Molecular phylogeny, divergence time estimates, and historical biogeography of *Circaea* (Onagraceae) in the Northern Hemisphere

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A R T I C L E   I N F O

A B S T R A C T

Circaea (Onagraceae) consists of eight species and six subspecies distributed in Eurasia and North America. The sister group of *Circaea* was recently shown to be *Fuchsia*, which comprises 107 species primarily distributed in montane Central and South America, including four species occurring in the South Pacific islands. Three plastid markers (*petB–petD*, *rpl16*, and *trnL-F*) and nrITS sequences from 13 of the 14 taxa of *Circaea* were sequenced and used to reconstruct the phylogenetic and biogeographic history of the genus. Parsimony and Bayesian analyses support that (1) *Circaea* is monophyletic; (2) the bilocular group is a weakly supported clade nested within the unilocular grade; (3) neither the *C. alpina* complex nor the *C. canadensis* complex is monophyletic; and (4) the western North American *C. alpina* subsp. *pacifica* diverged first in the genus. Divergence time estimates based on the Bayesian "relaxed" clock methods suggest that the earliest *Circaea* divergence occurred minimally at 16.17 mya (95% HPD: 7.69–24.53 mya). Biogeographic analyses using divergence–vicariance analysis (DIVA) and a likelihood method support the New World origin of *Circaea*. Three independent dispersal events between Eurasia and North America via the Bering land bridge were inferred within *Circaea*. Higher taxon diversity of *Circaea* in eastern Asia was probably caused by geologic and ecological changes during the late Tertiary in the Northern Hemisphere.

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1. Introduction

A primary aim of historical biogeography is to identify the causal factors or processes that have shaped the composition and distribution of biotas over time (Sanmartín, 2007). Another is to infer the evolution of geographic ranges of species and clades in a phylogenetic context (Ree and Smith, 2008). In seeking to understand how present-day biodiversity has been assembled, historical biogeography addresses important questions such as: Where did lineages originate? Where were ancestors distributed? Which processes (e.g., vicariance or dispersal) cause geographic ranges to evolve through time? Satisfying answers to these questions depend on robust estimates of phylogenetic relationships, ages of relevant clades, and the spatial and temporal occurrence of dispersal barriers (Sytsma et al., 2004). Many studies have recently addressed biogeographic questions using phylogenetic analyses, fossil-calibrated molecular dating, and reconstruction of ancestral geographic ranges (e.g., Xiang et al., 1998, 2000; Wen, 2001;

Sytsma et al., 2004; Nie et al., 2005, 2006; Bell, 2007; Sanmartín et al., 2008). New methods for estimating clade ages use "relaxed clock" models that allow variation in substitution rates among lineages (Drummond et al., 2006). Methods for reconstructing ancestral geographic ranges on phylogenetic trees commonly include those based on parsimony, e.g., tree fitting (Ronquist, 2003), event based divergence–vicariance analysis (DIVA, Ronquist, 1996, 1997), and likelihood method, e.g., Lagrange to infer biogeographic events (Ree et al., 2005; Ree and Smith, 2008).

Intercontinental disjunct distributions of plants in the Northern Hemisphere have attracted considerable attention since the time of Linnaeus (see Li, 1952; Graham, 1972; Boufford and Spongberg, 1983; Tiffney, 1985a, b; Wen, 1999, 2001; Tiffney and Manchester, 2001; Donoghue and Smith, 2004). Many temperate angiosperm genera exhibit disjunct distributional patterns in two or more of the following areas: eastern Asia, North America, western Asia, and southeastern Europe (Wood, 1972; Wu, 1983; Wen, 1999; Xiang and Soltis, 2001). The standard explanation put forward for these patterns is the existence of a widespread vegetation type (the 'mixed mesophytic forest') in the Tertiary followed by some extinctions due to climatic changes during the late Tertiary and

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the Quaternary (Wolfe, 1975; Tiffney, 1985a, b; Manchester, 1999; Wen, 1999; Sanmartín et al., 2001; Tiffney and Manchester, 2001; Milne and Abbott, 2002). These disjunct genera originated from several different centers at different latitudes that spread quickly to occupy the two continents via the North Atlantic land bridges, the Bering land bridge, and regions along the Tethys Sea Way (Wolfe, 1975; Tiffney, 1985a; Wen, 1999; Xiang and Soltis, 2001; Milne, 2006). A wide range of divergence times from molecular dating analyses among the disjunct groups between eastern Asia and North America suggests multiple and complex origins of the disjunctions in the Northern Hemisphere (Wen, 1999; Xiang et al., 2000; Donoghue et al., 2001).

Circaea is one of the two genera of Onagraceae (the other is Chamerion) exhibiting a wide distribution in Eurasia and North America. It is a small herbaceous genus with eight species and six additional subspecies distributed in the temperate to sub-arctic areas of the Northern Hemisphere (Table 1) spanning a wide range of latitudes (10–70°N) and altitudes (from sea level to 5000 m) (Boufford, 1982, 2005; Wagner et al., 2007). Species of Circaea occur in the mixed mesophytic and boreal forests and reach their greatest diversity in eastern Asia, where 11 of the 14 taxa are distributed (Boufford, 1982; Wagner et al., 2007).

The intercontinental disjunct patterns in Circaea involve two species complexes: the C. alpina complex and the C. canadensis complex (Table 1). Four of the six subspecies of the C. alpina complex are restricted to Asia (subsp. angustifolia, subsp. caulescens, subsp. imaicola, and subsp. micrantha); one is endemic to western North America (subsp. pacifica); and one (subsp. alpina) is distributed in the boreal and alpine zones of both North America and Eurasia (Table 1, Boufford, 1982). Each subspecies of this species complex exhibits different geographic or ecological preferences but with overlapping areas between two or more subspecies through parts of their distributional range. These subspecies form a reticulate pattern of morphologically intergrading populations, some of which are separated only by seemingly minute differences (Boufford, 1982; Boufford et al., 1990). The C. alpina complex seems to be continuously distributed in the vast area in the Northern Hemisphere. On the other hand, two subspecies of the Circaea canadensis complex are disjunctly distributed in eastern North America (subsp. canadensis), eastern Europe, the northern Altai Mountains, and Central to eastern Asia (subsp. quadrisulcata). The genus thus is an ideal group to study the evolution of the

intercontinental biogeographic disjunction in the Northern Hemisphere.

In general, eastern Asia harbors higher species diversity and possesses many more taxa than North America (Qian and Ricklefs, 2000, 2004; Xiang and Soltis, 2001). The recent molecular phylogenetic and biogeographic studies showed that intercontinental dispersal/migration of temperate taxa was biased toward one direction, largely from the Old World to the New World (Wen, 1999; Xiang and Soltis, 2001; Donoghue and Smith, 2004; Xiang et al., 2004). Boufford (1982), however, proposed that the ancestor of Circaea probably reached North America in the Paleocene from South America, the probable place of origin of the family. The relatively high diversity of taxa in eastern Asia was suggested to be related to the favorable climatic conditions prevailing there after Circaea reached eastern Asia, a well known center of survival for many Arcto-Tertiary plant groups (Axelrod, 1960), rather than the alternative hypothesis of an Asian origin for Circaea. Raven (1988) also suggested that the greater concentration of Circaea in eastern Asia is not due to the origin of the group there; instead it was due to the favorable conditions for survival in this region. Nevertheless, there have been no molecular phylogenetic analyses nor divergence time estimates conducted to test the biogeographic hypothesis of Circaea.

The sister group of Circaea has been suggested to be Fuchsia by recent molecular studies of Onagraceae (Bult and Zimmer, 1993; Conti et al., 1993; Ford and Gottlieb, 2007; Levin et al., 2003, 2004; Sytsma et al., 2004). In contrast to the herbaceous, circumboreal Circaea, the 107 species of Fuchsia are shrubs, trees, lianas, or epiphytes distributed primarily in Central and South America, especially in the tropical Andes, with two species in New Zealand and one on the Pacific island of Tahiti (Berry et al., 2004; Wagner et al., 2007). Despite the lack of unambiguous morphological synapomorphies, molecular data support the combination of Circaea and Fuchsia into a single tribe Circaeeae (Wagner et al., 2007).

Circaea has been classified into two "divisions", Uniloculares and Biloculares (Ascherson and Magnus, 1870), or two sections, sect. Uniloculares and sect. Lutetiana (= sect. Circaea) (Steinberg, 1949), based on the number of locules in the ovary. Circaea repens and C. alpina have just one locule, whereas the other six species have two locules (Table 1). Boufford (1982) considered C. repens to be intermediate between the unilocular and the bilocular groups due to its similar general appearance to the bilocular species, the

Table 1
Fourteen taxa of Circaea recognized by Boufford (1982, 2005) and Wagner et al. (2007), and their key characters and distribution.

Taxon	Locular number in the ovary	Position of the nectary	Geographic distribution
Circaea cordata Royle	2	At the base of the floral tube	Japan, E Russia, Korea, E China, Taiwan of China to W Himalaya.
C. glabrescens (Pamp.) Hand.-Mazz.	2	At the base of the floral tube	Central China, Taiwan of China.
C. mollis Sieb. & Zucc.	2	Exserted beyond the floral tube	Japan, E Russia, Korea, China to E Himalaya.
C. lutetiana L.	2	Exserted beyond the floral tube	Europe, North Africa, southwest Asia.
C. canadensis (L.) Hill			
subsp. canadensis	2	Exserted beyond the floral tube	E North America.
subsp. quadrisulcata (Maxim.) Boufford	2	Exserted beyond the floral tube	Japan, E Russia, Korea, northeast China, E Europe.
C. erubescens Franchet & Savatier	2	Exserted beyond the floral tube	Japan, Korea, north China to Taiwan of China.
C. repens Wallich ex Ascherson & Magnus	1	At the base of the floral tube	China to W Himalaya.
C. alpina L.			
subsp. alpina L.	1	At the base of the floral tube	Circumboreal
subsp. angustifolia (Hand.-Mazz.) Boufford	1	At the base of the floral tube	Southwest China
subsp. caulescens (Komarov) Tatewaki	1	At the base of the floral tube	Japan, Korea, China, southwest Asia
subsp. imaicola (Ascherson & Magnus) Kitamura	1	At the base of the floral tube	S China, Taiwan of China to W Himalaya, S Asia
subsp. micrantha (Skvortsov) Boufford	1	At the base of the floral tube	Southwest China to W Himalaya
subsp. pacifica (Ascherson & Magnus) Raven	1	At the base of the floral tube	W North America

presence of a trace of the second locule, and its flower position (also similar to that of the bilocular species). ith the consideration of *C. repens*, Boufford (1982) did not subdivide the genus into sections. In addition, based on a family-wide morphological comparison, Boufford (1982) proposed that the ancestral *Circaea* may have possessed the following character states: outcrossing, bilocular fruits, with nectary wholly within the floral tube, stolons without tubers, fruits not corrugated but with corky thickenings, and petals notched 1/3 to 2/3 their length. Two evolutionary "directions" were further suggested in the genus (Boufford, 1982). One is toward more efficient outcrossing through modification of the nectary so that it is more conveniently positioned for visitation by short-tongued insects such as syrphid flies. The other is toward self-pollination by having the anthers appressed to the stigma and dehiscing before the opening of the buds. The *C. alpina* complex is primarily self-pollinating, has only one locule in the ovary, lacks viscin threads on the pollen (unique in the family), and bears tubers at the tips of filiform rhizomes This species complex has therefore been considered to be a derived species in the genus. On the other hand, *C. cordata* was proposed as a more ancestral form due to its bilocular ovary and inferior nectary (Boufford, 1982). The base chromosome number of the genus is $n = 11$ with all species as diploids, but triploid hybrid individuals have been reported occasionally (Raven, 1963; Boufford, 1982; Seavey and Boufford, 1983). Inter-specific hybridization is common in *Circaea* and hybrids are highly sterile and reproduce vegetatively (Boufford, 1982). A cladistic analysis using morphological and anatomical characters was also conducted by Boufford et al. (1990). We therefore carry out this molecular study to infer phylogenetic relationships of *Circaea* and test the directionality of morphological evolution with an independent phylogenetic framework.

This study employs comprehensive sampling and DNA sequence data from both the nuclear and the plastid genomes to reconstruct the first molecular phylogeny of *Circaea* to test its species delimitations by previous studies (e.g., Boufford, 1982) based on morphology, to investigate the temporal and spatial diversification of the genus, and to provide insights into the divergence times and migration pathways of this disjunct group in the Northern Hemisphere.

2. Materials and methods

2.1. Taxon sampling

A total of 47 accessions belonging to 13 of the 14 currently recognized taxa of *Circaea* and representing all eight species, were included in this study (Table 2). Recent phylogenetic studies of Onagraceae show that *Fuchsia* is sister to *Circaea*, and *Hauya* is sister to the *Circaea–Fuchsia* clade or alternatively sister to the rest of the family except for *Ludwigia* L. (Bult and Zimmer, 1993; Conti et al., 1993; Ford and Gottlieb, 2007; Levin et al., 2003, 2004; Sytsma et al., 2004; Wagner et al., 2007). Because one of the main aims of this study is to reconstruct the ancestral area of *Circaea*, the sampling of its sister group *Fuchsia* needs to be broad. Thus 14 accessions of *Fuchsia* representing all currently recognized sections and covering all the geographic ranges of the genus, as well as one species of *Hauya* were included in the analysis. All samples of *Circaea* in this study were collected from natural populations.

2.2. DNA extractions, amplification, and sequencing

Total genomic DNA was extracted from silica-dried leaves using the DNeasy Plant Mini Kits (Qiagen Corporation, Valencia, California, USA). Polymerase chain reaction (PCR) of four genomic regions and cycle sequencing techniques were used to amplify and

sequence double-stranded DNA. The amplification and sequencing primers of the plastid regions were as follows: (1) the *petB–petD* spacer: primers "B" and "D" (primer forward: 5′-CTA TCG TCC RAC CGT TAC WGA GGC T-3′; primer reverse: 5′-CAA AYG GAT AYG CAG GTT CAC C-3′; Grivet et al., 2001); (2) the *trnL-F* spacer: primers "c" and "f" of Taberlet et al. (1991); and (3) the *rpl16* intron: primers F71 (5′-GCT ATG CTT AGT GTG TGA CTC GTT G-3′; Jordan et al., 1996) and R1516 (5′-CCC TTC ATT CTT CCT CTA TGT TG-3′; Kelchner and Clark, 1997).

Despite well-known potential flaws of nrITS region for inferring phylogeny (see Álvarez and Wendel, 2003), this region continues to be a widely used non-plastid region for species-level phylogenetic studies of plant groups (reviewed in Feliner and Rosselló, 2007). In this study, we followed the guidelines for obtaining a reliable ITS sequence in plants proposed by Feliner and Rosselló (2007) to amplify and analyze this region. The ITS region was amplified and sequenced with primers ITS4 and ITS5 (White et al., 1990).

The amplifications via polymerase chain reaction (PCR) were performed using 10 ng of genomic DNA, 4 pmol of each primer, 0.5 U *Taq* polymerase (Bioline), 2.5 mM MgCl$_2$ in a volume of 25 μL using a PTC-225 Peltier Thermal Cycler. The PCR cycling parameters for the ITS and the *petB–petD* spacer were as follows: a 95 °C initial hot start for 5 min with 38 cycles of 94 °C for 20 s, 50 °C for 30 s and 72 °C for 40 s followed by a final extension of 72 °C for 10 min. For the *trnL-F* spacer, the cycling parameters were 95 °C initial denaturing for 5 min with 38 cycles of 94 °C for 40 s, 50 °C for 60 s and 72 °C for 90 s and a final extension of 72 °C for 10 min. For the *rpl16* intron, cycling parameters consisted of a 95 °C initial hot start (5 min) and 30 cycles of 95 °C for 60 s, 50 °C for 60 s, an increase of 15 °C in 1 °C increments of 8 s each, 65° C for 4 min, followed by a final extension of 65 °C for 10 min (Kelchner and Clark, 1997). The PCR products were purified using the polyethylene glycol (PEG)/NaCl method of Kusukawa et al. (1990). Cycle sequencing was carried out directly on purified PCR product using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, California, USA), with 5 ng of primer, 1.5 μL of sequencing dilution buffer, and 1 μL of cycle sequencing mix in a 10 μL reaction volume. Cycle sequencing conditions were: 30 cycles of 30 s denaturation (96 °C), 30 s annealing (50 °C), and 4 min elongation (60 °C). Sequencing reactions were purified by gel filtration chromatography using the Sephadex columns (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and run on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA). We sequenced both strands of DNA with overlapping regions to ensure that each base is unambiguous. Electropherograms were assembled, ambiguous bases were corrected, and consensus sequences were generated with Sequencher 4.5 (GeneCodes, Ann Arbor, Michigan, USA). The sequences obtained in this study were deposited in GenBank (Table 2).

2.3. Sequence alignment and phylogenetic analyses

The sequences were aligned with ClustalX 1.83 (Thompson et al., 1997) and then adjusted manually using Se-Al 2.0 (Rambaut, 2002). Areas with ambiguous alignment or containing poly-N stretches were excluded from the analyses. The data matrix is available from the corresponding author.

Given that the plastid genome behaves as a single linked region and that the single regions exhibited low levels of variation, the three plastid markers (*trnL-F*, *rpl16*, and *petB–petD*) were concatenated *a priori*. Congruence between nrITS and the combined plastid data sets were tested using the incongruence length difference (ILD) test (Farris et al., 1994) as implemented by the partition homogeneity test in PAUP* for 100 replicates (heuristic

Table 2
Specimens sampled for phylogenetic analyses. Herbarium abbreviations follow standard herbarium acronyms from the Index Herbariorum.

Taxa	Source	Voucher	GenBank Accession No.			
			nrITS	trnL-F	rpl16	petB–petD
Circaea cordata Royle	Bomi Xian, Xizang Province, China	*J. Wen 9194* (US)	GQ232514	GQ232700	GQ232638	GQ232576
	Mt. Chiak, Wonju City, Kangwondo, South Korea	*K.O. Yoo.13* (US)	GQ232516	GQ232702	GQ232640	GQ232578
	Xinning Xian, Hunan Province, China	*J. Wen 9340* (US)	GQ232515	GQ232701	GQ232639	GQ232577
C. glabrescens (Pamp.) Hand-Mazz.	Xihe Xian, Gansu Province, China	*J. Wen 8028* (US)	GQ232521	GQ232707	GQ232645	GQ232583
	Shangrao Shi, Jiangxi Province, China	*J. Wen 9867* (US)	GQ232522	GQ232708	GQ232646	GQ232584
C. mollis Sieb. & Zucc.	Chengkou Xian, Chongqing Shi, China	*J. Wen 8087* (US)	GQ232528	GQ232714	GQ232652	GQ232590
	Nanchuan Xian, Chongqing Shi, China	*J. Wen 8174* (US)	GQ232529	GQ232715	GQ232653	GQ232591
	Xinning Xian, Hunan Province, China	*J. Wen 9286* (US)	GQ232530	GQ232716	GQ232654	GQ232592
	Xinning Xian, Hunan Province, China	*J. Wen 9358* (US)	GQ232531	GQ232717	GQ232655	GQ232593
	Mt. Maebong, Hongcheon Co., Kangwondo, South Korea	*K.O. Yoo 11* (US)	GQ232532	GQ232718	GQ232656	GQ232594
C. lutetiana L.	Zillertal, Tyrolia, Austria	*L.S. Ehrendorfer s.n.* (US)	GQ232523	GQ232709	GQ232647	GQ232585
	Germany	*F. Luebert 2946* (US)	GQ232524	GQ232710	GQ232648	GQ232586
	Near Tsagueri/ Cagveri, Borjomi, Republic of Georgia	*M. Merello et al. 2046* (MO)	GQ232525	GQ232711	GQ232649	GQ232587
	Caucasus, Russia	*J. Wen 10363* (US)	GQ232527	GQ232713	GQ232651	GQ232589
	Caucasus, Russia	*J. Wen 10352* (US)	GQ232526	GQ232712	GQ232650	GQ232588
C. canadensis (L.) Hill subsp. *canadensis*	Washington DC., USA	*J. Wen 9795* (US)	GQ232511	GQ232697	GQ232635	GQ232573
	Virginia, USA	*J. Wen 10385* (US)	GQ232508	GQ232694	GQ232632	GQ232570
	Quebec, Canada	*J. Wen 10476* (US)	GQ232509	GQ232695	GQ232633	GQ232571
	Pennsylvania, USA	*J. Wen 10497*(US)	GQ232510	GQ232696	GQ232634	GQ232572
C. canadensis subsp. *quadrisulcata* (Maxim.) Boufford	Fusong Xian, Jilin Province, China	*P. Peng 0702* (US)	GQ232512	GQ232698	GQ232636	GQ232574
	Qitaihe Shi, Heilongjiang Province, China	*Y.W. Wang 72031* (PE)	GQ232513	GQ232699	GQ232637	GQ232575
C. erubescens Franchet & Savatier	Japan	*H. Nagamasu 7369* (US)	GQ232517	GQ232703	GQ232641	GQ232579
	Japan	*H. Nagamasu 7370* (US)	GQ232518	GQ232704	GQ232642	GQ232580
	Xinning Xian, Hunan Province, China	*J. Wen 9313* (US)	GQ232519	GQ232705	GQ232643	GQ232581
	Shangrao Shi, Jiangxi Province, China	*J. Wen 9864* (US)	GQ232520	GQ232706	GQ232644	GQ232582
C. repens Wallich ex Ascherson & Magnus	Bomi Xian, Xizang Province, China	*J. Wen 9183* (US)	GQ232534	GQ232720	GQ232658	GQ232596
	Bomi Xian, Xizang Province, China	*J. Wen 9213* (US)	GQ232535	GQ232721	GQ232659	GQ232597
	Nyalam Xian, Xizang Province, China	*MacArthur-Tibet Expedition 709* (US)	GQ232533	GQ232719	GQ232657	GQ232595
C. alpina subsp. *caulescens* (Komarov) Tatewaki	Shangrao Shi, Jiangxi Province, China	*P. Peng 0701* (US)	GQ232494	GQ232680	GQ232618	GQ232556
C. alpina subsp. *imaicola* (Ascherson & Magnus) Kitamura	Jingdong Xian, Yunnan Province, China	*J. Wen 9094* (US)	GQ232498	GQ232684	GQ232622	GQ232560
	Bomi Xian, Xizang Province, China	*J. Wen 9212* (US)	GQ232501	GQ232687	GQ232625	GQ232563
	Gongbu Gyamdar Xian, Xizang Province, China	*J. Wen 9151* (US)	GQ232500	GQ232686	GQ232624	GQ232562
	Nyalam Xian, Xizang Province, China	*MacArthur-Tibet Expedition 879* (US)	GQ232496	GQ232682	GQ232620	GQ232558
	Songming Xian, Yunnan Province, China	*J. Wen 5754* (US)	GQ232497	GQ232683	GQ232621	GQ232559
	Yunnan Province, China	*J. Wen 9123* (US)	GQ232499	GQ232685	GQ232623	GQ232561
	Shangrao Shi, Jiangxi Province, China	*J. Wen 9845* (US)	GQ232502	GQ232688	GQ232626	GQ232564
	Xizang Province, China	*MacArthur-Tibet Expedition 1016* (US)	GQ232495	GQ232681	GQ232619	GQ232557

C. alpina subsp. *pacifica* (Ascherson & Magnus) Raven	Marin Co., California, USA	*B.G. Baldwin 1485* (US)	GQ232505	GQ232691	GQ232629	GQ232567
	Snow Bird, Utah, USA	*J. Wen 8000* (US)	GQ232507	GQ232693	GQ232631	GQ232569
	Tuolomne Co., California, USA	*J. Wen 7123* (US)	GQ232506	GQ232692	GQ232630	GQ232568
C. alpina L. subsp. *alpina*	Near Chulitna, Alaska, USA	*J. Wen 7214* (US)	GQ232493	GQ232679	GQ232617	GQ232555
	Rimouski Co., Quebec, Canada	*J. Wen 4729* (US)	GQ232489	GQ232675	GQ232613	GQ232551
	Near Sitka, Alaska, USA	*P.H. Raven s.n.* (US)	GQ232491	GQ232677	GQ232615	GQ232553
	Oberös Ferreich, Austria	*M. Hohla s.n.* (US)	GQ232490	GQ232676	GQ232614	GQ232552
	Quebec, Canada	*J. Wen 10442* (US)	GQ232492	GQ232678	GQ232616	GQ232554
C. alpina subsp. *micrantha* (Skvortsov) Boufford	Nyingchi Xian, Xizang Province, China	*MacArthur-Tibet Expedition 346* (US)	GQ232503	GQ232689	GQ232627	GQ232565
	Nyingchi Xian, Xizang Province, China	*J. Wen 9168* (US)	GQ232504	GQ232690	GQ232628	GQ232566
Fuchsia boliviana Carr.	La Paz, Bolivia	*S.D. Smith 447* (WIS)	GQ232536	GQ232722	GQ232660	GQ232598
F. bracelinae Munz	Minas Gerais, Brazil	*P. Berry 4525* (MO)	GQ232547	GQ232733	GQ232671	GQ232609
F. cylindracea Lindl.	Michoacán, Mexico	*P. Berry 5545* (MO)	GQ232537	GQ232723	GQ232661	GQ232599
F. denticulata Ruiz & Pav.	Santa Cruz, Bolivia	*M. Nee & J. Wen 53940* (US)	GQ232548	GQ232734	GQ232672	GQ232610
F. excorticata (J.R. & G. Forster) L. f.	New Zealand, South Island	*P. Berry & L. Brako 4623* (MO)	GQ232538	GQ232724	GQ232662	GQ232600
F. fulgens DC.	Cultivated, Seattle, Washington, USA	*O. De Graaf in July 1995* (MO)	GQ232539	GQ232725	GQ232663	GQ232601
F. insignis Hemsley	Azuay, Ecuador	*D. Green 1007* (MO)	GQ232540	GQ232726	GQ232664	GQ232602
F. lycioides Andr.	Cultivated in Missouri Botanical Garden M3649, St. Louis, Missouri, USA	*P. Berry 5546* (MO)	GQ232541	GQ232727	GQ232665	GQ232603
F. pachyrrhiza P.E. Berry & B.A. Stein	Cajamarca, Peru	*B. Stein et al. 4066* (MO)	GQ232542	GQ232728	GQ232666	GQ232604
F. procumbens Cunn.	New Zealand, North Island	*P. Berry & L. Brako 4625* (MO)	GQ232543	GQ232729	GQ232667	GQ232605
F. splendens Zucc.	Cultivated in Berkeley, California, USA	*UC-Berkeley Botanical Garden 81.0122*	GQ232544	GQ232730	GQ232668	GQ232606
F. thymifolia Kunth subsp. *thymifolia*	Puebla, Mexico	*J. Wen 8756* (US)	GQ232549	GQ232735	GQ232673	GQ232611
F. triphylla L.	Dominican Republic	*P. Berry 7760* (WIS)	GQ232545	GQ232731	GQ232669	GQ232607
F. verrucosa Hartw.	Táchira, Venezuela	*P. Berry 4581* (MO)	GQ232546	GQ232732	GQ232670	GQ232608
Hauya heydeana Donn. Sm.	Chiapas, Mexico	*J. Wen 8718* (US)	GQ232550	GQ232736	GQ232674	GQ232612

search, simple addition, TBR branching swapping), each saving a maximum of 100 trees per replicate. This method has been criticized recently (Siddall, 1997; Dolphin et al., 2000; Reeves et al., 2001; Yoder et al., 2001; Norup et al., 2006). Siddall (1997) points out that the ILD test does not actually reveal the amount of incongruence, and can be insensitive to small but significant topological differences suggested by the different data sets. Therefore we combined the two data sets to explore whether resolution and support would be improved by increasing the amount of sequencing data. This approach was performed by direct inspection of nodes with bootstrap scores above 70% in the separate analyses (Kluge, 1989; Nixon and Carpenter, 1996; Muellner et al., 2003; Acevedo-Rosas et al., 2004; Berry et al., 2004; Inda et al., 2008).

Phylogenetic analyses were undertaken for the nrITS, for the concatenated plastid data set, and for all molecular markers combined using maximum parsimony (MP) and Bayesian inference (BI) methods (Rannala and Yang, 1996). The most parsimonious trees were found by a heuristic search implemented with PAUP* 4.0b 10 (Swofford, 2003) using 1000 random addition sequence replicates, tree bisection-reconnection (TBR) branch swapping, MulTrees on. All characters were equally weighted and treated as unordered (Fitch, 1971). Gaps were treated as missing data. The amount of support for the clades revealed in the maximally parsimonious trees (MPTs) was examined with 1000 bootstrap replicates using the same options as above but only saving 100 trees per replicate.

Bayesian analyses were conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The best fit models were determined using the Akaike Information Criterion (Posada and Buckley, 2004) as implemented in Modeltest 3.7 (Posada and Crandall, 1998). This was the TrN + G model as best fitting the nrITS, the TVM + I + G model for the plastid data set, and the GTR + I + G model for the combined data set. For each analysis, the Markov chain Monte Carlo (MCMC) algorithm was run for 2,000,000 generations with four incrementally heated chains, starting from random trees and sampling one tree every 100 generations. Stability of the Markov chain was ascertained by plotting likelihood values against number of generations. The first 2000–5000 trees before stationary were discarded as burn-in, and the remaining trees were used to construct majority-rule consensus trees using PAUP*.

2.4. Divergence time estimation

To infer divergence times within *Circaea*, the combined nuclear and plastid data set was used. Extrapolations of divergence times across separate data set could be done to avoid the impact of heterogeneity. However, neither the nrITS nor the plastid data set provided enough phylogenetic information in the present study. The strict consensus trees from nrITS and plastid analyses differ only in some terminal clades (see below), which have no impact on the key results as discussed below. Thus we followed the procedures suggested by other authors that supported the use of more resolved but heterogeneous combined data set to infer the biogeographic history of plant lineages. Using a combined data set can also provide a better estimate of branch lengths and thus of divergence times (see Bell et al., 2005; Forest et al., 2007; Inda et al., 2008; Nie et al., 2008). A likelihood ratio test (Felsenstein, 1988) was carried out to test whether the combined markers evolved in a clock-like fashion. This test resulted in *P* < 0.05, suggesting that rate constancy in this data set was not supported. Therefore, the Bayesian dating method with a relaxed molecular clock was implemented using the program BEAST (Bayesian Evolutionary Analysis by Sampling Trees) 1.4.5 (Drummond and Rambaut, 2007) to estimate divergence times.

The Bayesian dating method uses a fully probabilistic model to describe rates of molecular sequence evolution in lineages over time and uses MCMC to derive the posterior distribution of rates and clade ages. For this analysis, rate variation among sites was modeled using a gamma distribution with four rate categories in the GTR model as suggested in Modeltest. We employed a relaxed molecular clock model with uncorrelated rates drawn from a log-normal distribution (Drummond et al., 2006). Posterior distributions of parameters were approximated using two independent MCMC analyses of 10,000,000 generations each, following a discarded burn-in of 1,000,000 generations. Samples from the two runs, which yielded similar results, were combined and convergence of the chains was checked using the program Tracer 1.3 (Rambaut and Drummond, 2004).

2.5. Calibrations

For fossil constraints, we checked all the pre-Quaternary fossil records of *Circaea* and its close allies. All known megafossils of *Circaea* are fossilized fruits from the Oligocene to the Pliocene in Europe and Siberia and did not extend beyond the current distribution areas of the genus (Reid and Reid, 1915; Dorofeev, 1963, 1969; Nikitin, 1957; Szafer, 1947). Pollen fossils are ample for *Fuchsia*, *Ludwigia* and their relatives in the Tertiary (Martin, 2003), suggesting an early diversification of these genera. To estimate the divergence of *Fuchsia*, Berry et al. (2004) used the *Circaea-Fuchsia* split estimated by Sytsma et al. (2004), which was based on calibrated *rbcL* and *ndhF* sequence data for the Myrtales using three fossil constraints. The three fossils of Myrtaceae, Melastomataceae, and Combretaceae produced similar estimates for the *Circaea–Fuchsia* split (42.7, 41.2 and 40.7 mya, respectively) (Berry et al., 2004). Because these times were consistent with the earliest fossil records of *Circaea* and *Fuchsia* (pollen from the Oligocene in Australia, Berry et al., 1990), Berry et al. (2004) used them to estimate divergence times of major clades of *Fuchsia*. In this paper, we followed Berry et al. (2004) and constrained the *Circaea-Fuchsia* node with the age of 41.5 mya (the average age from different fossil constraints). The root, i.e., the divergence time of *Hauya* and the *Circaea–Fuchsia* clade, was constrained to be 52.6 mya (the average of 54.1, 52.2 and 51.6) estimated by Berry et al. (2004). The time estimates of the nodes must be regarded as minimum ages.

2.6. Biogeographic analyses

To perform our analyses, we used the maximum clade credibility (MCC) phylogeny from the combined data set by BEAST, which provided the most resolved and best supported phylogeny, as well as a better estimation of the species tree as compared to the separate phylogenies. Distribution areas of *Circaea* and its allies were defined according to major taxonomic and geographic studies of these genera (Boufford, 1982; Berry et al., 2004; Wagner et al., 2007). Seven areas of endemism were defined, South Pacific islands (a), South America (b), Central America (c), western North America (d), eastern North America (e), western Eurasia (f), and eastern Eurasia (g) to cover all the endemic distributional areas of *Circaea* and its outgroups. Areas d–g represent distribution areas of *Circaea* (see Donoghue and Smith, 2004), whereas areas a–c correspond to those of *Fuchsia* and *Hauya* (Berry et al., 2004; Wagner et al., 2007). The biogeographic history of *Circaea* was investigated using dispersal–vicariance analysis (DIVA; Ronquist, 1996, 1997) and a maximum likelihood based method, LAGRANGE (Ree et al., 2005; Ree and Smith, 2008). Dispersal–vicariance analysis infers both ancestral distributions and biogeographic events based on a cost matrix derived from a simple biogeographic model by searching for a most-parsimonious solution, minimizing the total cost of the implied events. In LAGRANGE transitions between discrete states (ranges)
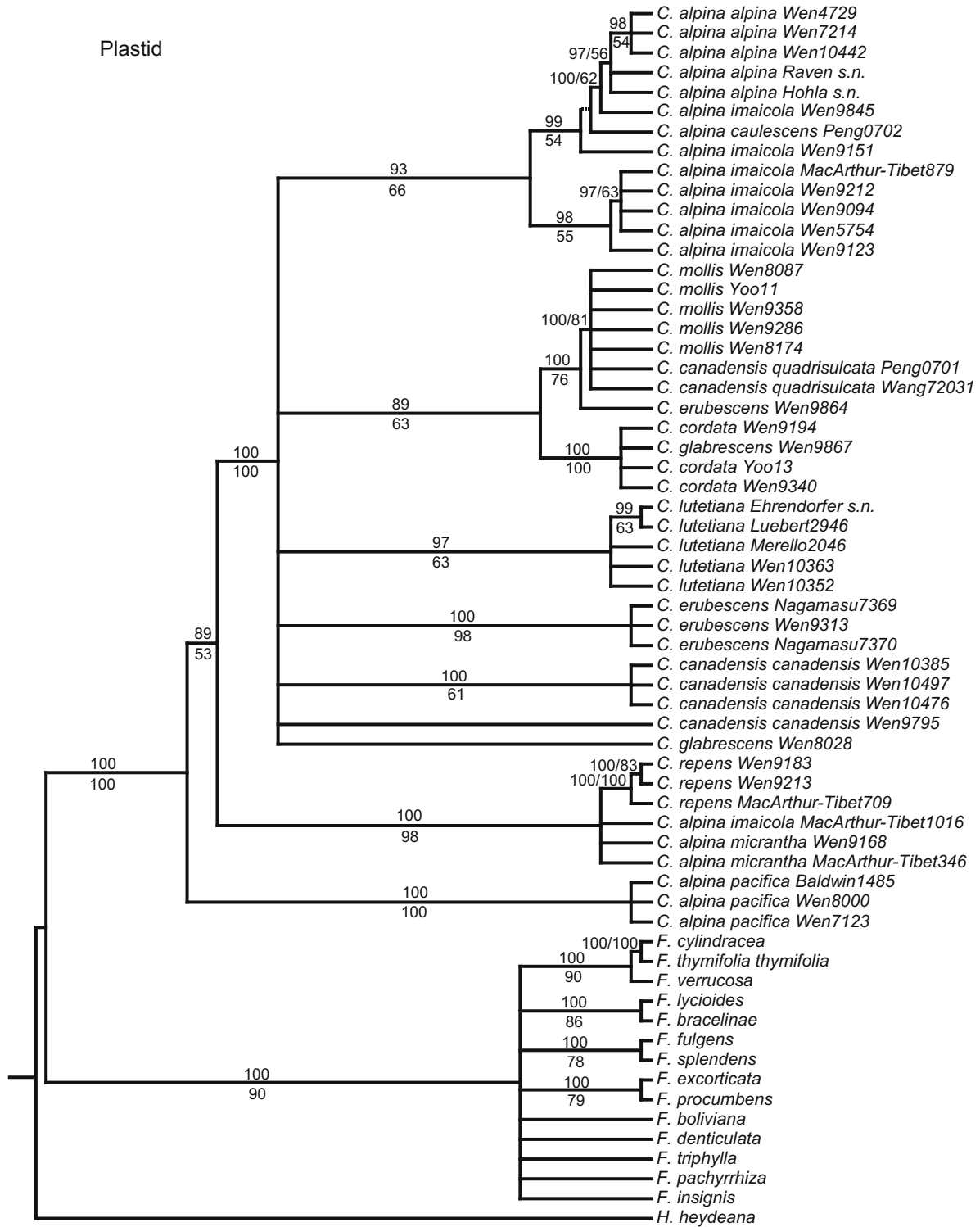
Plastid

Fig. 1. The strict consensus tree of 42 minimal-length trees derived from the maximum parsimony analysis of the combined plastid *trnL-F*, *rpl16*, and *petB–petD* data (tree length = 453 steps, CI = 0.79, and RI = 0.92). The bootstrap values (%) are shown below the lines and the Bayesian Markov chain Monte Carlo (MCMC) posterior probabilities (PP) (%) are indicated above the lines. Dashed lines indicate the clades that are not supported by both the BS and the BI analyses.

along phylogenetic branches are modeled as a function of time, thus enabling maximum likelihood estimation of the ancestral states (range inheritance scenarios) at cladogenesis events. This program finds the most likely ancestral areas at a node and the split of the areas in the two lineages, and it also calculates the probabilities of these most-likely areas at each node (Ree and Smith, 2008).

In the DIVA analysis, each taxon was scored as presence (1) or absence (0) for each of the seven areas. To explore influences of maximum number of areas constrained for each node, analyses with no constraint (C0), constraints of maximum areas of four (C1) and two (C2), were performed for comparison. The ML inferences of geographic range evolution using LAGRANGE were conducted for the same distribution matrix under the constraints of maximum areas
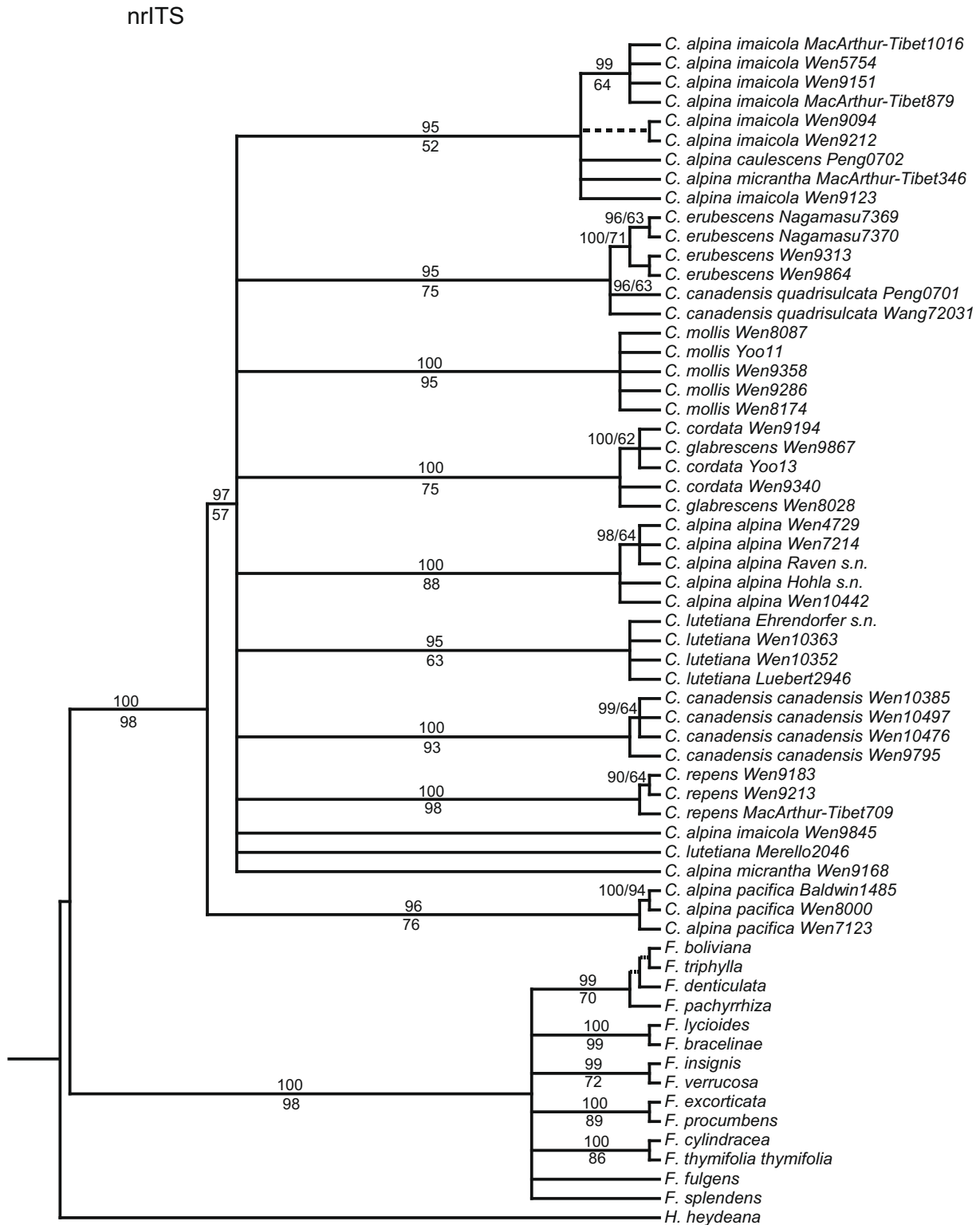
Fig. 2. The strict consensus tree of 5228 minimal-length trees derived from the maximum parsimony analysis of the nrITS data (tree length = 248 steps, CI = 0.79, and RI = 0.88). The bootstrap values (%) are shown below the lines and the PP values (%) are indicated above the lines. Dashed lines indicate the clades that are not supported by both the BS and the BI analyses.

of four (M1) and two (M2). To reduce computation time, the following areas were disallowed at all nodes: a–d, a–e, a–f, a–g, b–d, b–e, b–f, b–g, c–d, c–e, c–f, c–g, which requires prior extinction in their intervening areas, because both present and fossil species of *Circaea* (distributed in d–g) never occur in the natural areas (both present and fossil) of *Fuchsia* and *Hauya* (distributed in a–c).
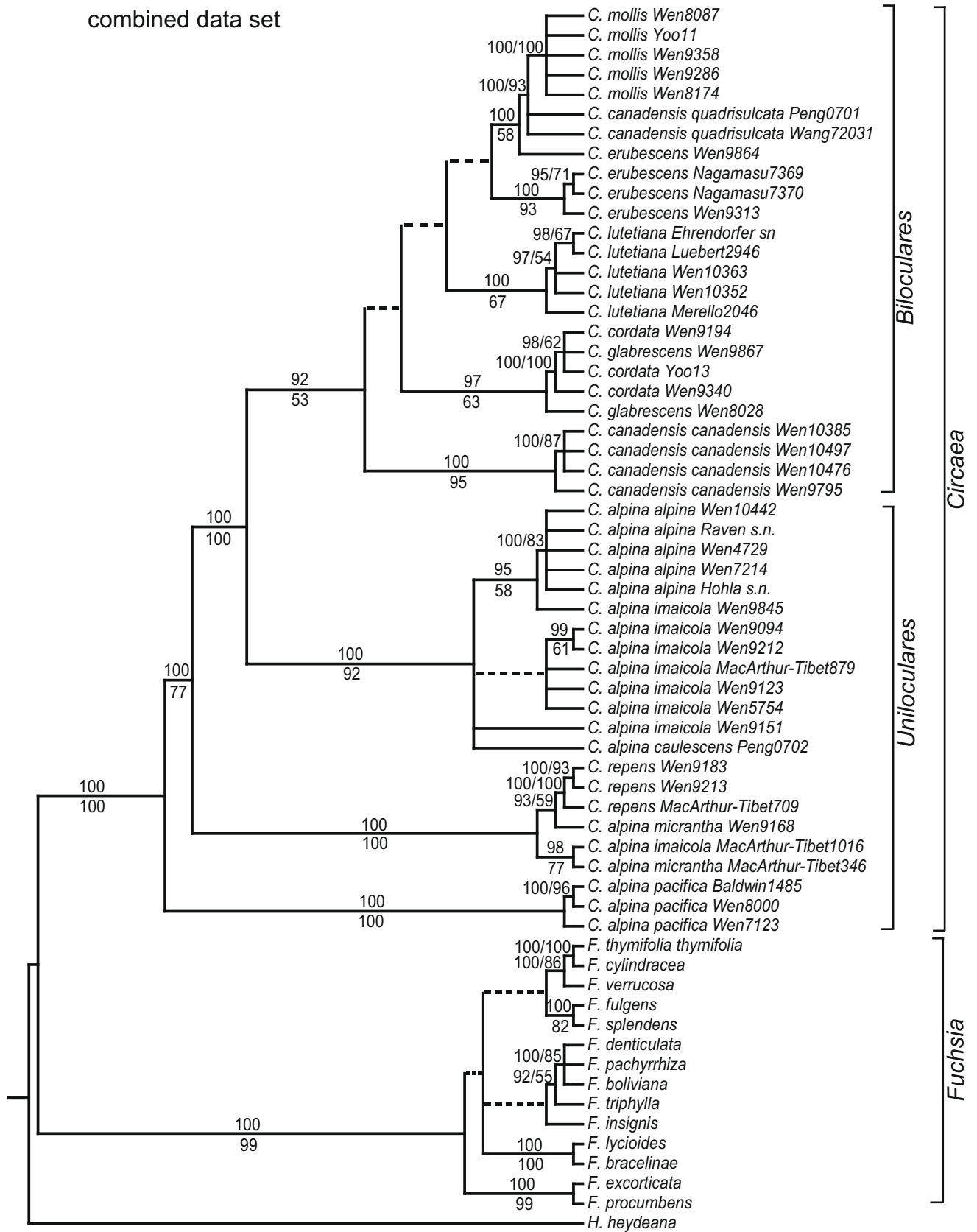
3. Results

3.1. Phylogenetic analyses

The separate *trnL-F*, *rpl16*, and *petB–petD* plastid data matrices consisted of 944, 1119 and 1529 aligned nucleotide positions,

combined data set

Fig. 3. The strict consensus tree of 385 minimal-length trees derived from the maximum parsimony analysis of the combined nuclear and plastid data set (tree length = 725 steps, CI = 0.77, and RI = 0.89). The bootstrap values (%) are shown below the lines and the PP values (%) are indicated above the lines. Dashed lines indicate the clades that are not supported by both the BS and the BI analyses.

respectively, with a concatenated 62-taxa plastid data matrix containing 3592 positions. Forty ambiguously aligned characters (14 from *trnL-F* and 26 from *rpl16* data set) were excluded from the analyses. Three hundred and thirty-eight (9.5%) of the 3552 non-excluded characters were variable and 150 (4.2%) were parsimony informative. The 62-taxa nrITS data matrix included 653 aligned

nucleotides of which 162 (25%) were variable and 76 (12%) were parsimony informative.

Parsimony-based and Bayesian (not shown) analyses of each data set (plastid, nrITS, and plastid/nrITS) produced similar topologies. The plastid and nuclear optimal trees were generally congruent concerning major clades, though the plastid topology was

Fig. 4. The maximum clade credibility (MCC) chronogram of *Circaea* and its relatives based on the combined nuclear and plastid data set using BEAST. Divergence time is given in million years before present. The tree was rooted using *Hauya* and the root was assigned with an estimated age of 52.6 my (node 1). The crown age of the *Circaea–Fuchsia* clade (node 2) was constrained with 41.5 my. Nodes 3–8 were discussed in the text. Biogeographic analyses of *Circaea* and its relatives were based on the DIVA (shown above the branches, constrained with maximum two areas C2) and the ML (shown below the branches, constrained with maximum two areas) analyses. Seven endemic areas were defined for both analyses: a. South Pacific islands, b. South America, c. Central America, d. western North America, e. eastern North America, f. western Eurasia, and g. eastern Eurasia. The optimal ancestral areas at each node presented under LAGRANGE are the ones with the highest likelihood scores and the highest probabilities among the alternatives. The probabilities of other solutions with likelihood scores nearly equal to the highest are much lower, thus are not presented. A slash in the result under LAGRANGE indicates the split of areas in two daughter lineages, i.e., left/right, where "left" and "right" are the ranges inherited by each descendant branch ("left" is the upper branch, and "right" the lower branch).

slightly more resolved than the nrITS topology (Figs. 1 and 2). The ILD test of the nrITS and the plastid data gave $p = 0.01$, suggesting heterogeneity of the two data sets. Conflicts among the topologies were detected in the following instances: *C. canadensis* subsp. *quadrisulcata* (two accessions), *C. erubescens* (*Wen 9864*), *C. alpina* subsp. *imaicola* (*MacArthur-Tibet 1016*) and *C. alpina* subsp. *micrantha* (*MacArthur-Tibet 346*) (Figs. 1 and 2). With the nrITS data set (Fig. 2), two accessions of *C. canadensis* subsp. *quadrisulcata* were nested within the *C. erubescens* clade with a BS of 75% and a PP of 95%; *C. erubescens* (*Wen 9864*) was also in the *C. erubescens* clade and clustered with the other three accessions of *C. erubescens* (BS = 71%, PP = 100%); *C. alpina* subsp. *imaicola* (*MacArthur-Tibet 1016*) was nested within a weakly supported clade (BS = 52%, PP = 95%) containing six accessions of *C. alpina* subsp. *imaicola*, one accession of *C. alpina* subsp. *caulescens* and one accession of *C. alpina* subsp. *micrantha* (*MacArthur-Tibet 346*). With the plastid data set, the two accessions of *C. canadensis* subsp. *quadrisulcata* were nested within the *C. mollis* clade with BS of 81% and PP of 100%; *C. erubescens* (*Wen 9864*) was grouped with the *C. mollis*–*C. canadensis* subsp. *quadrisulcata* clade (BS = 76%, PP = 100%); *C. alpina* subsp. *imaicola* (*MacArthur-Tibet 1016*) was clustered with a clade containing the *C. repens* clade and two accessions of *C. alpina* subsp. *micrantha* (BS = 98%, PP = 100%) including *MacArthur-Tibet 346*. The data sets were also significantly different based on the ILD test ($p = 0.01$) when the five conflicting accessions were excluded from the analysis. Therefore, our rationale for combining the nuclear and plastid data was based on this apparent non-related heterogeneity of the data with phylogenetic histories, given that the congruent sections of the tree still showed a significant conflict in the ILD test. These results corroborated the irrelevance of the significant ILD values in terms of combinability, as proposed by previous authors (e.g., Barker and Lutzoni, 2002; Berry et al., 2004; Inda et al., 2008). The combined tree (Fig. 3) was better resolved than the separate plastid and nrITS trees and the support values were substantially elevated. The topology of strict consensus tree from the combined data set was similar to that of the plastid strict consensus tree, with the main difference being that the bilocular group formed a weakly supported clade (BS = 53%, PP = 92%).

All data sets (plastid, nrITS, and plastid/nrITS) strongly supported the monophyly of *Circaea* (plastid: BS = 100%, PP = 100%; nrITS: BS = 100%, PP = 98%; plastid/nrITS: BS = 100%, PP = 100%) and a first-diverged *C. alpina* subsp. *pacifica* clade within the genus (plastid: BS = 100%, PP = 100%; nrITS: BS = 76%, PP = 96%; plastid/nrITS: BS = 100%, PP = 100%). In the combined analyses, the resolved clades (Fig. 3) roughly corresponded to the species and infra-specific taxa of *Circaea* delimited by Boufford (1982, 2005). For example, accessions of each of the following taxa: *C. mollis, C. lutetiana, C. canadensis* subsp. *canadensis, C. alpina* subsp. *alpina, C. repens*, and *C. alpina* subsp. *pacifica* grouped together, supporting the monophyly of these taxa. Accessions of *C. glabrescens* and *C. cordata* (Boufford, 1982) formed a weakly supported clade (BS = 63%, PP = 97%, Fig. 3). Two intercontinental disjunct subspecies of *C. canadensis* were separated, not showing a sister-group relationship, with *C. canadensis* subsp. *quadrisulcata* grouped with the eastern Asian species. Complex relationships were detected within the two unilocular species, *C. repens* and *C. alpina*, suggesting an unusual evolutionary history of these two species. The *Fuchsia* clade was largely unresolved in the combined analyses, congruent with the results of Berry et al. (2004).

3.2. Molecular dating

The topology of the MCC chronogram (Fig. 4) was nearly identical to the strict consensus tree of the combined data set (Fig. 3). The split of *C. alpina* subsp. *pacifica* from other species of *Circaea* was estimated at 16.17 mya (95% high posterior density (HPD) interval of

7.69–24.53 mya). The stem age of the bilocular group was dated at 8.02 mya (95% HPD: 3.83–12.27 mya). The Eurasian populations and the North American populations of *C. alpina* subsp. *alpina* diverged at 2.01 mya (95% HPD: 0.66–3.53 mya). The crown age of *Fuchsia* (node 3) was estimated to be 21.64 mya (95% HPD: 10.92–31.78 mya).

3.3. Biogeographic history

When left unconstrained (C0), DIVA often yields fairly uninterpretable results, with multiple ancestral areas for several nodes (Davis et al., 2002; Donoghue et al., 2001). This was also the case here, with widespread ancestral areas being inferred at nearly all basal nodes. Results with C2 (maximum two areas) were congruent with those with C1 (maximum four areas) and were shown in Fig. 4. Nodes of different results with C1 were: ancestor of all the accessions of *C. alpina* subsp. *alpina* (node 8, western North America–western Eurasia, eastern North America–western Eurasia, or western North America–eastern North America–western Eurasia; df, ef or def), ancestor of terminals *C. alpina* subsp. *imaicola Wen 5754*–*C. alpina* subsp. *imaicola Wen 9845* (western North America–eastern Eurasia, eastern North America–eastern Eurasia, western Eurasia–eastern Eurasia, western North America–eastern North America–eastern Eurasia, western North America–western Eurasia–eastern Eurasia, eastern North America–western Eurasia–eastern Eurasia, or western North America–eastern North America–western Eurasia–eastern Eurasia; dg, e.g., fg, deg, dfg, efg, or defg), ancestor of terminals *C. mollis Wen9358*–*F. excorticata* (node 2, South Pacific islands–South America–western North America, South Pacific islands–South America–eastern Eurasia, South Pacific islands–western North America–eastern Eurasia, South America–western North America–eastern Eurasia, or South Pacific islands–South America–western North America–eastern Eurasia; abd, abg, adg, bdg or abdg), and the ancestor of terminals *C. mollis Wen9358*–*H. heydeana* (node 1, South Pacific islands–South America–Central America–western North America, South Pacific islands–South America–Central America–eastern Eurasia, South Pacific islands–Central America–western North America–eastern Eurasia, or South America–Central America–western North America–eastern Eurasia; abcd, abcg, acdg, or bcdg). The DIVA analyses did not unambiguously estimate the ancestral area(s) of *Circaea* (Fig. 4).

For the ML analyses by LAGRANGE, the areas with the highest likelihood at each node were consistent among the two different constraints (M1, M2). The areas with highest likelihood were more numerous at basal nodes when maximum areas were constrained as four (M1) and contained the ancestral areas from maximum area constrained as two (M2), e.g., the most-likely areas of node 1: a + b + c + d/c (M1) and c/c (M2); node 2: d/a + b + c (M1) and d/c(M2); node 4: d + e + f + g/d (M1) and g/d (M2). M1 gave higher likelihood values than M2, suggesting a better fit to the data and thus a reason to prefer it. Inferences from M2 were also realistic biogeographically and agree with the results from DIVA (marked on Fig. 4), so M2 is also discussed in this paper. The ancestral area of *Circaea* was estimated to be in the New World, more specifically in western North America. The most likely ancestral areas of *Circaea* and *Fuchsia* (node 2, Fig. 4) were western North America (for the *Circaea* lineage) together with the south Pacific Islands + South America + Central America (for the *Fuchsia* lineage) (d/a + b + c) in M1, or western North America (for the *Circaea* lineage) together with Central America (for the *Fuchsia* lineage) (d/c) in M2.

4. Discussion

4.1. Phylogeny of Circaea

Our phylogenetic analyses (Figs. 1–3) strongly support the monophyly of *Circaea*. The morphological synapomorphies of the

genus include its 2-merous flowers with two stamens opposite the sepals, the indehiscent capsular fruits with hooked hairs, and pollen grains with reduced or no viscin threads (Boufford, 1982; Boufford et al., 1990; Skvarla et al., 1978, 2005). Thus, *Circaea* is clearly a well delimited genus in Onagraceae based on both morphological and molecular characters.

Our analyses further show that the bilocular group forms a weakly supported clade (BS = 53%, PP = 92%, in Fig. 3) nested within the unilocular group, and that the unilocular *C. alpina* subsp. *pacifica*, was the first extant taxon to diverge in the genus. In contrast, the cladistic analysis based on 22 morphological, anatomical and palynological characters suggested that the unilocular group was monophyletic and nested within the bilocular group, and that two bilocular species, *C. cordata* and *C. glabrescens*, diverged first in the genus (Boufford et al., 1990). Because *Circaea* was believed to be a highly isolated group in Onagraceae and its position in the family was uncertain at that time, a putative outgroup was used to root the trees (Boufford et al., 1990), with the polarity of the morphological characters determined based on the hypotheses proposed by Boufford (1982). The evolutionary polarity of 15 of the total 22 morphological characters used in their study was assigned and the character states in the outgroup were designated as "0". The polarity of the other seven characters was ambiguous, with their character states in the outgroup coded as "?". The differences between the phylogenies inferred from our molecular data here and Boufford et al. (1990)'s morphological data may be largely due to the lack of a "real" outgroup in the morphological cladistic analyses. The species relationships are similar in the molecular and in the morphological analyses, but the two analyses apparently differ mainly in the rooting position in the phylogeny.

Our analyses indicate that the two geographically widespread species complexes are each not monophyletic, namely, *C. canadensis* and *C. alpina*. The eastern Asian–eastern North American disjunct *C. canadensis* complex is characterized by its bilocular ovary, exserted nectary, prominently ribbed fruits, and nearly glabrous stems (Boufford, 1982, 2005). The major difference between the two subspecies is the presence/absence of minute bracteole at the base of the pedicel (Boufford, 1982, 2005). *Circaea canadensis* subsp. *canadensis* bears this bracteole, whereas *C. canadensis* subsp. *quadrisulcata* often lacks this bracteole. However, *C. canadensis* subsp. *quadrisulcata* shows a closer relationship with its eastern Asian allies than with the eastern North American counterpart in this study (Fig. 3). In spite of the morphological similarities of these two subspecies, it seems appropriate to treat *C. canadensis* subsp. *quadrisulcata* as a distinct species based on our analyses. It has been reported that several eastern Asian-eastern North American intercontinental disjunct plant taxa show a high level of morphological similarity, yet they are not sister to each other phylogenetically (Wen, 1999, 2001). This phenomenon has been reported for *Aralia* (Wen, 2000); *Liquidambar* (Hoey and Parks, 1991; Ickert-Bond and Wen, 2006), and *Magnolia* (Qiu et al., 1995a, b). The morphological similarity between two subspecies of *C. canadensis* may be largely due to convergence via morphological stasis, as suggested by Parks and Wendel (1990), Qiu et al. (1995a, b), and Wen (2001).

The *C. alpina* complex is also paraphyletic according to our analyses. *Circaea alpina* is characterized by its unilocular ovary, tuberous rhizomes, glabrous pedicels, and shallowly notched petals (less than half of their length). Subtle morphological differences such as development and shape of the inflorescence, presence of a minute bracteole at the base of pedicels, leaf shape, and habitats were used to delimitate subspecies within the complex (Boufford, 1982). Our combined analyses show that *C. alpina* subsp. *pacifica* is sister to the clade comprising all other taxa in the genus (Fig. 3). This taxon is characterized by its thin, translucent leaves with subentire margins and rounded bases, as well as conspicuously notched petals,

and it is geographically restricted to western North America. It was reported by Boufford (1982) to intergrade with another North American subspecies, *C. alpina* subsp. *alpina*, with intermediates found along the contact zone. Our data suggests that *C. alpina* subsp. *pacifica* is neither sister to *C. alpina* subsp. *alpina* nor to any other subspecies of *C. alpina*, and consequently should be treated as a distinct species. The morphological similarities among *C. alpina* subsp. *pacifica* and other subspecies of *C. alpina* may represent symplesiomorphies or convergences. The intergradation of *C. alpina* subsp. *pacifica* and *C. alpina* subsp. *alpina* as reported by Boufford (1982) may be best interpreted to be secondary, perhaps due to inter-specific hybridization, which is fairly common in the genus (Boufford, 1982). *Circaea alpina* subsp. *alpina* is the most wide-ranging taxon of the genus. It occurs from temperate to sub-arctic areas in the Northern Hemisphere, yet it is remarkably uniform throughout its range both morphologically and ecologically (Boufford, 1982). It differs from other subspecies of *C. alpina* by its inflorescence elongating after the opening of the flowers, glabrous stems, and conspicuously notched petals. Molecular analyses of samples from Alaska, Quebec, and Austria show that *C. alpina* subsp. *alpina* is a well defined taxonomic unit in spite of its disjunct distribution (BS = 83%, PP = 100%, in Fig. 3). Its closest relatives were found to be *C. alpina* subsp. *imaicola* and *C. alpina* subsp. *caulescens* from eastern Asia. All accessions of *C. alpina* subsp. *alpina*, *C. alpina* subsp. *caulescens* and seven of the eight accessions of *Circaea alpina* subsp. *imaicola* formed a core *C. alpina* clade in our combined analysis (BS = 92%, PP = 100%, in Fig. 3). *Circaea alpina* subsp. *caulescens* is distributed in cool temperate deciduous and mixed forests and in the southern part of boreal forests in eastern and central Asia (Table 1). It is distinguished by its pubescent stems, a usually glabrous inflorescence, dark-green leaves that are usually pubescent above and flowers that open after elongation of the raceme axis and are held on pedicels that diverge perpendicular to the axis of the raceme (Boufford, 1982). The distribution of *C. alpina* subsp. *caulescens* overlaps with the Asian part of distribution area of *C. alpina* subsp. *alpina* (Table 1). *Circaea alpina* subsp. *imaicola* is distinguished by the flowers being held on erect or ascending pedicels and opening before elongation of the raceme, pubescent stems, deep green or bluish-green opaque leaves, and the ovate to very broadly ovate leaves with usually rounded or truncate bases. It is distributed in cool, moist places in central and eastern Asia and in the mountains of southern India (Boufford, 1982), and it grows at lower elevations than *C. alpina* subsp. *micrantha* (Skvortsov, 1977), although there is a considerable area of overlap between 3000 and 4000 m (Boufford, 1982). It was claimed that plants of *C. alpina* subsp. *imaicola* often, but not always resemble subsp. *micrantha* to a greater degree as elevation increases (Boufford, 1982). Thus, we suspected that the sample of *C. alpina* subsp. *imaicola* (MacArthur-Tibet Expedition 1016), which was nested within the *C. alpina* subsp. *micrantha*–*C. repens* clade, may represent an individual from the overlap zone of these two subspecies. *Circaea alpina* subsp. *micrantha* is also morphologically similar to *C. alpina* subsp. *alpina*, and it grows at higher elevations than any of the other subspecies of *C. alpina* (Boufford, 1982). In this study, *C. alpina* subsp. *micrantha* was found to be closely related to *C. repens* rather than to the other subspecies of *C. alpina*. The unilocular *C. repens* is similar to the *C. alpina* complex in its floral characters, and it shares a large distribution area with *C. alpina* subsp. *micrantha* in the Himalayas and southwestern China. The phylogenetic pattern of the *C. repens*–*C. alpina* subsp. *micrantha*–*C. alpina* subsp. *imaicola* clade (BS = 100%, PP = 100%, in Fig. 3) may be due to the presence of inter-subspecific and inter-specific gene flow among these sympatric taxa.

The species relationships within the bilocular group are largely unresolved, possibly due to the evolutionary radiation of this group. Nevertheless, *Circaea glabrescens* is closely related to *C. cor-*

data based on our analyses (BS = 63%, PP = 97%, in Fig. 3). This relationship is also supported by morphology with their nectaries wholly included within the floral (Boufford, 1982; Boufford et al., 1990). In contrast, the nectaries of other species in the bilocular group are exserted beyond the floral tube (Table 1). *Circaea glabrescens* differs from *C. cordata* mainly by its glabrous inflorescence and obovoid to pyriform fruits. However, *C. glabrescens* and *C. cordata* are not well separated in our study. *Circaea glabrescens* is restricted to central China, with only one specimen collected in Taiwan, China (Boufford, 1982). The sample of *C. glabrescens* (*Wen 9867*) from Jiangxi Province is a new record in southeastern China and bridges the two previously known distribution areas. This specimen is morphologically consistent with *C. glabrescens*, but it did not group with the sample of this taxon from Gansu Province (Figs. 1–3). The relationship between *C. glabrescens* and *C. cordata* therefore needs to be further studied with a populational sampling scheme.

4.2. Historical biogeography and divergence times

Because the fossil records of *Circaea* did not extend beyond the current distribution areas of the genus, area relationships and range expansions of *Circaea* may be reasonably inferred by analyzing the extant taxa. Analyses of a number of disjunct taxa in the Northern Hemisphere have shown the common pattern of an Old World origin, e.g., in *Symplocarpus*, *Aralia*, *Calycanthus* (see Wen, 1999; Xiang and Soltis, 2001; Donoghue and Smith, 2004). In this study, although the DIVA analyses did not unambiguously infer the ancestral area of *Circaea*, the ML analyses suggest that *Circaea* originated in the New World (see results and Fig. 4). Our results support the hypotheses of the origin of *Circaea* proposed by Boufford (1982) and Raven (1988). Berry et al. (2004) also suggested that the more likely ancestral areas of the *Circaea–Fuchsia* clade were northern South America, Mesoamerica, or even southern North America. They proposed that *Fuchsia* and *Circaea* may have migrated south and north, respectively, and diversified in drastically different environments with their own distinctive morphologies. This scenario is consistent with our results. New World origins have been reported in several disjunct plant taxa in the Northern Hemisphere, e.g., *Fraxinus* (Jeandroz et al., 1997), *Phryma* (Nie et al., 2006), and *Ribes* subgenus *Grossularia* (Schultheis and Donoghue, 2004).

Within *Circaea*, our results suggest that the unilocular species diverged earlier than the bilocular group (Fig. 4). Skvortsov (1979) proposed that the unilocular group may have originated in southeastern Asia at the beginning of the Neogene. Our dating results are consistent to the time of origin of the group (*Circaea alpina* subsp. *pacifica* diverged first in the genus at 16.17 mya (95% HPD: 7.69–24.53 mya) during the Miocene), but the ancestral area was inferred to be in western North America (Fig. 4). The radiation of *Circaea* in eastern Eurasia, especially in southwestern China, is estimated to have occurred within the past 13 million years (node 5: 12.98 mya, 95% HPD: 6.21–19.61 mya), and the bilocular group is estimated to have origininated in eastern Eurasia at around 8.02 mya (node 6, 95% HPD: 3.87–12.27 mya) during the middle Miocene or the early Pliocene. This is consistent with geologic and ecological changes during the late Tertiary in the region (Axelrod et al., 1996). The changes include the recent major uplifts of the Qinghai–Tibetan Plateau between the early Miocene to the Pleistocene (Spicer et al., 2003; Guo et al., 2002), the extensive spreading of dry climates in both North America and Eurasia (Elias, 1942; Axelrod, 1980), and the onset of monsoonal conditions probably driven by the Himalayan uplift in the late Miocene in eastern Asia (Reiter and Ding, 1981; Ruddiman and Kutzbach, 1989; Monastersky, 1989). Significant increases in geologic and ecological diversity that accompanied the uplift of the Qinghai–Tibetan plateau most

likely promoted rapid allopatric speciations in small and isolated populations (Liu et al., 2006).

Our biogeographic analyses suggest three independent migrations between North America and Eurasia during the Tertiary to the Pleistocene, involving all three North American taxa. The first migration is related to the divergence of *C. alpina* subsp. *pacifica* and the common ancestor of the other taxa in the genus. Our LAGRANGE analyses suggested an initial diversification of *C. alpina* subsp. *pacifica* from the ancestor of the remaining *Circaea* taxa in the New World, with subsequent and independent movement into eastern Eurasia (Fig. 4). The eastern Eurasian species are thus proposed to have migrated from western North America. The divergence time of *C. alpina* subsp. *pacifica* and its Eurasian allies is in agreement with a migration path either across the North Atlantic land bridges connecting Europe to North America (>13 mya) (Parks and Wendel, 1990) or more likely across the Bering land bridge, since the time frame was toward the end of the availability of the North Atlantic land bridges via the stepping stones (Tiffney, 1985b).

The second migration involved the origin of the bilocular group. The ancestor of this group is estimated to have diverged initially at 8.02 mya (95% HPD: 3.87–12.27 mya), and the ancestral area was inferred to be eastern Eurasia by both DIVA and LAGRANGE analyses (node 6, Fig. 4). The eastern North American *C. canadensis* subsp. *canadensis* diverged from the bilocular group at ca. 6.37 mya (95% HPD: 2.99–9.68 mya) and was suggested to have migrated from eastern Eurasia (node 7, Fig. 4). This estimate is consistent with a possible migration route across the Bering land bridge, which was available at that time in the late Tertiary, whereas the North Atlantic land bridges (Tiffney, 1985b) were no longer available for plants by then.

The third intercontinental migration was within *C. alpina* subsp. *alpina*. This subspecies occurs widely in both continents in cool temperate and boreal forests between 30° and 65°N, but is restricted to high elevations at lower latitudes (Boufford, 1982). The divergence time between populations from Europe and North America is estimated to be ca. 2.01 mya (95% HPD: 0.66–3.53 mya) (node 8, Fig. 4). The ancestral area of *C. alpina* subsp. *alpina* was inferred to be Eurasia by LAGRANGE analysis when the maximum areas were constrained as two, but was not clearly determined by DIVA and ML analyses (f + d + e) when the maximum areas were constrained as four. The relatively recent divergence time of *C. alpina* subsp. *alpina* implies that this taxon may have dispersed shortly before the latest ice ages across the Northern Hemisphere, arguably from Eurasia to North America via the Bering land bridge. Long-distance dispersal of its epizoochoric fruits may also have played an important role during the course of migration.

5. Conclusions

Our molecular phylogenetic study has provided new insights into the systematics and evolutionary history of *Circaea*. We have demonstrated the monophyly of the genus and unraveled parts of its phylogenetic structure and biogeography. Our results indicate that the bilocular group is monophyletic and furthermore nested within a unilocular grade. Species relationships within the bilocular group, however, are still equivocal. *Circaea* and its sister genus *Fuchsia* were estimated to have diverged in the Paleogene in the New World. After its New World origin, a greater diversification of *Circaea* occurred in eastern Eurasia, with at least two subsequent migrations back into North America via the Bering land bridge. Long-distance dispersal may have played an important role in the formation of intercontinental disjunctions of the genus. A higher species diversity of *Circaea* in eastern Asia was probably caused by geologic and ecological changes during the late Tertiary in the Northern Hemisphere.

Acknowledgments

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