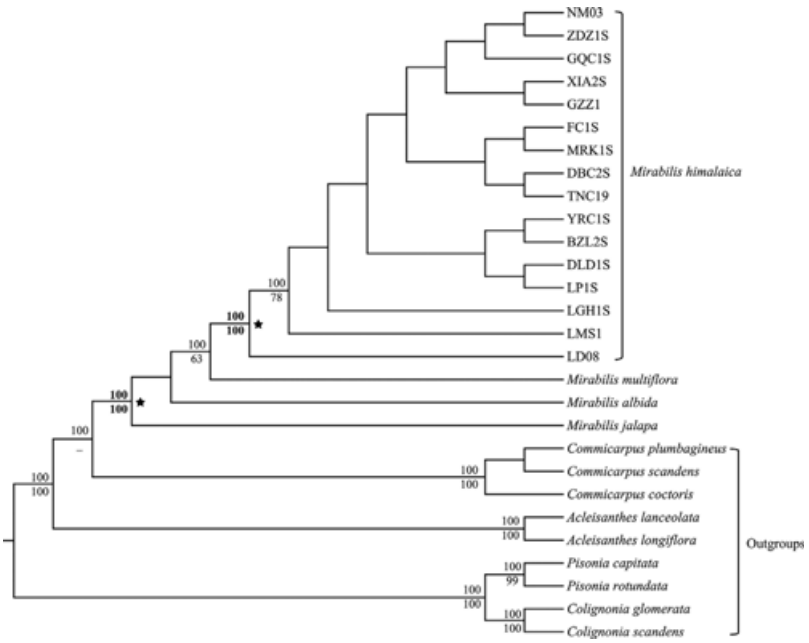

Fig. 1. Bayesian topology from the combined internal transcribed spacer and *rps16* data. Bayesian posterior probability values greater than 95% and bootstrap support greater than 50% are shown above and below the branches, respectively. –, Bootstrap support value <50; ★, major clade.

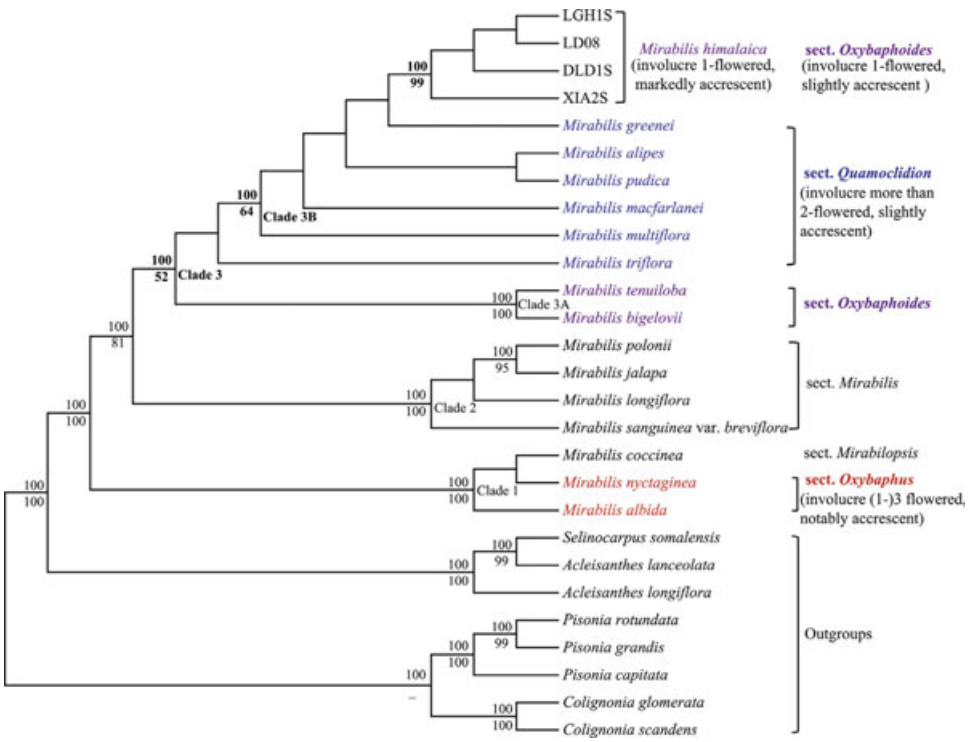

Fig. 2. Bayesian topology of internal transcribed spacer data. Posterior probability values above branches, bootstrap support values below branches. –, Bootstrap support value <50.

BS = 100), agreeing with the results of Levin (2000); and (iii) taxa from sections *Oxybaphoides* and *Quamoclidion* formed Clade 3 with a high posterior probability value, but low bootstrap support (PP = 100, BS = 52), but the two sections were paraphyletic. Within sect. *Oxybaphoides*, *M. tenuiloba* S. Watson and *M. bigelovii* A. Gray formed a strongly supported Clade 3A (PP = 100; BS = 100). The *M. himalaica* accessions together with sect. *Quamoclidion* sensu stricto (excluding

M. triflora Benth) formed Clade 3B with a high PP (PP = 100), but low BS (BS = 64).

3.2 Divergence time evaluation and ancestral area reconstruction

The BEAST analysis (Fig. 3) using ITS sequences and two fossil calibration points suggested a crown age for *Mirabilis* (sensu Spellenberg, 2003) of 13.13 Ma (D2 in Fig. 3; 95% highest posterior density [HPD], 6.91–20.62). The lineage containing *M. coccinea*, *M. albida*, and *M. nyctaginea* diverged first. The stem age for the divergence of *Mirabilis* was estimated at 39.76 Ma (D1 in Fig. 3; 95% HPD, 26.62–54.74). The crown age of Clade 3B (PP = 100, BS = 64 in Fig. 2), representing the divergence of *M. himalaica* from its North American counterparts, was estimated as 5.22 Ma (D3 in Fig. 3; 95% HPD, 2.53–8.18). Alignments of the ITS dataset and tree files of the beast analysis are deposited in the Dryad repository (Data S2).

Ancestral area reconstruction indicated a North American (A, 100%) origin for *Mirabilis* (node a in Fig. 3). Expansion from North America (A) to North America–Himalayas (AC) occurred at node b, albeit with lower support in the phylogeny at the section level. The ancestral area for clade 3B (node c, PP = 100, BS = 64 in Fig. 2) comprising *M. himalaica* and sect. *Quamoclidion* sensu stricto was inferred as North America–

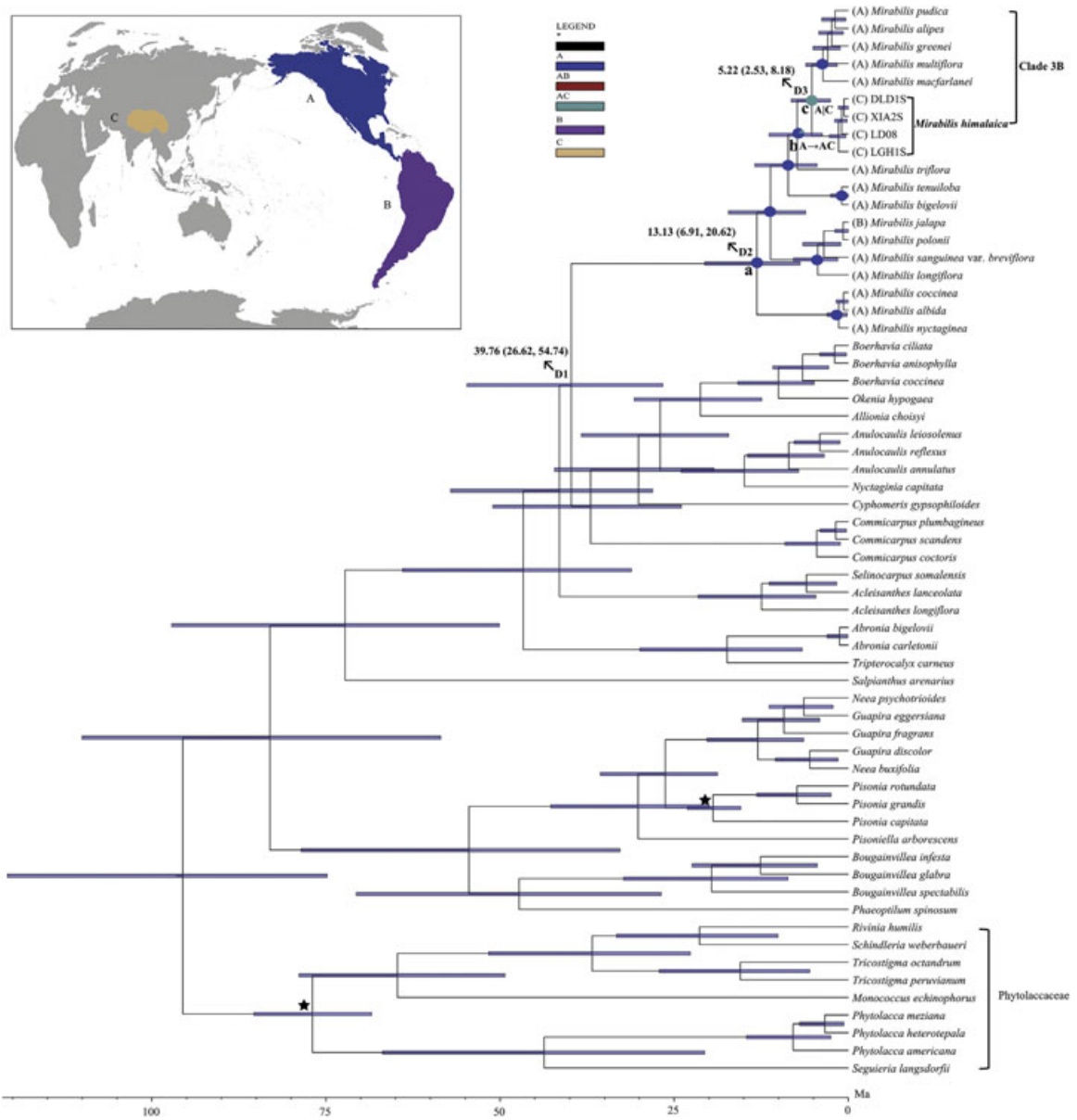

Fig. 3. Maximum clade credibility tree derived from BEAST analysis and topology derived from the dispersal–extinction–cladogenesis model of internal transcribed spacer sequences. Blue bars represent 95% credible interval for each node. The colored circles represent the ancestral areas reconstructed using dispersal–extinction–cladogenesis. ★, Calibration points; the time scale is at the bottom. A, North America; B, South America; C, the Himalayas. The map in the top left corner is derived from http://bzdt.nasg.gov.cn/jsp/browseMap.jsp?picId=%224028b0625501ad13015501ad2bfc0056%22.

Himalayas (AC, 100%), suggesting subsequent isolation (A|C) between the Himalayas (*M. himalaica*) and North America. In summary, the ancestor of *M. himalaica* migrated from North America to the Himalayas, evolving allopatrically into the extant species.

4 Discussion

4.1 Taxonomic status of *Mirabilis himalaica*

Mirabilis was monophyletic in both the ITS and ITS + *rps16* analyses (Figs. 1, 2). However, the three sampled species (*M. himalaica*, *M. albida*, and *M. nyctaginea*) that were placed in *Oxybaphus* by Sweet (1827) and Edgeworth (1846) were not monophyletic (Figs. 1, 2), supporting the relegation of *Oxybaphus* to synonymy within an inclusive *Mirabilis* (sensu Spellenberg, 2003).

Mirabilis himalaica was monophyletic with strong support (PP = 100, BS = 100) in the combined ITS + *rps16* analysis (Fig. 1), indicating that it is a well-defined species, but with no support for morphologically or geographically distinct lineages within it. Below the species level, although accession LD08 was sister to the remainder with moderate support (PP = 100; BS = 78), there was no obvious morphological difference or apparent geographical isolation from other accessions. *Mirabilis himalaica* var. *himalaica* occurs in the western Himalayas and also has been reported anecdotally from southern Xizang (Qamdo and Baxoi counties) (Guan, 1983; Tang, 1996; Lu & Gilbert, 2003). The two accessions YRC1S and TNC19 sampled from Qamdo and Baxoi counties, respectively, mixed with the other accessions (Fig. 1), and no distinct morphological traits were observed. The phylogenetic analysis combined with the stamen variation in the same individual listed above supports the idea that there is a single, morphologically variable species, although more comprehensive sampling across the western Himalayas is needed to investigate its phylogeography.

Our results were largely consistent with Levin (2000) at the section level, except that *M. albida*, *M. nyctaginea*, and *M. himalaica*. *Mirabilis albida*, *M. nyctaginea*, and *M. coccinea* together formed a sister clade to the remainder of the genus. Section *Oxybaphoides* was not monophyletic in our study, with *M. himalaica* placed with taxa from sect. *Quamoclidion* sensu stricto (excluding *M. triflora*). Sect. *Oxybaphoides* sensu lato is characterized by a suffruticose perennial habit and non-tuberous roots, a 1-flowered involucre that is only slightly enlarged in fruit and a mucilaginous anthocarp when wet, (Heimerl, 1932, 1934; Le Duc, 1995). However, sect. *Oxybaphoides* was paraphyletic in the ITS topology (Fig. 2) and the involucre of *M. himalaica* enlarges markedly in fruit, indicating that neither molecular nor morphological data support the inclusion of *M. himalaica* within sect. *Oxybaphoides*.

Section *Quamoclidion* (excluding *M. triflora* and including *M. himalaica*) was monophyletic (Fig. 2) and species in this section mostly possess an involucre with more than two flowers (Heimerl, 1932). Pilz (1978) regarded *Quamoclidion* as a subgenus based on its gamophyllous involucre that surrounds three or more flowers and is only slightly accrescent after anthesis. In contrast, *M. himalaica* has only one flower per involucre and the latter is strongly accrescent after anthesis, suggesting that traditional floral characters are less useful in *Mirabilis* than previously thought.

Species delimitation in sect. *Oxybaphus* is difficult due to extensive intergradation and intraspecific variation in almost all taxa (Spellenberg, 2002). Based on the morphological characteristics used to define the six sections within *Mirabilis* (Heimerl, 1934; Spellenberg, 2003), *M. himalaica* most closely resembles members of sect. *Oxybaphus*, possessing an enlarged 1-flowered involucre that is membranous in fruit. However, in our analyses sect. *Mirabilopsis* was embedded in sect. *Oxybaphus*, whereas *M. himalaica* was terminal in a sect. *Quamoclidion* species clade (Fig. 2), indicating that morphology does not mirror phylogeny in the genus. This conflict could result from adaptive phenotypic plasticity (Svanbäck & Schluter, 2012), as heterogeneous habitats can lead to divergent evolution between closely related taxa, whereas shared environments or pollination syndromes can result in convergent phenotypic evolution between distantly related taxa.

Taxonomic delimitation of sections in *Mirabilis* is not possible from our ITS phylogeny and Ledesma et al. (2011) similarly observed that there were no anatomical features to support the current sectional classification. As a result, the placement of *M. himalaica* into existing sections lacks support and a broadly defined *Mirabilis* without infrageneric sections seems to be preferable, pending more complete sampling of South American taxa.

4.2 Divergence timing and possible causes for disjunct distribution of *M. himalaica*

Research concerning divergence times in Nyctaginaceae is limited. Bell et al. (2010) used *rbcL*, *atpB* exons, and 18S rDNA sequences of 567 taxa from 335 angiosperm families, to evaluate divergence times in a BEAST analysis with 36 calibration points. They found that the crown age of the Nyctaginaceae clade (*Bougainvillea* + *Mirabilis*) was 23 (15–32) Ma. In contrast, the crown age of the equivalent, but better sampled clade in our study (Fig. 3) was 83 Ma (95% HPD, 59–110 Ma). Although this differs markedly from Bell et al. (2010), the very limited sampling for Nyctaginaceae in their study (two species) might account for this.

The Asia–North America biogeographic disjunction is usually explained by: (i) historical exchange by way of the North Atlantic land bridge before the middle Miocene; (ii) Beringia (mid–late Miocene); or (iii) long-distance dispersal (e.g., Tiffney, 1985; Wen, 1999; Tiffney & Manchester, 2001; Zhu et al., 2013), but Beringia is generally used to explain disjunctions for recently dispersed taxa. For example, Yi et al. (2015) inferred that *Osmorhiza* (Apiaceae) with a divergence age of 5.51 Ma (95% HPD, 2.81–8.37 Ma) migrated from Asia to North America by way of Beringia. Similarly, Zhu et al. (2013) found that *Astilbe* (Saxifragaceae) lineages in Asia to North America diverged 3.54 Ma (95% HPD, 1.29–6.18 Ma), arriving in North America either by Beringia or by long-distance dispersal.

The results of both the BEAST and ancestral area reconstruction analyses suggest that the ancestor of *M. himalaica* split from its North American counterparts at approximately 5.22 Ma (95% HPD, 2.53–8.18 Ma) in the late Miocene to early Pleistocene. The Bering land bridge was available for floristic exchanges until 3.5–5.0 Ma in the late Cenozoic (Wen, 1999; Zhu et al., 2013) and appears to be the

most likely migration route for the ancestor of *M. himalaica*. Nevertheless, long-distance dispersal events mediated by birds have also been hypothesized to explain disjunct distributions in plant species (e.g., Popp et al., 2011; Le Roux et al., 2014) and Viana et al. (2016) proposed direct evidence to confirm the effectiveness of the vectors of birds. The mucilaginous involucre around the fruit of *M. himalaica* is very sticky, enabling it to cling readily to feathers or fur, aiding in dispersal by birds or other animals. Therefore, long-distance dispersal might also have played a role in the migration of *M. himalaica* into Asia, consistent with the viewpoint of Douglas & Manos (2007).

In conclusion, we consider that *M. himalaica* is best placed into an inclusive *Mirabilis* (sensu Spellenberg, 2003) with no defined sections, rather than into *Oxybaphus* as a segregate genus. The species appears to have diverged from its North American counterparts in the late Miocene to early Pleistocene, spreading to Asia either by the Bering land bridge and/or through long-distance dispersal.

As a traditional medicinal species, market demand leads to pressure on the wild populations of *M. himalaica*. According to the IUCN Red List Categories and Criteria (version 3.1) (IUCN, 2001) and Guidelines for Application of IUCN Red List Criteria at Regional Levels (version 3.0) (IUCN, 2003), *M. himalaica* is "Near Threatened" on the scale of China, based on the field survey. At present the species is not listed for conservation in China, but the primary threat is serious human disturbance around villages, along roads, and in farmland. Damage or collection of individual plants, habitat disturbance or loss, and consumption by livestock were all observed, threatening the long-term survival of *M. himalaica* in many regions. Accordingly, the development of protocols for the ongoing protection of this species requires investigation, especially as *M. himalaica* breeds well both in the wild and under cultivation, suggesting that targeted habitat protection could be an effective method for its conservation.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12466/suppinfo:

Table S1. Taxa sampled in this study with their source publication references and their GenBank accession numbers.

Data S1. Sequences of *Mirabilis himalaica* from two loci, nuclear ribosomal internal transcribed spacer and *rps16*, available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.th0v8td

Data S2. Dataset alignment and tree files of phylogenetic analyses and BEAST analysis, available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.th0v8td