The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2013, **171**, 395–412. With 4 figures

Molecular phylogenetics and biogeography of the eastern Asian–eastern North American disjunct *Mitchella* and its close relative *Damnacanthus* (Rubiaceae, Mitchelleae)

WEI-PING HUANG[1,2], HANG SUN[1], TAO DENG[1], SYLVAIN G. RAZAFIMANDIMBISON[4], ZE-LONG NIE[1]* and JUN WEN[3]*

[1]*Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204, China*
[2]*University of Chinese Academy of Sciences, Beijing 100049, China*
[3]*Department of Botany, National Museum of Natural History, MRC 166, Smithsonian Institution, Washington DC, 20013-7012, USA*
[4]*Bergius Foundation, The Royal Swedish Academy of Sciences and Department of Botany, Stockholm University, SE-10691, Stockholm, Sweden*

Received 25 April 2012; revised 23 July 2012; accepted for publication 12 September 2012

Mitchella is a small genus of the Rubiaceae with only two species. It is the only herbaceous semishrub of the family showing a disjunct distribution in eastern Asia and eastern North America, extending to Central America. Its phylogeny and biogeographical diversification remain poorly understood. In this study, we conducted phylogenetic and biogeographical analyses for *Mitchella* and its close relative *Damnacanthus* based on sequences of the nuclear internal transcribed spacer (ITS) and four plastid markers (*rbcL*, *atpB-rbcL*, *rps16* and *trnL-F*). *Mitchella* is monophyletic, consisting of an eastern Asian *M. undulata* clade and a New World *M. repens* clade. Our results also support *Michella* as the closest relative to the eastern Asian *Damnacanthus*. The divergence time between the two intercontinental disjunct *Mitchella* species was dated to 7.73 Mya, with a 95% highest posterior density (HPD) of 3.14–12.53 Mya, using the Bayesian relaxed clock estimation. Ancestral area reconstructions suggest that the genus originated in eastern Asia. The semishrub *Mitchella* appears to have arisen from its woody ancestor in eastern Asia and then migrated to North America via the Bering land bridge in the late Miocene.

ADDITIONAL KEYWORDS: Bering land bridge – intercontinental disjunction.

INTRODUCTION

The well-known biogeographical disjunction between eastern Asia and eastern North America has attracted much attention from plant biologists not only because it exhibits a unique distribution pattern, but also because it offers an excellent opportunity to explore plant differentiation and evolution in allopatry (Boufford & Spongberg, 1983; Hong, 1993; Wen, 1999, 2001; Wen *et al*., 2010). Fossil, molecular phylogenetic and geological data all indicate that this disjunct pattern originated multiple times in multiple areas throughout the Tertiary (Tiffney, 1985a, b; Wen, 1999). Much progress has been made concerning the evolution of this pattern (Wen, 1998, 2001; von Dohlen, Kurosu & Aoki, 2002; Dane *et al*., 2003; Fu *et al*., 2005; Wen *et al*., 2010). Molecular data have been employed extensively to estimate divergence times (Xiang *et al*., 2000; Nie *et al*., 2006a, 2010; Meng *et al*., 2008; Bremer & Eriksson, 2009) and to infer ancestral areas of the disjunct groups (Wen, 2000; Xiang & Soltis, 2001). However, few studies have examined the evolution of the disjunct pattern

---

*Corresponding authors. E-mail: wenj@si.edu; niezl@mail.kib.ac.cn

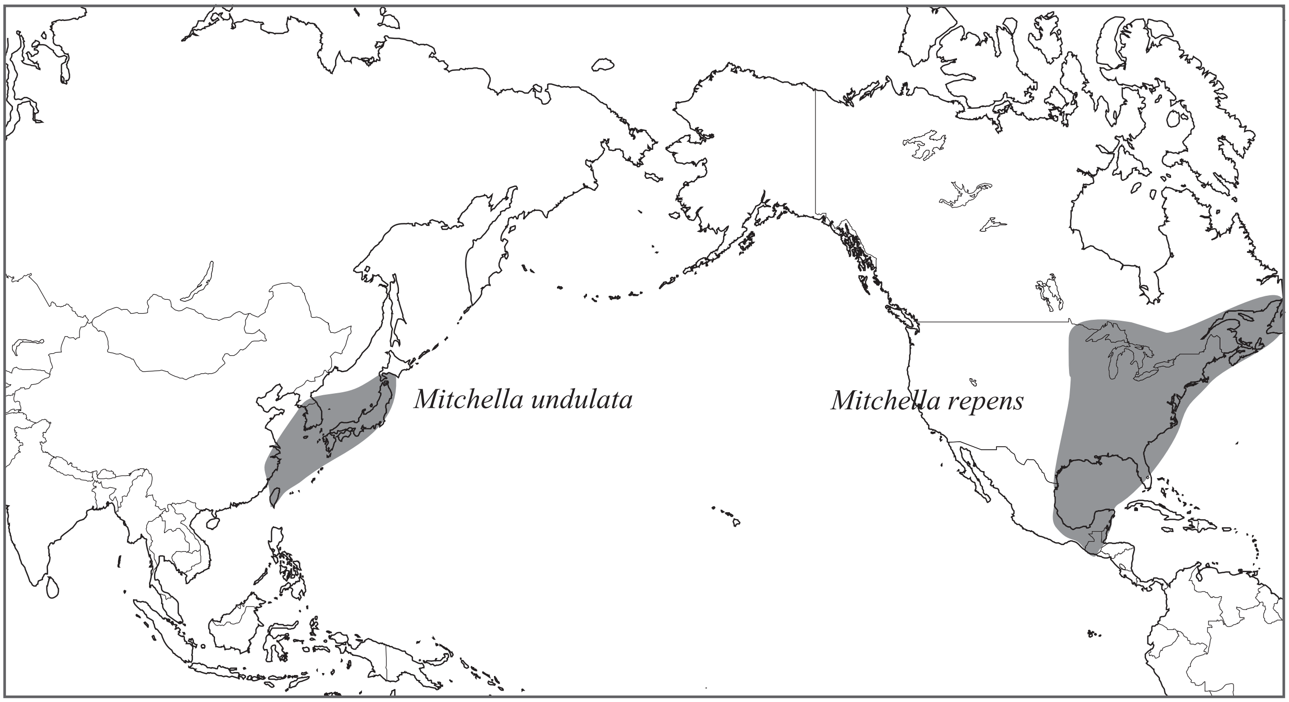

Figure 1. Distribution map of *Mitchella* showing disjunction between eastern Asia and eastern North America (including Central America).

in herbaceous taxa (Tiffney, 1985a, b; Wen, 1999; Nie *et al*., 2005).

Mitchella L. is a herbaceous semishrub genus in the mostly tropical and woody family Rubiaceae that exhibits the classical intercontinental disjunction between eastern Asia and eastern North America (Li, 1952). *Mitchella* is composed of only two species: the eastern Asian *M. undulata* Siebold & Zucc. and the eastern North American *M. repens* L., which extends to Central America (Li, 1952; Rogers, 2005; Chen *et al*., 2011). This genus can be distinguished from the herbaceous genera of Rubiaceae by a combination of several characters, including its rather long unbranched primary shoots, paired flowers on a short peduncle with the base of the calyx fused and two red drupaceous fused fruits with campylotropous ovules inserted in the upper part of the septum (Robbrecht, Puff & Igersheim, 1991; Yamazaki, 1993; Rogers, 2005; Chen *et al*., 2011). Both species are evergreen. The Asian *M. undulata* grows mostly on forest floors in Taiwan, Korea, Japan and southeastern China (Yamazaki, 1993; Liu & Yang, 1998; Chen *et al*., 2011), and the eastern North American *M. repens* occurs in moist or dry woods, along stream banks and on sandy slopes throughout eastern North America southwards to Martin County, Florida, with disjunct extensions to Central America (Li, 1952; Rogers, 2005) (Fig. 1).

Although the generic status of *Mitchella* has never been questioned, its phylogenetic position in Rubiaceae has been controversial. *Mitchella* has been placed in various tribes, such as 'Guettardidae' (Lindley, 1846), Anthospermeae (Hooker, 1873), Chiococceae (Baillon, 1880) and Paederieae (Puff, 1982). Based on a detailed morphological study, Robbrecht *et al*. (1991) pointed out that *Mitchella* is close to *Damnacanthus* Gaertn.f., a shrubby genus comprising about 13 species with evergreen leaves and a wide distribution in the understorey of natural laurel forests of south China, Taiwan, Japan, Korea, Vietnam, Laos, Myanmar and Assam, India (Yamazaki, 1993; Liu & Yang, 1998; Chen *et al*., 2011). A unique characteristic of *Damnacanthus* is heterophylly associated with sympodial growth and paired thorns (Robbrecht *et al*., 1991; Naiki & Nagamasu, 2003, 2004). Recent molecular phylogenetic studies also support the close relationships between *Mitchella* and *Damnacanthus* (Andersson & Rova, 1999; Bremer & Manen, 2000; Razafimandimbison, Rydin & Bremer, 2008). A new tribe Mitchelleae Razafim. & B.Bremer, including only these two genera, was established by Razafimandimbison *et al*. (2008), which belongs to the subfamily Rubioideae.

The New World *M. repens* and the eastern Asian *M. undulata* are morphologically similar (Robbrecht *et al*., 1991). No molecular studies have focused particularly on this genus and almost all previous molecular analyses have included only one species of *Mitchella*. Xiang *et al*. (2000) suggested the divergence between eastern Asian and eastern North American species of *Mitchella* at about 5.89 ± 2.38 Mya based on *rbcL* sequences. Because taxa once suspected as sister disjunct species based on morphological similarities may not represent real sister species (Wen, 1999), whether the two species of *Mitchella* are phylogenetically closest to each other or genetically distant from each other needs to be examined in a broader phylogenetic framework, especially with a comprehensive sampling scheme including its close relative *Damnacanthus*.

Here, we used four plastid fragments (*atpB-rbcL*, *rbcL*, *trnL-F* and *rps16*) and the nuclear ribosomal internal transcribed spacer (ITS) region to address the following questions. (1) Is *Mitchella* a monophyletic genus? (2) What is the phylogenetic relationship between *Mitchella* and *Damnacanthus*? (3) What are the most likely hypotheses to explain the biogeographical disjunction of *Mitchella* between eastern Asia and the New World? The molecular markers selected and most sequences in the dating analysis have been used widely in previous studies in Rubiaceae (e.g. Bremer, Andreasen & Olsson, 1995; Andersson & Rova, 1999; Rova *et al*., 2002; Church, 2003; Razafimandimbison, Kellogg & Bremer, 2004; Nie *et al*., 2005; Razafimandimbison *et al*., 2008, 2009; Bremer & Eriksson, 2009).

MATERIAL AND METHODS

TAXON SAMPLING

The voucher information for all the materials and GenBank accessions are presented in Table 1. Our sampling included both species of *Mitchella*: *M. repens* from North and Central America (seven accessions) and *M. undulata* from eastern Asia (three accessions). *Damnacanthus* is supported to be the closest relative of *Mitchella* (Robbrecht *et al*., 1991; Razafimandimbison *et al*., 2008) and eight of the 13 species were sampled in this study. To test the monophyly of *Mitchella* with all available data, sequences of two *Mitchella* accessions from GenBank were combined with our dataset (Table 1). Based on Razafimandimbison *et al*. (2008), five species of Morindeae and three species from Gaertnereae (sequences from GenBank) were chosen as outgroup taxa in the phylogenetic analysis (Table 1).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Total DNA of field-collected material was extracted from silica gel-dried leaf tissue using the Plant Total DNA Extraction Kit (BioTeKe, Beijing, China). Isolated DNA was amplified and sequenced following Rydin *et al*. (2008) for the *atpB-rbcL* spacer, Bremer *et al*. (1995) for *rbcL*, Oxelman *et al*. (1997) for *rps16*, Razafimandimbison & Bremer (2002) and Razafimandimbison, Kellogg & Bremer (2004) for ITS and Razafimandimbison & Bremer (2002) for *trnL-F*. All polymerase chain reactions (PCRs) were run in a PTC-100 thermocycler (MJ Research, Ramsey, MN, USA). PCR products were purified using an agarose gel DNA purification kit (Takara, Shiga, Japan), following the manufacturer's instructions. Sequencing was performed with BigDye Terminator 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3730 Sequencer using the same primers as employed for the PCR amplifications. All sequences were analysed and assembled with Sequencher ver.4.14 (Gene Code, Ann Arbor, MI, USA).

PHYLOGENETIC ANALYSES

The computer program CLUSTALX (Thompson *et al*., 1997) was used for an initial alignment of all the sequences, followed by manual alignment using BioEdit (Hall, 1999). All datasets were analysed under maximum parsimony (MP) with PAUP 4.0b10 (Swofford, 2003) and Bayesian inference (BI) with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). For the MP analyses, we used heuristic searches with tree bisection–reconnection (TBR) branch swapping, MULTREES option on and 1000 replicates of random taxon addition. All characters were unordered and equally weighted, and gaps were treated as missing data in the analyses. Bootstrap tests of the data used 1000 pseudoreplicates to evaluate clade support.

For the Bayesian analyses, the best-fitting models of sequence evolution for the plastid and ITS datasets were chosen by MrModeltest v. 3.7 (Nylander, 2004) under the Akaike information criterion (Akaike, 1973). Bayesian analyses were conducted under four independent Markov chain runs for 10 million Metropolis-coupled generations, sampling trees every 1000 generations. The first 10% of trees were discarded as burn-in (average split deviations between parallel runs < 0.01). In the combined plastid and ITS analyses, we set the matrices into two unlinked partitions (the plastid data and the ITS data). All Bayesian analyses were run twice with random starting trees, and a consensus tree was constructed using the saved trees by the two independent runs. Clades with posterior probabilities (PPs) over 95% were regarded as strongly supported.

DIVERGENCE TIME ESTIMATION

After assessing the sequences generated, and those available from GenBank, we chose to use the combined ITS, *atpB-rbcL*, *rbcL*, *rps16* and *trnL-F* data to estimate the divergence time of *Mitchella* between the New World and eastern Asia. A likelihood ratio test

Table 1. Voucher information and GenBank accessions of the taxa used in the phylogenetic study

Taxon	Locality	Voucher information	*rbcL*	*rps*16	*atpB-rbcL*	*trnL-F*	ITS
Appunia guatemalensis Donn.Sm.	–	–	AJ288593[1]	AM945306[2]	AJ234009[1]	AM945332[2]	AM945191[2]
Caelospermum monticola Baill. ex Guillaumin	–	–	AF331644[3]	AF001438[4]	AM945221[2]	AM945334[2]	AM945194[2]
Damnacanthus giganteus (Mak.) Nakai	China, Sichuan	*Nie 2093* (KUN)	JX412457	JX412437	JX412395	JX412374	JX412416
	China, Jiangxi	*Xie 428* (KUN)	JX412458	JX412438	JX412396	JX412375	JX412417
Damnacanthus henryi (H.Lév.) H.S.Lo	China, Yunnan	*Nie 3546* (KUN)	JX412459	JX412439	JX412397	JX412376	JX412418
	China, Yunnan	*Nie 3941* (KUN)	JX412460	–	JX412398	JX412377	JX412419
Damnacanthus indicus C.F.Gaertn	China, Jiangxi	*Huang 054* (KUN)	JX412461	JX412440	JX412399	JX412378	JX412420
	China, Jiangxi	*Huang 042* (KUN)	JX412462	JX412441	JX412400	JX412379	JX412421
Damnacanthus labordei (H.Lév.) H.S.Lo	China, Hunan	*Zhang 484* (KUN)	JX412463	JX412442	JX412401	JX412380	JX412422
Damnacanthus macrophyllus Siebold ex Miq.	China, Guizhou	*Nie 2241* (KUN)	JX412464	JX412443	JX412402	JX412381	JX412423
	China, Zhejiang	*Deng 109* (KUN)	JX412465	JX412444	JX412403	JX412382	JX412424
	China, Zhejiang	*Nie2240* (KUN)	JX412466	JX412445	JX412404	JX412383	JX412425
Damnacanthus major Sieb. & Zucc.	China, Zhejiang	*Deng 108* (KUN)	JX412467	JX412446	JX412405	JX412384	JX412426
Damnacanthus officinarum C.C.Huang	China, Sichuan	*Xie* 426 (KUN)	JX412468	JX412447	JX412406	JX412385	JX412427
Damnncanthus hananensis (Lo) Lo ex Y. Z. Ruan	China, Hainan	*Nie 3967* (KUN)	JX412469	JX412448	JX412407	JX412386	JX412428
Gaertnera phyllosepala Baker	–	–	AM117227[5]	AM117307[5]	AM945226[2]	DQ662139[6]	AM945199[2]
Gaertnera sp.	–	–	AM945288[2]	AM945311[2]	AM945227[2]	AM945340[2]	AM945200[2]
Gynochthodes coriacea Blume	–	–	AJ288603[1]	AM117311[5]	AM945219[2]	AJ847407[7]	AM945192[2]
Gynochthodes sp.	–	–	AM945284[2]	AM945307[2]	AM945220[2]	AM945333[2]	AM945193[2]
Mitchella repens L.	USA, South Carolina	*Wen10020* (US)	JX412470	JX412449	JX412408	JX412387	JX412429
	USA, Maryland	*Wen10102* (US)	JX412471	JX412450	JX412409	JX412388	JX412430
	USA, Delaware	*Wen10421* (US)	JX412472	JX412451	JX412410	JX412389	JX412431
	Canada, Quebec	*Wen10478* (US)	JX412473	JX412452	JX412411	JX412390	JX412432
	Mexico	*Breedlove et al. 32460* (MEXU)	JX412474	JX412453	JX412412	JX412391	JX412433
	Mexico	*Patterson and Mayfield 7369* (MEXU)	JX412475	JX412454	JX412413	JX412392	JX412434
	–	–	Z68805[8]	AF001441[4]	AM945223[2]	AM945337[2]	AF072019[9]
Mitchella undulata Sieb. & Zucc.	China, Zhejiang	*Deng 081* (KUN)	JX412476	JX412455	JX412414	JX412393	JX412435
	China, Zhejiang	*Deng 085* (KUN)	JX412477	JX412456	JX412415	JX412394	JX412436
	–	–	AF190445[10]	–	AJ234016[1]	–	AB103533[11] AB103532[11]
Morinda citrifolia L.	–	–	AJ318448[12]	AJ320078[12]	AJ234013[1]	AF152616[13]	AY762843[14]
Pagamea guianensis Aubl.	–	–	AM945290[2]	AF002744[4]	AM945229[2]	AM945342[2]	AF333846[15]

ITS, internal transcribed spacer.

Sequences obtained from other studies: [1]Bremer & Manen, 2000; [2]Razafimandimbison *et al.*, 2008; [3]L. Andersson, unpublished; [4]Andersson & Rova, 1999; [5]Bremer & Eriksson, 2009; [6]Backlund, Bremer & Thulin, 2007; [7]Alejandro, Razafimandimbison & Liede-Schumann, 2005; [8]Bremer, 1996; [9]Nepokroeff, Bremer & Sytsma, 1999; [10]Xiang *et al.*, 2000; [11]Yokoyama, Fukuda & Tsukaya, 2003; [12]Novotny *et al.*, 2002; [13]Rova *et al.*, 2002; [14]A. D. Proujansky and D. L. Stern, unpublished; [15]Malcomber, 2002 rejected the molecular clock hypothesis ($P < 0.05$). We thus used a Bayesian relaxed method implemented in BEAST 1.7.1 (Drummond & Rambaut, 2007) to estimate the divergence times. With our focus on the divergence time of genus *Mitchella*, and with the consideration of minimizing the influence from topological uncertainties in our analyses on dating of the phylogeny, we excluded some *Damnacanthus* taxa. To allow multiple fossil calibrations in a broad phylogenetic framework of Rubiaceae, sequences of 63 additional taxa were obtained from GenBank (see Appendix). *Gelsemium sempervirens* (L.) J.St.-Hil. (Gelsemiaceae) was selected as the outgroup in our dating analysis.

We used the GTR model of nuclear substitution, gamma distribution for four rate categories, uncorrelated log-normal relaxed clock model and Yule process tree prior in the Bayesian dating analyses. Two separate BEAST runs were set to perform 50 million generations with 10% burn-in, and each run was checked for convergence with Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/).

Fossils of Rubiaceae have been widely used to estimate the divergence time of the family or certain clades in the family (Nie *et al*., 2005; Antonelli *et al*., 2009; Bremer & Eriksson, 2009; Smedmark, Eriksson & Bremer, 2010). Although there have been many described leaves and pollen fossils of Rubiaceae since the Cretaceous and Palaeocene (Graham, 2009), the most convincing fossil of Rubiaceae is from *Cephalanthus* L., which was reported from the late Eocene to the Pliocene in almost 20 fossil sites (Dorofeev, 1960, 1963; Friis, 1985; Mai & Walther, 1985; Antonelli *et al*., 2009). We followed Antonelli *et al*. (2009) in using the oldest fossil of this genus to place a minimum age constraint of 33.9 Ma, which was fixed by using the ending point of the geological epoch to which the fossil belongs as the stem age of *Cephalanthus*. The pollen fossils of *Faramea* Aubl. have been reported from the late Eocene (~34–40 Ma) in Panama to the Pliocene in Veracruz, Mexico (Graham, 2009); we thus used 37Ma, the mean age of the late Eocene, to set a minimum age of the *Faramea* stem node. Saenger (1998) reported two pollen fossil ages of *Scyphiphora* C.F.Gaertn.: 16 Ma from Japan and 23 Ma from the Marshall Islands. *Scyphiphora* is the only extant genus of Rubiaceae that belongs to mangrove vegetation (Bremer & Eriksson, 2009), and its pollen characters are well defined and unique in Rubiaceae. We thus used 23 Ma as a minimum age prior for the *Scyphiphora* stem node.

Morinda chinensis Shi, Liu & Jin was recently described as a well-preserved fossil dated back to the late early to the early late Eocene (Shi *et al*., 2012). This fossil has a head-shaped infructescence (multiple fruits or syncarps), which is developed from a capitulum composed of about 20–30 flowers, the fruits of which are fused into one unit (Shi *et al*., 2012), and these characters fit well with *Morinda*. Shi *et al*. (2012) argued that, based on its shape and number of simple fruits of the infructescence, the fossil fruit should be placed in *Morinda* section *Roioc* DC. However, the authors also point out that its infructescence contains fewer simple fruits than other species of this section. Molecular phylogenetic analysis also suggests that *Morinda* is paraphyletic (Razafimandimbison *et al*., 2009). As the phylogenetic position of this species is unclear, we used this fossil to calibrate the stem age of *Morinda* with the prior set to 44.5 + 3 Ma, between 40.6 and 48.4 Ma (late early Eocene–early late Eocene).

Four fossils were selected as calibration points in our analyses, three of which (*Cephalanthus*, *Faramea* and *Scyphiphora*) were the same as in Bremer & Eriksson (2009). We used the new fossil to calibrate the stem age of *Morinda* to enhance the accuracy for the dating of *Mitchella*, because these two genera belong to the sister tribes Morindeae and Mitchelleae, respectively (Razafimandimbison *et al*., 2009). To root the tree, 78 Ma was enforced as the split time between Rubiaceae and other Gentianales, based on Bremer, Friis & Bremer (2004), who used a broad sampling of asterids and multiple fossils.

BIOGEOGRAPHICAL ANALYSES

We defined two areas of endemism to assess the historical biogeography of the *Mitchella* clade, eastern Asia (A) and North America to Central America (B), based on the extant distributions of the species in the Bayesian tree and geological history. Although many analyses on the disjunct taxa (Baird *et al*., 2010; Nie *et al*., 2010; Xu *et al*., 2010) have used dispersal–vicariance analysis (DIVA; Ronquist, 1996) to infer ancestral distributions, DIVA requires fully bifurcated trees. Because our Bayesian trees were not fully resolved, we used RASP 1.1 (Yu, Harris & He, 2011), which implements the S-DIVA (statistical dispersal–vicariance analysis) method (Yu, Harris & He, 2010) and allows uncertainties in the phylogenetic trees. We used Bayesian trees from the phylogenetic analyses (10 000 trees, excluding the remote outgroup of Gaertnereae) as input for S-DIVA. The condensed tree was computed using these 9000 trees (excluding the burn-in 1000 trees); the 'maxarea' was set to two and state frequencies were estimated.

RESULTS

The statistics of the sequences are shown in Table 2. The ITS dataset had the highest percentage of potentially parsimony informative (PI) sites (21.16%),

Table 2. Sequence characteristics of *Mitchella* and its relatives used in this study

	ITS	*rbcL*	*rps16*	*atpB-rbcL*	*trnL-F*	Plastid	Plastid + ITS
Length of aligned matrices (bp)	534	1318	985	694	1103	4100	4634
Number of potentially parsimony-informative characters (PICs)	113	70	59	42	59	230	343
Percentage of potentially parsimony-informative sites	21.16	5.31	5.99	6.05	5.35	5.61	7.40
Retention index	0.7789	0.8079	0.9160	0.8968	0.9167	0.8466	0.8098
Consistency index	0.7500	0.7706	0.9346	0.8807	0.9353	0.8500	0.8100
Best tree length	352	170	153	109	170	618	979

ITS, internal transcribed spacer.

although ITS was the shortest of the fragments. As the individual plastid markers had limited PI (5.31%–6.05%, Table 2) and generated unresolved trees in our separate analyses, we combined these four regions to reconstruct a combined plastid phylogeny. As there were no statistically supported conflicts of the ITS and plastid trees, our discussion is based on results from concatenated plastid and ITS data. The MP and BI analyses produced similar results, and only the Bayesian tree, with parsimony bootstrap (PB) and Bayesian PPs, is presented in Fig 2.

Mitchella was strongly supported as monophyletic (PP = 1.00, PB = 87%; Fig. 2) with two well-supported groups: the New World *M. repens* (PP = 1.00, PB = 98%) and the eastern Asian *M. undulata* (PP = 1.00, PB = 92%). In all analyses, tribe Mitchelleae (*Mitchella* and *Damnacanthus*) was supported as monophyletic (PP = 1.00, PB = 100%). In *Damnacanthus*, four lineages were recognizable with strong support (Fig. 2): (1) *D. giganteus* Nakai, *D. labordei* (H.Lév.) H.S.Lo, *D. officinarum* C.C.Huang and *D. macrophyllus* Siebold ex Miq. (accessions of Nie2241 and Nie2240); (2) *D. henryi* (H.Lév.) H.S.Lo and *D. hainanensis* (H.S.Lo) Y.Z.Ruan (PP = 1.00, PB = 100%); (3) *D. macrophyllus* Deng109 and *D. major* Siebold & Zucc. (PP = 1.00, PB = 100%); and (4) the two accessions of *D. indicus* C.F.Gaertn.

The chronogram and estimated divergence times from the dating analyses at the family level are shown in Fig. 3. The combined tree resolved Rubiaceae into three major lineages, formally recognized as subfamilies Cinchonoideae, Ixoroideae and Rubioideae. The divergence between the eastern North American and the eastern Asian species was estimated at 7.33 Ma in the late Miocene, with a 95% highest posterior density (HPD) of 3.14–12.53 Ma, covering a period from the late–middle Miocene to the Pliocene. The S-DIVA analyses clearly inferred eastern Asia as the ancestral area of *Mitchella* (Fig. 4).

DISCUSSION

PHYLOGENETIC RELATIONSHIPS

Our results based on the combined analysis of plastid and nuclear data support the monophyly of *Mitchella* (Fig 2), which comprises two widely disjunct species: *M. undulata* from eastern Asia and *M. repens* from eastern North America extending to Central America. These two species share many features, such as a creeping habit with dark evergreen leaves and rooting at the nodes (Rogers, 2005; Chen *et al.*, 2011), and usually heterostylous flowers (i.e. some individuals have exserted stamens and an included style, whereas others possess included stamens and an exserted style) (Blaser, 1954; Ganders, 1975; Hicks, Wyatt & Meagher, 1985; Yamazaki, 1993; Chen *et al.*, 2011). These two species are morphologically so similar that *M. undulata* has sometimes been reduced to infraspecific rank as *M. repens* var. *undulata* (Sieb. & Zucc.) Makino (Makino, 1909; Robbrecht *et al.*, 1991). In spite of the low level of morphological variation, molecular results support a clear separation of the eastern Asian and the New World clades (Fig. 2). Nevertheless, minor morphological and phenological differences can be observed between them. *Mitchella repens* usually has leaves obtuse at the apex and entire margins, and flowers in April to June (Miller & Miller, 2005; Rogers, 2005), whereas *M. undulata* has leaves acuminate to rounded at the apex and sometimes undulate at the margins, and usually flowers in June to August (Makino, 1909; Yamazaki, 1993; Liu & Yang, 1998; Chen *et al.*, 2011).

Our results also strongly support *Mitchella* as the closest relative of *Damnacanthus* (PP = 1.00,

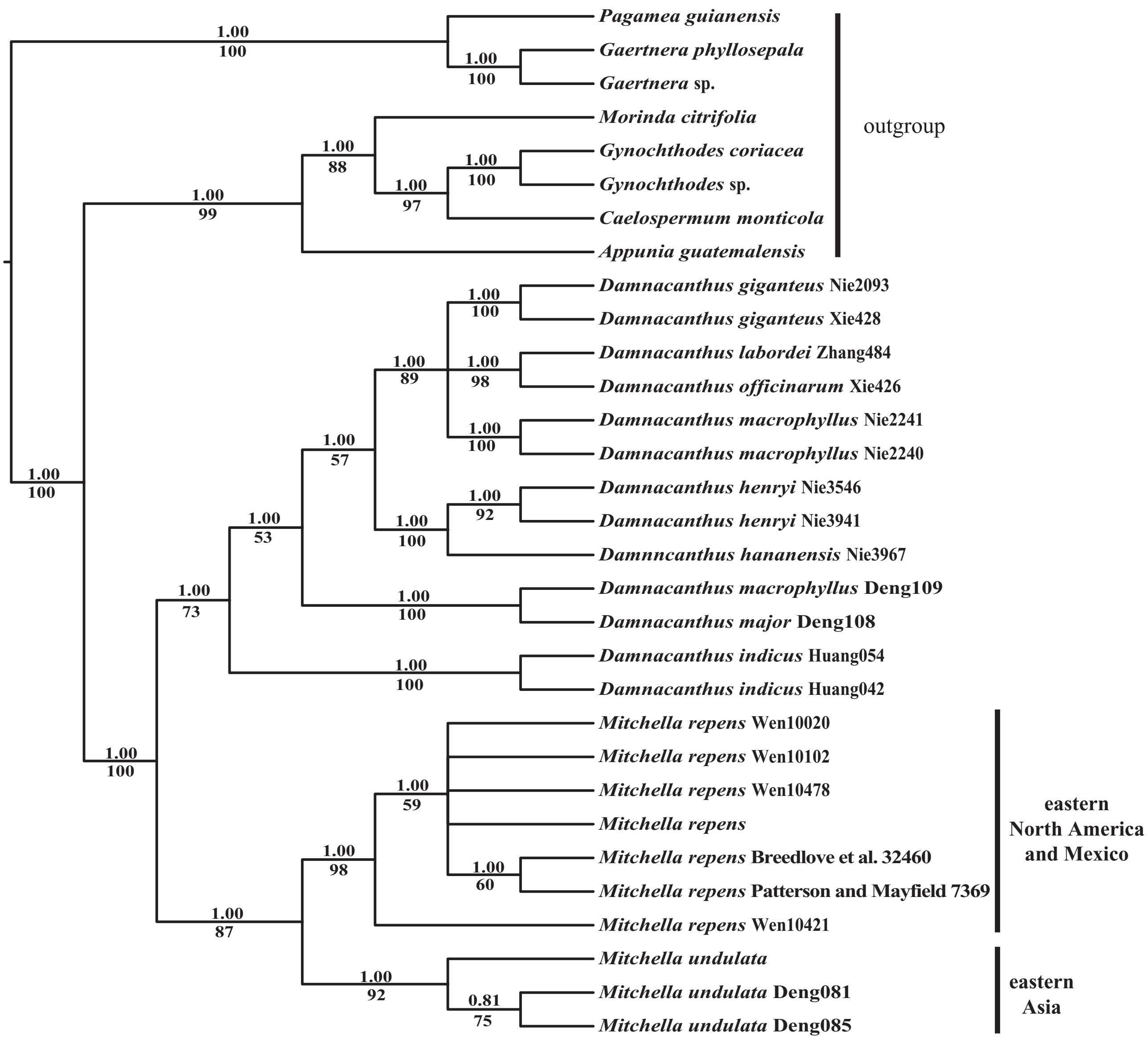

Figure 2. Bayesian majority-rule consensus tree of *Mitchella*, inferred from combined sequence data of four plastid markers (*rbcL*, *atpB-rbcL*, *rps16* and *trnL-F*) and internal transcribed spacer (ITS). Bayesian posterior probabilities are shown above the branches and maximum parsimony (MP) bootstrap values are shown below.

PB = 100%, Fig. 2) and confirm the monophyly of tribe Mitchelleae as proposed by Razafimandimbison *et al.* (2008). Baillon (1880) first suggested the close affinities between *Damnacanthus* and *Mitchella*. Robbrecht *et al.* (1991) further pointed out that *Mitchella* and *Damnacanthus* were closely related based on a detailed morphological study. Recently, molecular data supported the close relationship of *Mitchella* and *Damnacanthus* (Bremer, 1996; Andersson & Rova, 1999; Bremer & Manen, 2000; Razafimandimbison *et al.*, 2008, 2009). The two genera share a number of morphological characters: campylotropous (rarely seen in other genera of Rubiaceae), pitted endocarp, red syncarpous fruits, fused two flowers with each sole flower having four carpels, placenta inserted in the upper part of the septum and chromosome number of $2n = 22$ (Robbrecht *et al.*, 1991).

Robbrecht *et al.* (1991) also stressed differences in various important character states which support the separation of *Damnacanthus* and *Mitchella* as two distinct genera. *Damnacanthus* is a shrubby genus with conspicuously heterophyllous evergreen leaves, whereas *Mitchella* spp. are small creeping semi-shrubs (herbaceous-like) with green uniform leaves. In *Mitchella*, lateral branches originate on older parts of rather long unbranched primary shoots, whereas a

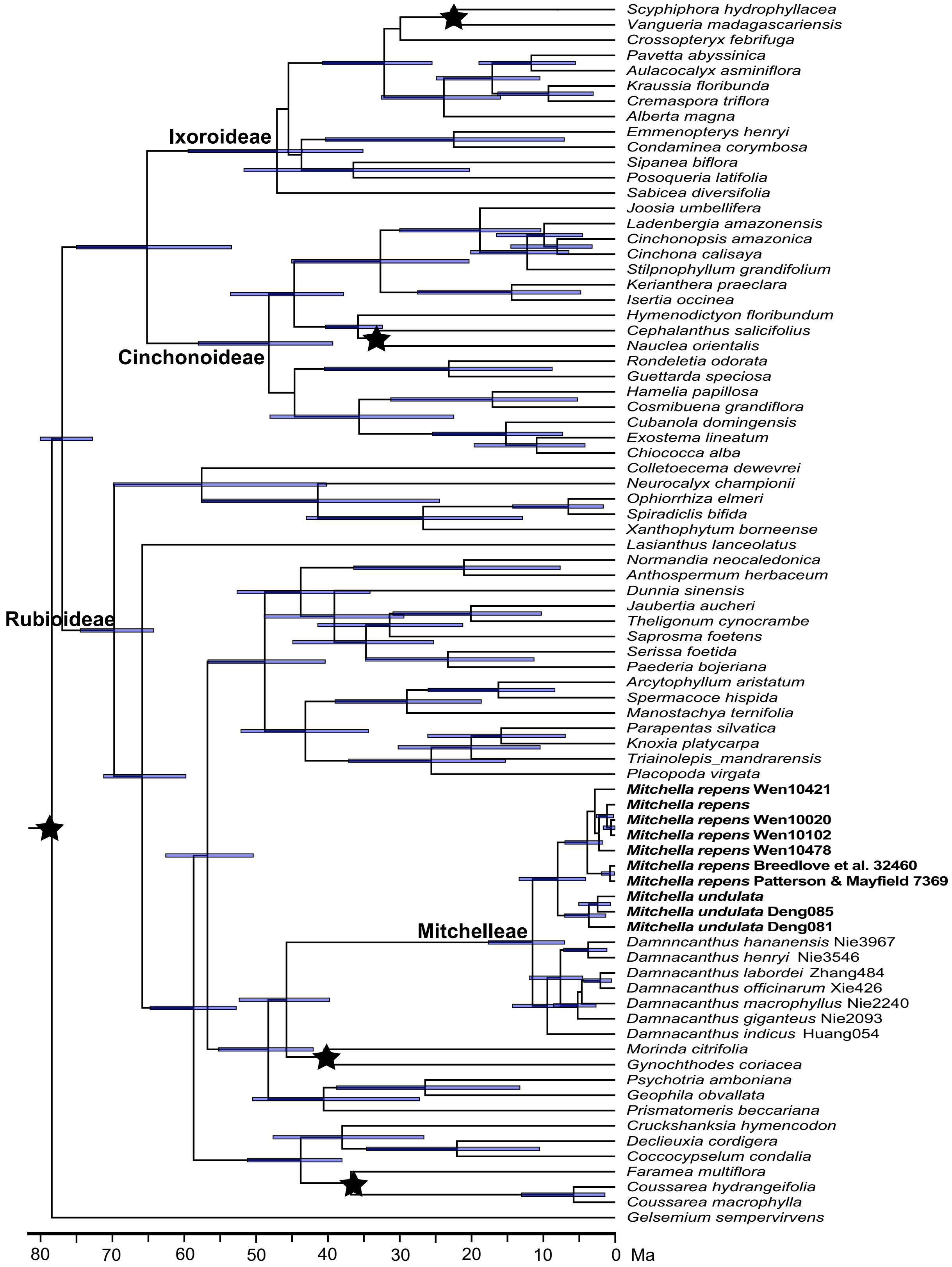

Figure 3. Chronogram of *Mitchella* and its relatives from Rubiaceae based on the combined internal transcribed spacer (ITS), *atpB-rbcL*, *rbcL*, *rps16* and *trnL-F* data estimated from BEAST. Calibration points are indicated with black stars. Node bars represent 95% highest posterior distribution of node age estimates.

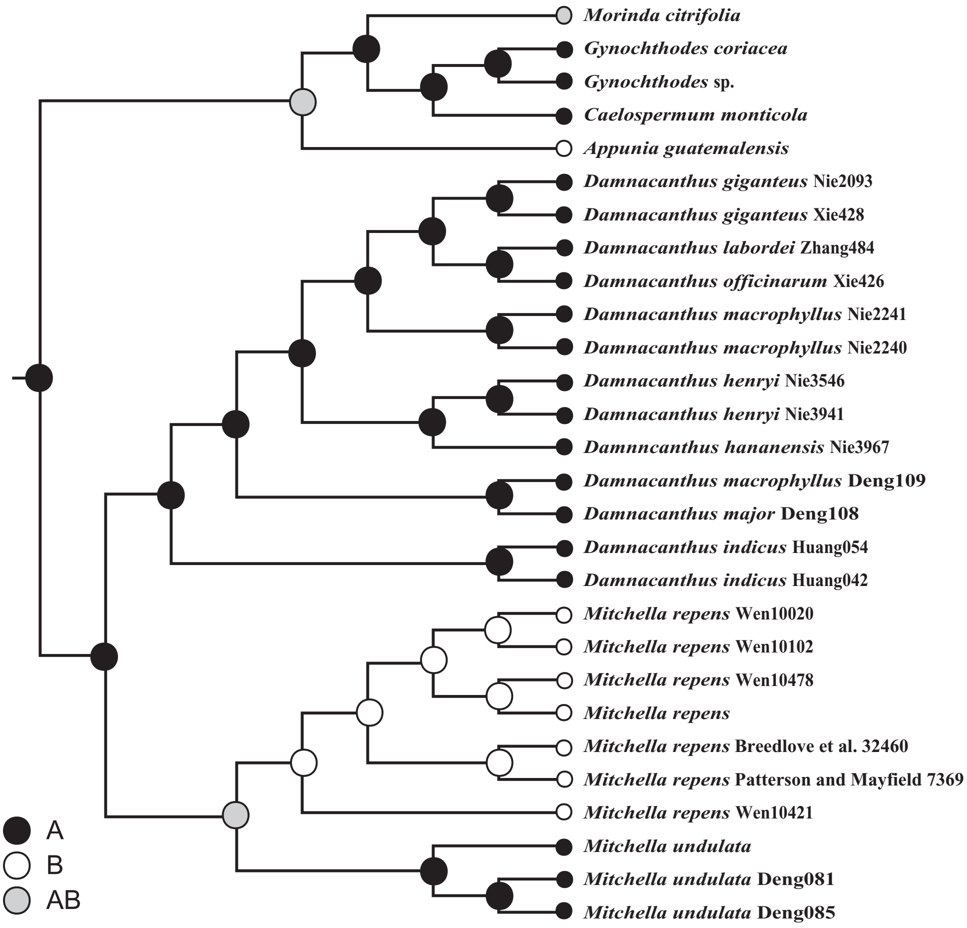

Figure 4. Ancestral area reconstruction of *Mitchella* using statistical dispersal–vicariance (S-DIVA) in RASP: A, eastern Asia; B, eastern North America and Central America.

regular sympodial branching pattern prevails in *Damnacanthus*. The two neighbouring flowers are fused by their ovaries in *Mitchella*, but separate in *Damnacanthus*. *Mitchella* has 'compound' drupes, whereas fruits of *Damnacanthus* are mostly paired, but have separate pedicels. The morphological differences mentioned above are consistent with our molecular results of the clear separation of *Damnacanthus* and *Mitchella* (Fig. 2). However, Razafimandimbison *et al*. (2008) suggested that *Damnacanthus* is paraphyletic with *Mitchella* nested in it. Further studies with complete sampling of *Damnacanthus* and further molecular data are needed to test the relationships between these two genera and to circumscribe species of *Damnacanthus*.

BIOGEOGRAPHICAL DIVERSIFICATION OF *MITCHELLA*

Our dating results, calibrated with four fossils, are similar to those of Antonelli *et al*. (2009), but much younger than those of Bremer & Eriksson (2009). The difference in the root age set may explain the difference, as we set the root of the family at 78 Ma, whereas Bremer & Eriksson (2009) set 45 Ma as the minimum age prior for the family. The divergence time between the New World *M. repens* and the eastern Asian *M. undulata* was estimated to be about 7.33 Ma (95% HPD, 3.14–12.53 Ma) in the late Miocene (Fig. 3). This estimate is similar to that of Xiang *et al*. (2000), which was 5.89 ± 2.38 Ma based on the *rbcL* gene using an average synonymous substitution rate of 22 species belonging to 11 plant groups [$R_s = (1.23 \pm 0.128) \times 10^{-9}$ substitutions per site per year].

Wen *et al*. (2010) reported that the divergence time of most Asian–North American temperate disjunct lineages is between 3 and 25 Ma. The divergence time of *Mitchella* in our study is consistent with results of other studies on eastern Asian and eastern North American disjunct taxa dating back to the late Tertiary to early Pleistocene (Wen, 2000; Dane *et al*., 2003; Nie *et al*., 2005; Baird *et al*., 2010).

The ancestors of modern eastern Asian–eastern North American disjunct genera have been hypothesized to have originated in various areas and attained their present distribution via multiple pathways. At least three hypotheses have been proposed: migration through the North Atlantic land bridges (Tiffney, 1985a); migration across the Bering land bridge (Tiffney, 1985b); and long-distance dispersal (Tiffney, 1985b; Wen, 1999; Wen *et al*., 2010). Our biogeographical analyses inferred the ancestral area of *Mitchella* as eastern Asia (Fig. 2) with a divergence time of the disjunction in the late Miocene (Fig. 3). The intercontinental disjunction of *Mitchella* is most likely to be explained as a migration from Asia to North America via the Bering land bridge. During the late Miocene and early Pliocene, the Bering land bridge was available for floristic exchanges of temperate plants until about 3.5 Ma (Hopkins, 1967; Wen, 1999).

The North Atlantic land bridge is a less likely route for the *Mitchella* disjunction, because this route mostly contributed to the dispersal of more tropical elements, and this floristic connection was no longer viable by the middle Miocene (Parks & Wendel, 1990; Tiffney & Manchester, 2001). *Mitchella* is a small semishrub with red drupes, which are often dispersed by small mammals over only short distances (Eriksson & Bremer, 1991; Willson, 1993; Bremer & Eriksson, 1992). Long-distance dispersal is thus considered quite unlikely to explain the intercontinental disjunction in *Mitchella*. We favour a hypothesis based on a migration scenario across the Bering land bridge, which has been proposed in a number of other temperate groups in the late Miocene and the Pliocene. For instance, *Phryma* L. (Phrymaceae) shows a classical intercontinental disjunction between eastern Asia and eastern North America, and was explained by the Beringian migration in the late Miocene with the divergence time estimated as 3.68 ± 2.25–5.23 ± 1.37 Ma (Nie *et al*., 2006a). Similar cases can also be found in *Penthorum* L. (Xiang *et al*., 2000), *Circaea* L. (Xie *et al*., 2009), *Saxifraga rivularis* L. (Westergaard *et al*., 2010), *Symplocarpus* Salisb. (Nie *et al*., 2006b) and *Astilbe* Buch.-Ham. ex D.Don (Kim *et al*., 2009; Zhu *et al*., in press).

Mitchella mostly occupies the subtropical to temperate region, whereas most genera of Rubiaceae are distributed in tropical regions (Ehrendorfer, Manen & Natali, 1994; Manen, Natali & Ehrendorfer, 1994; Bremer & Eriksson, 2009). However, some populations of *M. repens* are found in Central America, which can be explained by a southward expansion from eastern North America, as accessions from Mexico are nested in the eastern North American clade (Fig. 2), although they are sister to the eastern North American samples in the BEAST phylogenetic tree, as shown in Fig. 4. Except for some *M. repens* populations from Central America, *Mitchella* has a more northern distribution than most *Damnacanthus* spp. (Chen *et al*., 2011). Our phylogenetic results suggest that *Mitchella* may have adapted to a cold climate and evolved to the herbaceous life form from its woody *Damnacanthus*-like ancestor (Fig. 2), as indicated by the woody basal stem of *Mitchella*. Palaeontological evidence suggests that, in the early Tertiary, the Boreotropical flora was continuously distributed across the north temperate zone (Leopold & MacGinitie, 1972; Wolfe, 1972; Hong, 1993; Graham, 1972). With the global cooling in the late Tertiary, thermophilic plants, including Rubiaceae, moved southwards, except a few taxa, such as *Mitchella*, which most probably survived as relict herbaceous elements in the temperate regions of the Northern Hemisphere. This type of adaptation has also been reported in other taxa, such as *Parthenocissus* Planch., one of the few temperate genera of Vitaceae, which was most likely a derivative of the Eocene Boreotropical element (Nie *et al*., 2010).

ACKNOWLEDGEMENTS

This study was supported by grants from the Natural Sciences Foundation of China (NSFC 30970193 to Z.-L. Nie, 30828001 and 30625004 to J. Wen and T. Yi, 40930209 and 31061160184 to H. Sun), the One Hundred Talents Program of the Chinese Academy of Sciences (2011312D11022 to H. Sun), the United Fund of the NSFC and Yunnan Natural Science Foundation (U1136601 to H. Sun) and the John D. and Catherine T. MacArthur Foundation to J. Wen, R. Ree and G. Mueller. Laboratory work was performed at and partially supported by the Laboratory of Analytical Biology of the National Museum of Natural History, Smithsonian Institution. Fieldwork in North America was supported by the Small Grants Program of the National Museum of Natural History, the Smithsonian Institution. We thank the MEXU herbarium for loaning the sampled Mexican *Mitchella repens* used in this study.

REFERENCES

Akaike H. 1973. Information theory and an extension of the maximum likelihood principle. In: Petrov BN, Csáki F, eds. *Second International Symposium on Information Theory, Vol. 1*. Budapest: Akademiai Kiado Press, 267–281.

Alejandro GD, Razafimandimbison SG, Liede-Schumann S. 2005. Polyphyly of *Mussaenda* inferred from ITS and *trnT-F* data and its implication for generic limits in Mussaendeae (Rubiaceae). *American Journal of Botany* **92:** 544–557.

Andersson L, Antonelli A. 2005. Phylogeny of the tribe Cinchoneae (Rubiaceae), its position in Cinchonoideae, and description of a new genus, *Ciliosemina*. *Taxon* **54:** 17–28.

Andersson L, Rova JHE. 1999. The *rps16* intron and the phylogeny of the Rubioideae (Rubiaceae). *Plant Systematics and Evolution* **214:** 161–186.

Andersson L, Rova JHE, Guarin FA. 2002. Relationships, circumscription, and biogeography of *Arcytophyllum* (Rubiaceae) based on evidence from cpDNA. *Brittonia* **54:** 40–49.

Andreasen K, Baldwin BG, Bremer B. 1999. Phylogenetic utility of the nuclear rDNA ITS region in subfamily Ixoroideae (Rubiaceae): comparisons with cpDNA *rbcL* sequence data. *Plant Systematics and Evolution* **217:** 119–135.

Andreasen K, Bremer B. 1996. Phylogeny of the subfamily Ixoroideae (Rubiaceae). *Opera Botanica Belgica* **7:** 119–138.

Antonelli A, Nylander JAA, Persson C, Sanmartín I. 2009. Tracing the impact of the Andean uplift on Neotropical plant evolution. *Proceedings of the National Academy of Sciences* **106:** 9749.

Backlund M, Bremer B, Thulin M. 2007. Paraphyly of Paederieae, recognition of *Putorieae* and expansion of *Plocama* (Rubiaceae-Rubioideae). *Taxon* **56:** 315–328.

Baillon H. 1880. *Histoire des plantes* 7. Paris: Hachette.

Baird KE, Funk VA, Wen J, Weeks A. 2010. Molecular phylogenetic analysis of *Leibnitzia Cass.*(Asteraceae: Mutisieae: *Gerbera*-complex), an Asian–North American disjunct genus. *Journal of Systematics and Evolution* **48:** 161–174.

Blaser JLeC. 1954. The morphology of the flower and inflorescence of *Mitchella repens*. *American Journal of Botany* **41:** 533–539.

Boufford DE, Spongberg SA. 1983. Eastern Asian–eastern North American phytogeographical relationships—a history from the time of Linnaeus to the twentieth century. *Annals of the Missouri Botanical Garden* **70:** 423–439.

Bremer B. 1996. Phylogenetic studies within Rubiaceae and relationships to other families based on molecular data. *Opera Botanica Belgica* **7:** 33–50.

Bremer B, Andreasen K, Olsson D. 1995. Subfamilial and tribal relationships in the Rubiaceae based on *rbcL* sequence data. *Annals of the Missouri Botanical Garden* **82:** 383–397.

Bremer B, Eriksson T. 2009. Time tree of Rubiaceae: phylogeny and dating the family, subfamilies, and tribes. *International Journal of Plant Sciences* **170:** 766–793.

Bremer K, Friis EM, Bremer B. 2004. Molecular phylogenetic dating of asterid flowering plants shows early Cretaceous diversification. *Systematic Biology* **53:** 496–505.

Bremer B, Manen JF. 2000. Phylogeny and classification of the subfamily Rubioideae (Rubiaceae). *Plant Systematics and Evolution* **225:** 43–72.

Bremer B, Thulin M. 1998. Collapse of Isertieae, reestablishment of Mussaendeae, and a new genus of Sabiceeae (Rubiaceae); phylogenetic relationships based on rbcL data. *Plant Systematics and Evolution* **211:** 71–92.

Chen T, Zhu H, Chen J, Taylor CM, Ehrendorfer F, Lantz H, Funston AM, Puff C. 2011. Rubiaceae. In: Wu CY, Raven PH, eds. *Flora of China*, *Vol. 19*. Beijing: Science Press and St. Louis, MO: Missouri Botanical Garden Press, 93–217.

Church SA. 2003. Molecular phylogenetics of *Houstonia* (Rubiaceae): descending aneuploidy and breeding system evolution in the radiation of the lineage across North America. *Molecular Phylogenetics and Evolution* **27:** 223–238.

Cortés-B R, Delprete PG, Motley TJ. 2009. Phylogenetic placement of the tribe Retiniphylleae among the subfamily Ixoroideae (Rubiaceae). *Annals of the Missouri Botanical Garden* **96:** 61–67.

Delprete PG, Cortes-B R. 2004. A phylogenetic study of the tribe Sipaneeae (Rubiaceae, Ixoroideae), using trnL-F and ITS sequence data. *Taxon* **53:** 347–356.

Dane F, Lang P, Huang H, Fu Y. 2003. Intercontinental genetic divergence of *Castanea* species in eastern Asia and eastern North America. *Heredity* **91:** 314–321.

von Dohlen CD, Kurosu U, Aoki S. 2002. Phylogenetics and evolution of the eastern Asian–eastern North American disjunct aphid tribe, Hormaphidini (Hemiptera: Aphididae). *Molecular Phylogenetics and Evolution* **23:** 257–267.

Dorofeev PI. 1960. New data about Tertiary floras of Kirrenskiy ravine on the Ob River. *Doklady Akademii Nauk SSSR* **133:** 211–213.

Dorofeev PI. 1963. *Tertiary floras of Western Siberia*. Moscow: Izd-vo Akademii nauk SSSR.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7:** 214.

Eriksson O, Bremer B. 1991. Fruit characteristics, life forms, and species richness in the plant family Rubiaceae. *American Naturalist* **138:** 751–761.

Eriksson O, Bremer B. 1992. Pollination systems, dispersal modes, life forms, and diversification rates in angiosperm families. *Evolution* **46:** 258–266.

Ehrendorfer F, Manen JF, Natali A. 1994. cpDNA intergene sequences corroborate restriction site data for reconstructing Rubiaceae phylogeny. *Plant Systematics and Evolution* **190:** 245–248.

Friis EM. 1985. Angiosperm fruits and seeds from the Middle Miocene of Jutland, Denmark. *Biologiske Skrifter* **24:** 1–165.

Fu C, Kong H, Qiu Y, Cameron KM. 2005. Molecular phylogeny of the east Asian–North American disjunct *Smilax* sect. *Nemexia* (Smilacaceae). *International Journal of Plant Sciences* **166:** 301–309.

Ganders FR. 1975. Fecundity in distylous and self-incompatible homostylous plants of *Mitchella repens* (Rubiaceae). *Evolution* **29:** 186–188.

Graham, A. 1972. Outline of the origin and historical recognition of floristic affinities between Asia and eastern North America. In: Graham A, ed. *Floristics and Paleofloristics of Asia and Eastern North America*. Amsterdam: Elsevier, 1–18.

Graham A. 2009. Fossil record of the Rubiaceae. *Annals of the Missouri Botanical Garden* **96:** 90–108.

Groeninckx I, Dessein S, Ochoterena H, Persson C, Motley TJ, Kårehed J, Bremer B, Huysmans S, Smets

E. 2009. Phylogeny of the herbaceous tribe Spermacoceae (Rubiaceae) based on plastid DNA data. *Annals of the Missouri Botanical Garden* **96:** 109–132.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Hicks DJ, Wyatt R, Meagher TR. 1985. Reproductive biology of distylous partridgeberry, *Mitchella repens*. *American Journal of Botany* **72:** 1503–1514.

Hong DY. 1993. Eastern Asian–North American disjunctions and their biological significance. *Cathaya* **5:** 1–39.

Hooker JD. 1873. Ordo LXXXIV. Rubiaceae. In: Bentham G, Hooker JD, eds. *Genera plantarum ad exemplaria imprimis in herbariis kewensibus servata defirmmata*. London: 2. 7–151.

Hopkins DM. 1967. *The Bering land bridge*. Stanford, CT: Stanford University Press.

Huelsenbeck J, Ronquist F. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17:** 754–755.

Kainulainen K, Mouly A, Khodabandeh A, Bremer B. 2009. Molecular phylogenetic analysis of the tribe Alberteae (Rubiaceae), with description of a new genus, *Razafimandimbisonia*. *Taxon* **58:** 757–768.

Kårehed J, Bremer B. 2007. The systematics of Knoxieae (Rubiaceae): molecular data and their taxonomic consequences. *Taxon* **56:** 1051–1076.

Kårehed J, Groeninckx I, Dessein S, Motley TJ, Bremer B. 2008. The phylogenetic utility of chloroplast and nuclear DNA markers and the phylogeny of the Rubiaceae tribe Spermacoceae. *Molecular Phylogenetics and Evolution* **49:** 843–866.

Khan SA, Razafimandimbison SG, Bremer B, Liede-Schumann S. 2008. Sabiceeae and Virectarieae (Rubiaceae, Ixoroideae): one or two tribes? New tribal and generic circumscriptions of Sabiceeae and biogeography of *Sabicea* s.l. *Taxon* **57:** 7–23.

Kim S, Kim S, Shin H, Kim Y. 2009. Molecular phylogeny of *Astilbe*: implications for phylogeography and morphological evolution. *Korean Journal of Plant Taxonomy* **39:** 35–41.

Leopold EB, MacGinitie HD. 1972. Development and affinities of Tertiary floras in the Rocky Mountains. In: Graham A, ed. *Floristics and Palaeofloristics of Asia and Eastern North America*. Amsterdam: Elsevier, 147–200.

Li HL. 1952. Floristic relationships between eastern Asia and eastern North America. *Transactions of the American Philosophical Society* **42:** 371–429.

Lindley J. 1846. *The vegetable kingdom*. London: Bradbury & Evans.

Liu H-Y, Yang T. 1998. Rubiaceae. In: **Editorial Committee of the Flora of Taiwan, ed.** *Flora of Taiwan*, 2nd edn, Vol. *4*. Taipei: National Taiwan University, 245–340.

Mai DH, Walther H. 1985. The upper Eocene floras of the Weisselster basin and adjacent areas. *Abhandlungen des Staatlichen Museums für Mineralogie und Geologie zu Dresden* **33:** 1–260.

Makino T. 1909. Observations on the flora of Japan. *The Botanical Magazine* **XXIII:** 68–69.

Malcomber ST. 2002. Phylogeny of *Gaertnera* Lam. (Rubiaceae) based on multiple DNA markers: evidence of a rapid radiation in a widespread, morphologically diverse genus. *Evolution* **56:** 42–57.

Manen JF, Natali A, Ehrendorfer F. 1994. Phylogeny ofRubiaceae-Rubieae inferred from the sequence of a cpDNA intergene region. *Plant Systematics and Evolution* **190:** 195–211.

Manns U, Bremer B. 2010. Towards a better understanding of intertribal relationships and stable tribal delimitations within Cinchonoideae s.s. (Rubiaceae). *Molecular Phylogenetics and Evolution* **56:** 21–39.

Meng Y, Wen J, Nie ZL, Sun H, Yang YP. 2008. Phylogeny and biogeographic diversification of *Maianthemum* (Ruscaceae: Polygonatae). *Molecular Phylogenetics and Evolution* **49:** 424–434.

Miller JH, Miller KV. 2005. *Forest plants of the southeast and their wildlife uses*. Athens, GA: University of Georgia Press.

Motley TJ, Wurdack KJ, Delprete PG. 2005. Molecular systematics of the Catesbaeeae-Chiococceae complex (Rubiaceae): flower and fruit evolution and biogeographic implications. *American Journal of Botany* **92:** 316–329.

Motohashi K, Inaba S, Anzai K, Takamatsu S, Nakashima C. 2009. Phylogenetic analyses of Japanese species of *Phyllosticta* sensu stricto. *Mycoscience* **50:** 291–302.

Mouly A, Razafimandimbison SG, Achille F, Haevermans T, Bremer B. 2007. Phylogenetic placement of *Rhopalobrachium fragrans* (Rubiaceae): evidence from molecular (*rps16* and *trnT-F*) and morphological data. *Systematic Botany* **32:** 872–882.

Mouly A, Razafimandimbison SG, Florence J, Jérémie J, Bremer B. 2009. Paraphyly of *Ixora* and new tribal delimitation of Ixoreae (Rubiaceae): inference from combined chloroplast (*rps16*, *rbcL*, and *trnT-F*) sequence data. *Annals of the Missouri Botanical Garden* **96:** 146–160.

Naiki A, Nagamasu H. 2003. Distyly and pollen dimorphism in *Damnacanthus* (Rubiaceae). *Journal of Plant Research* **116:** 105–113.

Naiki A, Nagamasu H. 2004. Correlation between distyly and ploidy level in *Damnacanthus* (Rubiaceae). *American Journal of Botany* **91:** 664–671.

Nepokroeff M, Bremer B, Sytsma KJ. 1999. Reorganization of the genus *Psychotria* and tribe Psychotrieae (Rubiaceae) inferred from ITS and *rbcL* sequence data. *Systematic Botany* **24:** 5–27.

Nie Z-L, Sun H, Beardsley PM, Olmstead RG, Wen J. 2006a. Evolution of biogeographic disjunction between eastern Asia and eastern North America in *Phryma* (Phrymaceae). *American Journal of Botany* **93:** 1343–1356.

Nie Z-L, Sun H, Chen Z-D, Meng Y, Manchester SR, Wen J. 2010. Molecular phylogeny and biogeographic diversification of *Parthenocissus* (Vitaceae) disjunct between Asia and North America. *American Journal of Botany* **97:** 1342–1353.

Nie Z-L, Sun H, Li H, Wen J. 2006b. Intercontinental biogeography of subfamily Orontioideae (*Symplocarpus*, *Lysichiton*, and *Orontium*) of Araceae in eastern Asia and North America. *Molecular Phylogenetics and Evolution* **40:** 155–165.

Nie Z-L, Wen J, Sun H, Bartholomew B. 2005. Monophyly of *Kelloggia* Torrey ex Benth.(Rubiaceae) and evolution of its intercontinental disjunction between western North America and eastern Asia. *American Journal of Botany* **92:** 642–652.

Novotny V, Basset Y, Miller SE, Weiblen GD, Bremer B, Cizek L, Drozd P. 2002. Low host specificity of herbivorous insects in a tropical forest. *Nature* **416:** 841–844.

Nylander J. 2004. *Mrmodeltest v2*. Program distributed by the author. Uppsala: Evolutionary Biology Centre, Uppsala University.

Olmstead RG, Bremer B, Scott KM, Palmer JD. 1993. A parsimony analysis of the Asteridae sensu lato based on *rbcL* sequences. *Annals of the Missouri Botanical Garden* **80:** 700–722.

Oxelman B, Lidén M, Berglund D. 1997. Chloroplast *rps16* intron phylogeny of the tribe Sileneae (Caryophyllaceae). *Plant Systematics and Evolution* **206:** 393–410.

Parks CR, Wendel JF. 1990. Molecular divergence between Asian and North American species of *Liriodendron* (Magnoliaceae) with implications for interpretation of fossil floras. *American Journal of Botany* **77:** 1243–1256.

Persson C. 2000. Phylogeny of Gardenieae (Rubiaceae) based on chloroplast DNA sequences from the *rps16* intron and *trnL* (UAA)-*F* (GAA) intergenic spacer. *Nordic Journal of Botany* **20:** 257–270.

Piesschaert F, Andersson L, Jansen S, Dessein S, Robbrecht E, Smets E. 2000. Searching for the taxonomic position of the African genus *Colletoecema* (Rubiaceae): morphology and anatomy compared to an *rps16* intron analysis of the Rubioideae. *Canadian Journal of Botany* **78:** 288–304.

Puff C. 1982. The delimitation of the tribe Anthospermeae and its affinities to the Paederieae (Rubiaceae). *Botanical Journal of the Linnean Society* **84:** 355–377.

Razafimandimbison SG, Bremer B. 2002. Phylogeny and classification of Naucleeae s.l. (Rubiaceae) inferred from molecular (ITS, *rbcL*, and *trnT-F*) and morphological data. *American Journal of Botany* **89:** 1027–1041.

Razafimandimbison SG, Kellogg EA, Bremer B. 2004. Recent origin and phylogenetic utility of divergent ITS putative pseudogenes: a case study from Naucleeae (Rubiaceae). *Systematic Biology* **53:** 177–192.

Razafimandimbison SG, McDowell TD, Halford DA, Bremer B. 2009. Molecular phylogenetics and generic assessment in the tribe Morindeae (Rubiaceae–Rubioideae): how to circumscribe *Morinda* L. to be monophyletic? *Molecular Phylogenetics and Evolution* **52:** 879–886.

Razafimandimbison SG, Rydin C, Bremer B. 2008. Evolution and trends in the Psychotrieae alliance (Rubiaceae)—a rarely reported evolutionary change of many-seeded carpels from one-seeded carpels. *Molecular Phylogenetics and Evolution* **48:** 207–223.

Robbrecht E, Puff C, Igersheim A. 1991. The genera *Mitchella* and *Damnacanthus*: evidence for their close alliance; comments on the campylotropy in the Rubiaceae and circumscription of the Morindeae. *Blumea* **35:** 307–345.

Rogers GK. 2005. The genera of Rubiaceae in the southeastern United States, Part II. Subfamily Rubioideae, and subfamily Cinchonoideae revisited(*Chiococca*, *Erithalis*, and *Guettarda*) 1. *Harvard Papers in Botany* **10:** 1–45.

Ronquist F. 1996. *DIVA: dispersal–vicariance analysis, version 1.1*. Uppsala: Uppsala University.

Rova JHE, Delprete PG, Andersson L, Albert VA. 2002. A *trn*L-F cpDNA sequence study of the Condamineeae–Rondeletieae–Sipaneeae complex with implications on the phylogeny of the Rubiaceae. *American Journal of Botany* **89:** 145–159.

Rydin C, Kainulainen K, Razafimandimbison SG, Smedmark JEE, Bremer B. 2009a. Deep divergences in the coffee family and the systematic position of *Acranthera*. *Plant Systematics and Evolution* **278:** 101–123.

Rydin C, Razafimandimbison SG, Bremer B. 2008. Rare and enigmatic genera (*Dunnia*, *Schizocolea*, *Colletoecema*), sisters to species-rich clades: phylogeny and aspects of conservation biology in the coffee family. *Molecular Phylogenetics and Evolution* **48:** 74–83.

Rydin C, Razafimandimbison SG, Khodabandeh A, Bremer B. 2009b. Evolutionary relationships in the Spermacoceae alliance (Rubiaceae) using information from six molecular loci: insights into systematic affinities of *Neohymenopogon* and *Mouretia*. *Taxon* **58:** 793–810.

Saenger P. 1998. Mangrove vegetation: an evolutionary perspective. *Marine and Freshwater Research* **49:** 277–286.

Shi X, Jin J, Ye C, Liu W. 2012. First fruit fossil record of *Morinda* (Rubiaceae) from China. *Review of Palaeobotany and Palynology* **179:** 13–16.

Simões AO, Livshultz T, Conti E, Endress ME. 2007. Phylogeny and systematics of the Rauvolfioideae (Apocynaceae) based on molecular and morphological evidence. *Annals of the Missouri Botanical Garden* **94:** 268–297.

Smedmark JEE, Eriksson T, Bremer B. 2010. Divergence time uncertainty and historical biogeography reconstruction—an example from *Urophylleae* (Rubiaceae). *Journal of Biogeography* **37:** 2260–2274.

Sonké B, Dessein S, Taedoumg H, Groeninckx I, Robbrecht E. 2008. A new species of *Colletoecema* (Rubiaceae) from southern Cameroon with a discussion of relationships among basal Rubioideae. *Blumea* **53:** 533–547.

Struwe L, Thiv M, Kadereit JW, Pepper ASR, Motley TJ, White PJ, Rova JHE, Potgieter K, Albert VA. 1998. *Saccifolium* (Saccifoliaceae), an endemic of Sierra de la Neblina on the Brazilian-Venezuelan border, is related to a temperate-alpine lineage of Gentianaceae 1. *Harvard Papers in Botany* **3:** 199–214.

Swofford DL. 2003. *PAUP*: phylogenetic analysis using parsimony, version 4.0 b10*. Sunderland, MA: Sinauer.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25:** 4876–4882.

Tiffney BH. 1985a. The Eocene North Atlantic land bridge: its importance in Tertiary and modern phytogeography of the Northern Hemisphere. *Journal of the Arnold Arboretum* **66:** 243–273.

Tiffney BH. 1985b. Perspectives on the origin of the floristic similarity between eastern Asia and eastern North America. *Journal of the Arnold Arboretum* **66:** 73–94.

Tiffney BH, Manchester SR. 2001. The use of geological and paleontological evidence in evaluating plant phylogeographic hypotheses in the Northern Hemisphere Tertiary. *International Journal of Plant Sciences* **162:** S3–S17.

Wen J. 1998. Evolution of the Eastern Asian and Eastern North American disjunct pattern: insights from phylogenetic studies. *Korean Journal of Plant Taxonomy* **28:** 63–81.

Wen J. 1999. Evolution of eastern Asian and eastern North American disjunct distributions in flowering plants. *Annual Review of Ecology and Systematics* **30:** 421–455.

Wen J. 2000. Internal transcribed spacer phylogeny of the Asian and eastern North American disjunct *Aralia* sect. *Dimorphanthus* (Araliaceae) and its biogeographic implications. *International Journal of Plant Sciences* **161:** 959–966.

Wen J. 2001. Evolution of eastern Asian–eastern North American biogeographic disjunctions: a few additional issues. *International Journal of Plant Sciences* **162:** S117–S122.

Wen J, Ickert-Bond S, Nie Z-L, Li R. 2010. Timing and modes of evolution of eastern Asian–North American biogeographic disjunctions in seed plants. In: Long M, Gu H, Zhou Z, eds. *Darwin's heritage today: Proceedings of the Darwin 2010 Beijing International Conference*. Beijing: Higher Education Press, 252–269.

Westergaard KB, Jørgensen MH, Gabrielsen TM, Alsos IG, Brochmann C. 2010. The extreme Beringian/Atlantic disjunction in *Saxifraga rivularis* (Saxifragaceae) has formed at least twice. *Journal of Biogeography* **37:** 1262–1276.

Willson MF. 1993. Mammals as seed-dispersal mutualists in North America. *Oikos* **67:** 159–176.

Wolfe, JA. 1972. An interpretation of Alaskan Tertiary floras In: Graham A, ed. *Floristics and paleofloristics of Asia and eastern North America*. Amsterdam: Elsevier, 201–233.

Wolff D, Liede-Schumann S. 2007. Evolution of flower morphology, pollen dimorphism, and nectar composition in *Arcytophyllum*, a distylous genus of Rubiaceae. *Organisms Diversity and Evolution* **7:** 106–123.

Xiang QY, Soltis DE. 2001. Dispersal–vicariance analyses of intercontinental disjuncts: historical biogeographical implications for angiosperms in the Northern Hemisphere. *International Journal of Plant Sciences* **162:** 29–39.

Xiang QY, Soltis DE, Soltis PS, Manchester SR, Crawford DJ. 2000. Timing the eastern Asian–eastern North American floristic disjunction: molecular clock corroborates paleontological estimates. *Molecular Phylogenetics and Evolution* **15:** 462–472.

Xie L, Wagner WL, Ree RH, Berry PE, Wen J. 2009. Molecular phylogeny, divergence time estimates, and historical biogeography of *Circaea* (Onagraceae) in the Northern Hemisphere. *Molecular Phylogenetics and Evolution* **53:** 995–1009.

Xu X, Walters C, Antolin MF, Alexander ML, Lutz S, Ge S, Wen J. 2010. Phylogeny and biogeography of the eastern Asian–North American disjunct wild-rice genus (*Zizania* L., Poaceae). *Molecular Phylogenetics and Evolution* **55:** 1008–1017.

Yamazaki T. 1993. Rubiaceae. In: Iwatsuki KT, Yamazaki K, Boufford DE, Ohba H, eds. *Flora of Japan*, *Vol. 3a*. Tokyo: Koudansha, 206–240.

Yokoyama J, Fukuda T, Tsukaya H. 2003. Morphological and molecular variation in *Mitchella undulata*, with special reference to the systematic treatment of the dwarf form from Yakushima. *Journal of Plant Research* **116:** 309–315.

Yu Y, Harris A, He X. 2010. S-DIVA (statistical dispersal–vicariance analysis): a tool for inferring biogeographic histories. *Molecular Phylogenetics and Evolution* **56:** 848–850.

Yu Y, Harris A, He X. 2011. *RASP (reconstruct ancestral state in phylogenies) v1.1*. Available at: http://mnh.scu.edu.cn/soft/blog/RASP

Zhu W-D, Nie Z-L, Wen J, Sun H. in press. Molecular phylogeny and biogeography of *Astilbe* (Saxifragaceae) in Asia and eastern North America. *Botanical Journal of the Linnean Society* **171:** DOI: 10.1111/j.1095-8339.2012.01318.x.

APPENDIX

SEQUENCES OBTAINED FROM GENBANK AND USED IN THE DIVERGENCE TIME ANALYSES

Species	ITS	*atpB-rbcL*	*rbcL*	*rps16*	*trnL-F*	Reference
Alberta magna E.mey.	AJ224842	–	Y18708	EU145491	AJ620118	Andersson & Rova, 1999; Rydin *et al*., 2008; Kainulainen *et al*., 2009
Anthospermum herbaceum L.f.	FM204677	AJ234028	X83623	EU145496	EU145544	Bremer *et al*., 1995; Bremer & Manen, 2000; Rydin *et al*., 2008; Kainulainen *et al*., 2009
Arcytophyllum aristatum Standl.	AM182061	FJ695343	AJ288595	AF333348	AF333349	Bremer & Manen, 2000; Andersson *et al*., 2002; Wolff & Liede-Schumann, 2007; Rydin *et al*., 2009b

APPENDIX *Continued*

Species	ITS	*atpB-rbcL*	*rbcL*	*rps16*	*trnL-F*	Reference
Aulacocalyx jasminiflora Hook.f.	FM204688	DQ131704*	EU817413	EF205639	EU817455	Mouly *et al*., 2007, 2009; Kainulainen *et al*., 2009
Cephalanthus salicifolius Humb. & Bonpl.	AJ346886	GQ851993	AJ346975	GQ852381	AJ346920	Razafimandimbison & Bremer, 2002; Manns & Bremer, 2010
Chiococca alba (L.) Hitchc.	AY763882	–	L14394	AF004034	AY763813	Olmstead *et al*., 1993; Motley *et al*., 2005; Manns & Bremer, 2010
Cinchona calisaya Weed.	AY538352	GQ852003	AY538478	AF242927	GQ852482	Rova *et al*., 2002; Andersson & Antonelli, 2005; Manns & Bremer, 2010
Cinchonopsis amazonica (Stand.) L.Andersson	AY538357	GQ852002	AY538482	AY538428	AY538452	Andersson & Antonelli, 2005; Manns & Bremer, 2010
Coccocypselum condalia Pers.	EU145358	EU145420	AM117217	EU145499	EU145547	Rydin *et al*., 2008, 2009a; Bremer & Eriksson, 2009
Colletoecema dewevrei (De Wild.) E.M.A.Petit	EU145353	DQ131713*	FJ209067	AF191491	EU145532	Piesschaert *et al*., 2000; Rydin *et al*., 2008, 2009a; Sonké *et al*., 2008
Condaminea corymbosa (Ruiz & Pav.) DC.	FJ984973	–	Y18713	FJ884645	AF102406	Andersson & Rova, 1999; Motley *et al*., 2005
Cosmibuena grandiflora (Ruiz & Pav.) Rusby	GQ852120	GQ852007	AY538483	AF242929	AF152686	Rova *et al*., 2002; Andersson & Antonelli, 2005; Manns & Bremer, 2010
Coussarea hydrangeifolia (Benth.) Benth. & Hook.f. ex Müll.Arg.	EU145360	EU145326	EU145460	EU145501	EU145549	Rydin *et al*., 2008, 2009a
Coussarea macrophylla (Mart.) Müll.Arg.	–	–	Y11847	AF004040	–	Bremer & Thulin, 1998; Andersson & Rova, 1999
Cremaspora triflora (Thonn.) K.Schum.	AJ224824	DQ131718*	Z68856	AF200990	AF201040	Andreasen & Bremer, 1996; Andreasen *et al*., 1999; Persson, 2000
Crossopteryx febrifuga (Afzel. ex G.Don) Benth.	FM204689	DQ131719*	JF265372	FM204717	FM207123	Kainulainen *et al*., 2009
Cruckshanksia hymenodon Hook. & Arn.	–	AJ234004	AJ288599	EU145502	EU145550	Bremer & Manen, 2000; Rydin *et al*., 2008
Cubanola domingensis (Britton) Aiello	AY763891	DQ131720*	X83632	AF004044	AF152701	Rova *et al*., 2002; Motley *et al*., 2005
Declieuxia cordigera Mart. & Zucc. ex Schult. & Schult.f.	EU145361	EU145327	AM117224	AM117298	EU145551	Rydin *et al*., 2008, 2009a; Bremer & Eriksson, 2009
Dunnia sinensis Tutcher	EU145393	EU145343	EU145471	EU145519	EU145587	Rydin *et al*., 2008, 2009a

APPENDIX *Continued*

Species	ITS	*atpB-rbcL*	*rbcL*	*rps16*	*trnL-F*	Reference
Emmenopterys henryi Oliv.	FJ984985	DQ131728*	|Y18715	AF242941	AF152637	Bremer *et al.*, 1999; Rova *et al.*, 2002
Exostema lineatum (Vahl) Schult.	AY763901	DQ131732*	AY538484	AF242944	AY763833	Andersson & Antonelli, 2005; Motley *et al.*, 2005
Faramea multiflora A.Rich.	EU145363	EU145328	Z68796	AF004048	AF102422	Andreasen & Bremer, 1996; Struwe *et al.*, 1998; Andersson & Rova, 1999; Rydin *et al.*, 2008, 2009a
Gelsemium sempervirens (L.) J.St.-Hil.	AB454364	AJ233985	L14397	DQ660581	AF159696	Olmstead *et al.*, 1993; Bremer & Manen, 2000; Rova *et al.*, 2002; Simões *et al.*, 2007; Motohashi *et al.*, 2009
Geophila obvallata Didr.	AM945196	–	AM117228	JN643111*	JN643390	Bremer & Eriksson, 2009
Guettarda speciosa L.	AY763904	GQ852025	JF738600*	AF242964*	AY763835	Motley *et al.*, 2005; Manns & Bremer, 2010
Gynochthodes coriacea Blume	AM945192	AM945219	AJ288603	AM117311	AJ847407	Bremer & Manen, 2000; Alejandro *et al.*, 2005; Razafimandimbison *et al.*, 2008; Bremer & Eriksson, 2009
Hamelia papillosa Urb.	GQ852134	AJ233992	AY538487	AF004053	AF102439*	Andersson & Rova, 1999; Bremer & Manen, 2000; Andersson & Antonelli, 2005; Manns & Bremer, 2010
Hymenodictyon floribundum (Hochst. & Steud.) B.L.Rob.	AJ346905	DQ131742*	AY538488	AF004058	AY538454	Razafimandimbison & Bremer, 2002; Andersson & Antonelli, 2005
Isertia coccinea (Aubl.) J.F.Gmel.	GQ852140	–	GQ852337	GQ852405	AF152689	Rova *et al.*, 2002; Manns & Bremer, 2010
Jaubertia aucheri Guill.	FJ695456	FJ695383	DQ662178	DQ662202	DQ662145	Backlund *et al.*, 2007; Rydin *et al.*, 2009b
Joosia umbellifera H.Karst.	AY538361	–	AY538492	AY538433	GQ852521	Andersson & Antonelli, 2005; Manns & Bremer, 2010
Kerianthera praeclara J.H.Kirkbr.	AY538362	–	AY538493	AF242970*	AY538459	Andersson & Antonelli, 2005
Knoxia platycarpa Arn.	AM267002	FJ695363	AJ288631	AM266826	AM266915	Bremer & Manen, 2000; Kårehed & Bremer, 2007; Rydin *et al.*, 2009b
Kraussia floribunda Harv.	–	DQ131746*	JF265494*	AM117325	AM117368	Bremer & Eriksson, 2009
Ladenbergia amazonensis Ducke	AY538363	–	AY538494	AY538434	AY538460	Andersson & Antonelli, 2005
Lasianthus lanceolatus (Griseb.) Urb.	EU145367	EU145331	AM117238	AF004062	EU145554	Andersson & Rova, 1999; Rydin *et al.*, 2008, 2009a; Bremer & Eriksson, 2009

APPENDIX *Continued*

Species	ITS	*atpB-rbcL*	*rbcL*	*rps16*	*trnL-F*	Reference
Manostachya ternifolia E.S.Martins	FJ695446	EU542973	AM117246	EU543042	EU543127	Bremer & Eriksson, 2009; Groeninckx *et al*., 2009; Rydin *et al*., 2009b
Mitchella repens L.	AF072019*	AM945223	Z68805	AF001441	FJ906973	Bremer, 1996; Andersson & Rova, 1999; Razafimandimbison *et al*., 2008; Razafimandimbison *et al*., 2009
Morinda citrifolia L.	GU222395*	AJ234003	X83651	AJ320078	AF152616	Bremer *et al*., 1995; Bremer & Manen, 2000; Novotny *et al*., 2002; Rova *et al*., 2002
Nauclea orientalis (L.) L.	AJ346897	EU145320	X83653	AY538440	AJ346958	Bremer *et al*., 1995; Razafimandimbison & Bremer, 2002; Andersson & Antonelli, 2005; Rydin *et al*., 2008
Neurocalyx championii Benth. ex Thwaites	EU145376	–	EU145463	EU145509	EU145563	Rydin *et al*., 2008, 2009a
Normandia neocaledonica Hook.f.	AF257930*	FJ695375	FJ695375	AF257931*	AM409177	Khan *et al*., 2008; Rydin *et al*., 2009b
Ophiorrhiza elmeri Merr.	EU145378	–	EU145464	EU145510	EU145564	Rydin *et al*., 2008, 2009a
Paederia bojeriana (A.Rich. ex DC.) Drake	FJ695454	DQ131757*	DQ662181	DQ662206	DQ662152	Backlund *et al*., 2007; Rydin *et al*., 2009b
Parapentas silvatica (K.Schum.) Bremek.	AM267023	AJ234021	X83657	AM266849	AM266937	Bremer *et al*., 1995; Bremer & Manen, 2000; Kårehed & Bremer, 2007
Pavetta abyssinica Fresen.	FM204696	–	Z68863	FM204726	FM207133	Andreasen & Bremer, 1996; Kainulainen *et al*., 2009
Placopoda virgata Balf.f.	AM267064	FJ695382	Z68815	AM266894	AM266980	Bremer, 1996; Kårehed & Bremer, 2007; Rydin *et al*., 2009b
Posoqueria latifolia (Rudge) Schult.	DQ787409*	–	Z68850	AF242998*	AF152680	Andreasen & Bremer, 1996; Rova *et al*., 2002
Prismatomeris beccariana (Baill. ex K.Schum.) J.T.Johanss.	AM945206	AM945238	AF331651*	AF331652*	–	Razafimandimbison *et al*., 2008
Psychotria amboniana K.Schum.	AM945215	AM945248	AM945302	AM945328	AJ847409	Alejandro *et al*., 2005; Razafimandimbison *et al*., 2008
Rondeletia odorata Jacq.	AY730307*	EU145321	Y11857	EU145490	AF152741	Bremer & Thulin, 1998; Rova *et al*., 2002, Rydin *et al*., 2008
Sabicea diversifolia Pers.	AJ846883	DQ131781*	AM117268	EU145494	AJ847396	Alejandro *et al*., 2005; Bremer & Eriksson, 2009
Saprosma foetens (Wight) K.Schum.	FJ695460	FJ695386	DQ662193	DQ662218	DQ662168	Backlund *et al*., 2007; Rydin *et al*., 2009b

APPENDIX *Continued*

Species	ITS	*atpB-rbcL*	*rbcL*	*rps16*	*trnL-F*	Reference
Scyphiphora hydrophyllacea C.F.Gaertn.	–	–	Y18717	DQ923045	FM207140	Bremer *et al*., 1999; Kainulainen *et al*., 2009
Serissa foetida (L.f.) Lam.	FJ980385*	AJ234034	Z68822	AF004081	AF152618	Bremer, 1996; Andersson & Rova, 1999; Bremer & Manen, 2000
Sipanea biflora (L.f.) Cham. & Schltdl.	AY555116	DQ131788*	AY538509	AF004085	AF152675	Andersson & Rova, 1999; Delprete & Cortes, 2004; Andersson & Antonelli, 2005
Spermacoce hispida L.	AM939540	EU543011	AJ288623	EU543073	EU543162	Rova *et al*., 2002; Kårehed *et al*., 2008; Groeninckx *et al*., 2009; Rydin *et al*., 2009b
Spiradiclis bifida Kurz	EU145379	–	EU145465	EU145511	EU145565	Rydin *et al*., 2008, 2009a
Stilpnophyllum grandifolium L.Andersson	AY538375	GQ852090	AY538510	AY538446	AY538476	Andersson & Antonelli, 2005; Manns & Bremer, 2010
Theligonum cynocrambe L.	FJ695470	FJ695393	X83668	AF004087	FJ695427	Bremer *et al*., 1995; Andersson & Rova, 1999; Rydin *et al*., 2009b
Triainolepis mandrarensis Homolle ex Bremek.	AM267068	FJ695394	FJ695250	AM266899	AM266985	Kårehed & Bremer, 2007; Rydin *et al*., 2009b
Vangueria madagascariensis J.F.Gmel.	AJ224839	–	X83670	EU821636	FM207146	Bremer *et al*., 1995; Andreasen *et al*., 1999; Cortés-B *et al*., 2009
Xanthophytum borneense (Valeton) Axelius	EU145381	EU145335	EU145466	EU145513	EU145567	Rydin *et al*., 2008, 2009a

*Sequence unpublished.