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Phylogeny and biogeographic diversification of *Maianthemum* (Ruscaceae: Polygonatae)

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ABSTRACT

Maianthemum (Ruscaceae) comprises 28–38 species and includes the two traditionally recognized genera: *Maianthemum sensu stricto* and *Smilacina*. Thirty-seven samples representing 22 species of *Maianthemum* and six closely related outgroup taxa were sequenced for eight chloroplast and nuclear markers (*trnL-F*, *rps16*, *rpl16*, *psbA-trnH*, *rbcL*, *ndhF*, *trnK*, and ITS) with a total length of nearly 10,000 base pairs. Phylogenetic analyses supported the monophyly of *Maianthemum* with *Maianthemum sensu stricto* nested within *Smilacina*. Almost all species from the eastern Himalayan region in SW China except for *Maianthemum tatsienense* and *M. stenolobum* form a well supported clade. This clade is characterized morphologically by short filaments and large anthers, relatively large flowers, and pubescent stems and leaves. *Maianthemum tatsienense* and *M. stenolobum* from SW to central China form another clade. The other species from eastern Asia (central to NE China and Japan) and the New World fall into several clades. The intercontinental disjunction between eastern Asia and North America in *Maianthemum sensu stricto* is estimated to be at 1.68 million years ago (mya) with the Bayesian relaxed clock relying on uncorrelated rates. A recent radiation at about 2.04 mya is suggested in the high mountains of SW China, corresponding to the geographical heterogeneity in that region after the uplift of the Himalayas. Long distance dispersal by birds may have facilitated the evolution of their intercontinental disjunction and their biogeographic diversifications in SW China.

1. Introduction

Taxa of *Maianthemum* Wiggers are rhizomatous understory herbs of Polygonatae in Ruscaceae. The genus was formerly placed in Liliaceae (Engler, 1888) and Convallariaceae (Dahlgren et al., 1985). Convallariaceae are polyphyletic, and the family was merged with Ruscaceae based on DNA sequence data and the absence of phytomelan (the carbonaceous, opaque material usually covering the testa) in their seeds (Chase et al., 1995a,b, 1996, 2000; Rudall et al., 1997, 2000; Fay et al., 2000).

Maianthemum has often been split into two separate genera based on their flower morphology: the dimerous *Maianthemum sensu stricto* (four tepals, four stamens, and two carpels) and the trimerous *Smilacina* Desf. (six tepals, six stamens, and three carpels). These two traditionally recognized genera are similar morphologically except for the number of flower parts (LaFrankie, 1986a), but the merge of them into one genus has long been highly controversial (Pursh, 1814; Link, 1821; Greene, 1888; Therman, 1956; Kawano and Suzuki, 1971; LaFrankie, 1986a,b; Shinwari et al., 1994; Shinwari, 2000). LaFrankie (1986a,b) conducted morphological analyses on the New World species, and supported combining the two genera, with *Maianthemum* having the nomenclatural priority over *Smilacina*. In this study, we follow the classification of LaFrankie (1986a,b).

Species delimitation and infrageneric classification within *Maianthemum* have been problematic (Emons, 1945; Galway, 1945; Hara, 1987; LaFrankie, 1986a; Li, 1990). Species are largely recognized based on rhizome morphology, number of leaves, and characters of the inflorescence and flowers (Hara, 1987; Li, 1990). Many of these characters are highly variable within species. For example, in *Maianthemum purpureum* (Wallich) LaFrankie, individual rhizomes are thick and tuberous, as well as subspherical, or horizontal and cylindrical, whereas they are elongated and stoloniferous in some other populations. Hara (1987) recognized four sections of Asian taxa (*Maianthemum sensu stricto* not included). His classification was based on corolla shape, insertion of stamens, sexuality of flowers, and pollen shape. Li (1990) independently

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proposed a classification of the genus (including both *Maianthemum sensu stricto* and *Smilacina*) with two subgenera and five sections based on rhizome shape, leaf number, inflorescence morphology, flower color, and number of floral parts.

Maianthemum comprises 28–38 species widely distributed in the Northern Hemisphere with ca. 23 species in eastern Asia (from Russia and Japan to the Himalaya) and 17 species in Central and North America (Fig. 1), of which two species [*M. trifolium* (L.) Sloboda and *M. dilatatum* (Alph. Wood) A. Nelson & J.F. Macbride] occur in both eastern Asia and North America (Li and Chen, 1983; LaFrankie, 1986c; Li and Huang, 1990; García-Arévalo, 1992; Lee, 1993; López-Ferrari and Espejo, 1993; Noltie, 1993; Liang, 1995; Espejo et al., 1996; Takahashi, 1997; Kim and Lee, 1998; Chen and Kawano, 2000a,b; Li and Li, 2002; Judd, 2003). In addition, the range of the Asian species *M. bifolium* (L.) F.W. Schmidt also extends to northern Europe (Chen and Kawano, 2000b). About one-third of the species (including three species with 2-merous flowers) are widely distributed in the north temperate zone from the arctic to the region near the Rio Grande in southwestern North America in the New World and from Russia and Japan to Sichuan province of Central China in the Old World (Fig. 1). The remainder of the species is restricted to two regions on each continent, with about 50% of the species in the high mountains of SW China (e.g., the Hengduan Mountains) to the eastern Himalaya and the other 50% of the species in the mountains of Central America from central Mexico to western Panama.

The species from Central and North America have highly distinctive morphology (LaFrankie, 1986b). Taxa of *Maianthemum* in the Old World, especially in SW China, are, however, more variable among and within species than those of their New World congeners (LaFrankie, 1986a,b; Li, 1990). The Asian species show a relatively high level of morphological variation among species, and even within a species (such as the variable rhizome shapes of *M. purpureum* as described above). Flower color also varies from whitish to deep purple-red in the Old World species, whereas it is usually white in the New World taxa. A wide range of variation in flower color has also been observed in the Sino-Himalayan region (Hara, 1987; Li, 1990; pers. obs. by Y. Meng).

Previous molecular studies have recognized two clades in Polygonatae: one comprising *Disporopsis*, *Heteropolygonatum* and *Polygonatum*, and the other, *Maianthemum sensu stricto* and *Smilacina* based on the restriction site analysis of the plastid *trnK* region (including the *trnK* gene) and DNA sequences of *trnK* and *rbcL* gene (Tamura et al., 1997; Yamashita and Tamura, 2000; Jang and Pfosser, 2002). These previous studies focused on the relationships at the tribal level and sampled only a few species of *Maianthemum*. Kim and Lee (2007) supported the monophyly of the broadly circumscribed *Maianthemum* based on partial *trnK* sequences, but their study had very limited sampling and the single chloroplast marker provided low resolution of relationships within the genus.

With a broader taxon sampling and increased character sampling from several DNA regions, our paper intends to (1) test if *Maianthemum sensu lato* is monophyletic; (2) reconstruct the phylogeny of the genus; and (3) construct the biogeographic diversification of the genus in the Northern Hemisphere, with an emphasis on their diversification in the eastern Himalayan mountains of SW China.

2. Materials and methods

2.1. Taxon sampling

Thirty-seven accessions representing 22 species of *Maianthemum* and six outgroup taxa were sequenced (Table 1). Our sampling covers the taxonomic diversity of the genus with 17 of the 19–25 recognized species in eastern Asia (including all 13 species from SW China), three of the five species from North America, and two of the 10–12 species of Central America. Three species from *Polygonatum*, two from *Disporopsis* (also in Polygonatae), and one from *Convaralia* were selected as outgroups because of their close relationship with *Maianthemum* (Yamashita and Tamura, 2000).

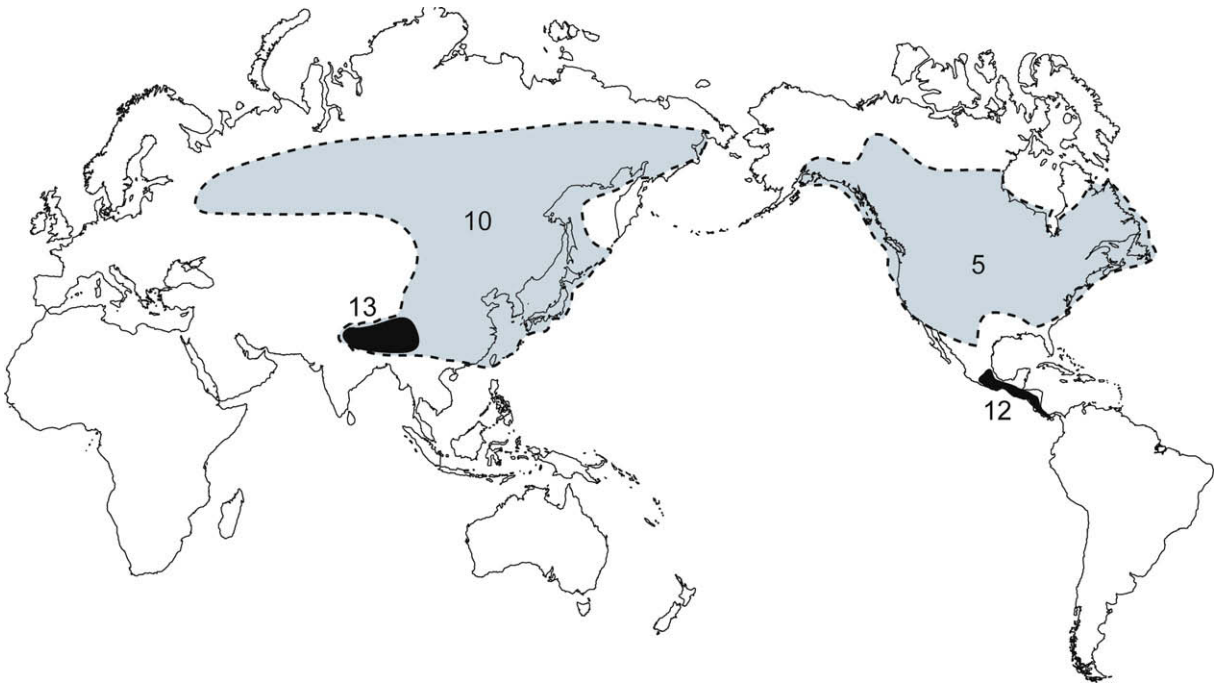

Fig. 1. Distribution of *Maianthemum* in the Northern Hemisphere (dashed line shaded with a light gray) with species numbers shown in each geographic area. The two dark shaded areas show the current centers of species diversity in SW China and Central America.

Table 1
Voucher information and GenBank accession numbers for *Maianthemum* and related taxa used in this study

Taxa	Voucher	Locality	ITS	*ndhF*	*rbcL*	*rpl16*	*rps16*	*trnLF*	*psbA_trnH*	*trnK*
M. atropurpureum (Franch.) LaFrankie	*Nie & Meng 309* (KUN)	China: Yunnan, Gongshan	EU850016	EU850049	EU850082	EU850115	EU850149	EU850186	EU850223	EU850255
	Tibet expedition 767 (KUN, US)	China: Xizang	EU850011	EU850045	EU850080	—	EU850145	EU850181	EU850218	EU850250
	Tibet expedition 781 (KUN, US)	China: Xizang	EU850012	—	—	EU850111	—	EU850182	EU850219	EU850251
M. bifolium (L.) F. W. Schmidt	*Wen 8530* (US)	China: Beijing	EU850027	EU850060	EU850093	EU850124	EU850160	EU850197	EU850234	EU850265
	Meng 211 (KUN)	China: Changbai Mountain	EU850028	EU850061	EU850094	EU850125	EU850161	EU850198	EU850235	EU850266
M. canadense Desf.	*Nie & Meng 525* (US)	USA: Virginia	EU850029	EU850062	—	EU850126	EU850162	EU850199	EU850236	EU850267
M. dahuricum (Turczaninow ex Fischer & C.A. Meyer) LaFrankie	*Meng 224* (KUN)	China: Heilongjian	EU850026	EU850059	EU850092	EU850123	EU850159	EU850196	EU850233	EU850264
M. forrestii (W. W. Smith) LaFrankie	*Meng 245* (KUN)	China: Yunnan, Lushui	EU850015	EU850048	—	EU850114	EU850148	EU850185	EU850222	EU850254
M. fusciduliflorum (Kawano) S. C. Chen & Kawano	*Nie & Meng 395* (KUN)	China: Yunnan, Gongshan	EU850019	EU850052	EU850085	EU850117	EU850152	EU850189	EU850226	EU850258
M. fuscum (Wallich) LaFrankie	*Nie & Meng 312* (KUN)	China: Yunnan, Gongshan	EU850008	EU850043	EU850077	EU850109	EU850142	EU850178	EU850215	EU850247
M. gigas (Woodson) LaFrankie	*Martinez 39002 (US)*	Mexico: Jalisco	EU850034	EU850067	EU850099	EU850131	EU850167	EU850204	EU850241	EU850272
M. gongshanense (S. Yun Liang) H. Li	*Nie & Meng 301* (KUN)	China: Yunnan, Gongshan	EU850007	EU850042	EU850076	EU850108	EU850141	EU850177	EU850214	EU850246
M. henryi (Baker) LaFrankie	*Meng 244* (KUN)	China: Yunnan, Lushui	EU850006	—	EU850075	EU850107	EU850140	EU850176	EU850213	—
	Nie & Meng 311 (KUN)	China: Yunnan, Gongshan	EU850017	EU850050	EU850083	EU850116	EU850150	EU850187	EU850224	EU850256
	Wen 9017 (US)	China: Shaanxi, Taibaishan	EU850010	—	EU850079	—	EU850144	EU850180	EU850217	EU850249
M. japonicum (A. Gray) LaFrankie	*Meng 223* (KUN)	China: Heilongjiang, Yuchun	EU850024	EU850057	EU850090	EU850121	EU850157	EU850194	EU850231	EU850263
	Meng 213 (KUN)	China: Jilin, Changbaishan	EU850025	EU850058	EU850091	EU850122	EU850158	EU850195	EU850232	—
	Murata s.n.	Japan: Tokyo	EU850033	EU850066	EU850098	EU850130	EU850166	EU850203	EU850240	EU850271
M. lichiangense (W. W. Smith) LaFrankie	*Meng 247* (KUN)	China: Yunnan, Zhongdian	EU850013	EU850046	—	EU850112	EU850146	EU850183	EU850220	EU850252
M. nanchuanense H. Li & J. L. Huang	*Nie & Meng 201* (KUN)	China: Chongqing, Jingfushan	EU850023	EU850056	EU850089	EU850120	EU850156	EU850193	EU850230	EU850262
M. oleraceum (Baker) LaFrankie	*Nie & Meng 307* (KUN)	China: Yunnan, Gongshan	EU850009	EU850044	EU850078	EU850110	EU850143	EU850179	EU850216	EU850248
M. paniculatum (Martens & Galeotti) LaFrankie	*Martinez 39110* (US)	Mexico: Jalisco	EU850035	EU850068	EU850100	EU850132	EU850168	EU850205	EU850242	—
	Martinez 39109 (US)	Mexico: Jalisco	EU850036	EU850069	EU850101	EU850133	EU850169	EU850206	EU850243	—
M. purpureum (Wallich) LaFrankie	*Meng 05-1 (KUN)*	China: Sichuan, Mt.Emei	EU850014	EU850047	EU850081	EU850113	EU850147	EU850184	EU850221	EU850253
M. racemosum (L.) Link	*Wen 8562* (US)	USA: Virginia	EU850030	EU850063	EU850095	EU850127	EU850163	EU850200	EU850237	EU850268
	Nie & Meng 524 (US)	USA: Virginia	EU850031	EU850064	EU850096	EU850128	EU850164	EU850201	EU850238	EU850269
M. stellatum (L.) Link	*Nie s.n.* (US)	USA: Chicago	EU850032	EU850065	EU850097	EU850129	EU850165	EU850202	EU850239	EU850270
M. stenolobum (Franchet) S. C. Chen & Kawano	*Nie & Meng 418* (KUN)	China: Yunnan, Lijiang	EU850022	EU850055	EU850088	EU850119	EU850155	EU850192	EU850229	EU850261
M. szechuanicum (F. T. Wang & Tang) H. Li	*Nie & Meng 468* (KUN)	China: Sichuan, Maoxian	EU850020	EU850053	EU850086	—	EU850153	EU850190	EU850227	EU850259
M. tatsienense (Franch.) LaFrankie	*Nie & Meng 313* (KUN)	China: Yunnan, Gongshan	EU850021	EU850054	EU850087	EU850118	EU850154	EU850191	EU850228	EU850260
M. tubiferum (Batalin) LaFrankie	*Nie & Meng 466* (KUN)	China: Sichuan, Maoxian	EU850018	EU850051	EU850084	—	EU850151	EU850188	EU850225	EU850257
Convallaria majalis L.	*Meng 201* (KUN)	China: Heilongjiang, Yuchun	EU850000	—	EU850070	—	EU850134	EU850171	EU850207	—
Disporopsis aspera (Hua) Engl. & Krause	*Li H 22773* (KUN)	China: Yunnan, Lushui	EU850002	EU850038	EU850072	EU850103	EU850136	EU850172	EU850209	—
D. fuscopicta Hance	*Nie & Meng 202* (KUN)	China: Chongqi, Jingfushan	EU850004	EU850040	EU850073	EU850105	EU850138	EU850174	EU850211	EU850245
Polygonatum cyrtonema Hua	*Nie & Meng 203* (KUN)	China: Chongqing, Jingfushan	EU850001	EU850037	EU850071	EU850102	EU850135	EU850170	EU850208	—
P. cirrhifolium (Wall.) Royle	*Tibet expendition 844* (KUN, US)	China: Xizang	EU850005	EU850041	EU850074	EU850106	EU850139	EU850175	EU850212	—
P. sibiricum F. Delaroche	*Nie & Meng 315* (KUN)	China: Yunnan	EU850003	EU850039	—	EU850104	EU850137	EU850173	EU850210	EU850244

2.2. DNA extraction and sequencing

Total DNA was extracted from about 15 mg silica-gel dried leaf material using the modified CTAB method of Doyle and Doyle (1987) or the DNeasy plant mini kits (QIAGEN, Mississauga, Ontario, Canada) following the manufacturer's protocol. Polymerase chain reaction (PCR) amplifications were performed using 10 ng of genomic DNA, 4 pmol of each primer, 0.5 U *Taq* polymerase (Promega), 2.5 mM $MgCl_2$ in a volume of 20 μl under the following conditions: 3 min at 95 °C, followed by 30 cycles of 20 s at 94 °C, 30 s at 50 °C, and 40 s at 72 °C, and then a final 5 min extension at 72 °C. Amplifications were carried out in an Eppendorf Mastercycler (Perkin-Elmer Corp., Foster City, California, USA).

The primers used in the amplification and sequencing were as below. For *trnL-F* regions, primers c, d, e, and f as in Taberlet et al. (1991) were used with the internal primers d and e used only for degraded DNA. For *rbcL*, primers Z1 and 3′ (Zurawski et al., 1981; Olmstead et al., 1993) were used. In taxa for which the primer 3′ failed to produce any readable sequences, another primer Z1204R was used (Zurawski et al., 1981). The following pairs were employed for the markers: *rps16*: primers F and R2 (Oxelman et al., 1997; Andersson and Rova, 1999); *psbA-trnH*: primers *psbA* and *trnH* (Sang et al., 1997; Hamilton, 1999); *rpl16*: primers *rpl16*_F and R (Asmussen, 1999); *ndhF*: primers 15F and 2110R or 2070R (Olmstead and Sweere, 1994; Oxelman et al., 1999; Bremer et al., 2002); *trnK*: primers *trnK*_685F (GTATCGCACTATGTATGATTTGA) and 2R (AACTAGTCGGATGGAGTAG) are modified from Lavin et al. (2000) and an internal primer *trnK*_maiR (GACTTGA AAGATAACCCAGAA) was designed for sequencing; ITS: primers MF (TCGAGACCCGAACGGACRAT) and MR (GTGCTCGGC ATGGGTTCCTT), which were designed in this study based on the ITS sequences of *Maianthemum racemosum* from GenBank (U23982 and U24041). When amplification of the ITS region was unsuccessful, two internal primers ITS2 and ITS3 (White et al., 1990) were used in the following combinations: MF and ITS2, and ITS3 and MR to obtain PCR products in two shorter fragments.

The PCR products were purified using the polyethylene glycol (PEG) precipitation procedure following the manufacturer's protocols. Cycle sequencing was carried out using the following profile: 35 cycles of 97 °C for 15 s, 50 °C for 5 s, and 60 °C for 4 min. The products of cycle-sequencing reactions were cleaned using the Sephadex columns (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and dried at 60 °C in a rotary vacuum evaporator. The sequences were generated on an ABI prism 3100 capillary sequencer (Applied Biosystems, Foster City, California, USA). Sequences were then aligned with ClustalX version 1.83 (PC version, Thompson et al., 1997), followed by manual adjustments in BioEdit (Hall, 1999).

2.3. Phylogenetic analysis

Parsimony analyses used heuristic searches with 10 random taxon addition replicates in PAUP* 4.0b10 (Swofford, 2003). Bootstrap support (BS) for the clades (Felsenstein, 1985) revealed in the maximally parsimonious tree(s) (MPTs) was examined with 500 bootstrap replicates and heuristic search options. Maximum likelihood (ML) was implemented in GARLI ver. 0.951 (Zwickl, 2006; http://www.zo.utexas.edu/faculty/antisense/Garli.html) starting from random trees and using 10,000,000 generations per search. ML bootstrap values were estimated from 100 bootstrap replicates in GARLI.

The TVM + G model of DNA substitutions for the maximum likelihood analysis was determined by the Akaike information criterion (AIC) in Modeltest version 3.6 (Posada and Crandall, 1998; Posada and Buckley, 2004). Bayesian inference was conducted using MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001) with the model as estimated above. The Bayesian Markov chain Monte Carlo (MCMC) algorithm was run for 2,000,000 generations with 4 incrementally heated chains, starting from random trees and sampling one out of every 100 generations. The first 2000–5000 trees were discarded as burn-in, depending on when chains appeared to have become stationary. The trees sampled from within the burn-in stage were excluded, and the remaining trees were assumed to be representative of the posterior probability distribution. The majority rule consensus tree was calculated in PAUP*, and the resulting branch values represent the posterior probabilities (PP). Internodes with PP $\geqslant$ 95% were considered statistically significant.

Sequences of the seven chloroplast genes or noncoding regions (*rps16*, *ndhF*, *rbcL*, *psbA-trnH*, *rpl16*, *trnK*, and *trnL-F*) were directly combined because they are linked and to our knowledge there is no report on recombination of cpDNA in the study group. The incongruence length difference (ILD) test of ITS vs. the combined chloroplast sequences was implemented in PAUP* (Farris et al., 1994) to assess potential conflicts from different DNA fragments. Taxa with entire markers missing were excluded from the matrix in the ILD test.

2.4. Dating the times of divergence

There is no reliable fossil record for *Maianthemum* or even Ruscaceae. We conducted a broad analysis of *rbcL* sequences of *Maianthemum* (i.e., representatives from SW China and New World, and disjunct lineage of *Maianthemum s. str.*) together with samples from the order Asparagales and other monocots. We rooted the tree with *Acorus americanus* (Rafinesque) Rafinesque (Acoraceae). A matrix of 80 *rbcL* sequences with 71 obtained from GenBank (see Appendix) was used for the molecular dating. We tested for clock-like behavior of the data set with a likelihood ratio test (Felsenstein, 1988). The test resulted in $P < 0.05$, suggesting that rate constancy in this data set was not supported.

We used a Bayesian approach to estimate the time to the most recent common ancestor of several lineages in *Maianthemum* and their credibility intervals were implemented in BEAST version 1.4.7, which employs the MCMC to co-estimate topology, substitution rates and node ages (Drummond and Rambaut, 2007). Rate variation among sites was modeled using a gamma distribution with four rate categories in the GTR model. We employed a relaxed molecular clock model relying on uncorrelated rates drawn from a log-normal distribution (Drummond et al., 2006). Posterior distributions of parameters were approximated using two independent MCMC analyses of 20,000,000 steps each, following a discarded burn-in of 2,000,000 steps. Samples from the two chains, which yielded similar results, were combined. Convergence of the chains was checked using the program Tracer 1.3 (Rambaut and Drummond, 2004).

Relatively few reliable fossils have been reported from the monocots primarily due to problems of preservation (Herendeen and Crane, 1995; Gandolfo et al., 2000; Crepet et al., 2004). A few fossils of the Asparagales have been reported from the late Eocene (Couper, 1960; Muller, 1981; Herendeen and Crane, 1995), but all need to be carefully re-examined as they may be too young to calibrate the crown clade of the order (Wikström et al., 2001; Janssen and Bremer, 2004). The age of the crown Asparagales was estimated as 92–101 mya by Wikström et al. (2001), and 119 mya by Janssen and Bremer (2004). Due to the criticisms over the estimates of the former (e.g., Bremer, 2002; Chase, 2004), we set the minimum crown age of the order at 119 mya based on the oldest age of the order. The split between *Acorus* and the remaining monocots was estimated to be more than 134 mya by Bremer (2000) and between 127–141 mya by Wikström et al. (2001) based on chloroplast data and multiple fossil calibrations. This result is in

agreement with Good-Avila et al. (2006) based on *rbcL* and *trnL-F* data and have been adopted by Janssen and Bremer (2004). We, thus, calibrated the crown age of monocots as 134 mya in our analyses. We also used fossil evidence to set the minimum age of Arecaeae at 84 mya (Daghlian, 1981), following Magallón and Sanderson (2001) and Good-Avila et al. (2006).

3. Results

The statistics of chloroplast sequences (*trnL-F*, *rps16*, *rpl16*, *psbA-trnH*, *rbcL*, *trnK*, and *ndhF*) and nuclear ITS are shown in Table 2. The ILD test indicated that our cpDNA and ITS data sets were congruent ($P = 0.18$). We thus performed a combined analysis of all chloroplast and ITS sequences. Because of the low variation of all markers sequenced for *Maianthemum* and the congruence among them, we present the combined results.

The strict consensus tree with BS and PP support is shown in Fig. 2. The topology of the maximum likelihood tree is similar to that of the strict consensus tree (see Fig. 3). The monophyly of *Maianthemum* including both the traditional *Smilacina* and *Maiahtmeum sensu stricto* is reconfirmed in all of our analyses (Figs. 2 and 3). Our results also support the following relationships within *Maianthemum*: (1) a clade including almost all species from the high mountains of SW China to the eastern Himalaya (clade A in Fig. 2; BS = 100, PP = 1.00); (2) *Maianthemum tatsienense* and *M. stenolobum* as clade B (Fig. 2); (3) a clade of *M. japonicum* and *M. nanchuanense* from central to NE China and Japan with BS = 100 and PP = 1.00 (clade C in Fig. 2); (4) the monophyly of *Maianthemum sensu stricto* (clade D in Fig. 2); and (5) *M. stellatum* as the possible sister to the clade of the Central American taxa with BS = 73% and PP = 1.00 (Fig. 2). The position of the Old World *M. dahuricum* and New World *M. racemosum* remained unresolved (Fig. 2).

Based on the *rbcL* gene sequence data sampled from the Asparagales and other monocots, divergence times of *Maianthemum* were estimated to be no earlier than the middle Miocene (8.30 mya with 95% high posterior density [HPD] interval of 3.92–13. 28) using the Bayesian method with a relaxed molecular clock. The disjunction of *Maianthemum sensu stricto* between eastern Asia and North America was estimated at 1.68 mya (95% HPD: 0.03–3.88 mya). The divergence time of the clade from SW China (Clade A in Fig. 2) was estimated as 2.04 mya (95% HPD: 0.40–4.11 mya).

4. Discussion

4.1. Monophyly of Maianthemum

Our results robustly support the monophyly of *Maianthemum sensu lato*, including both *Maianthemum sensu stricto* and *Smilacina* (Figs. 2 and 3). The monophyly of the genus is also supported by the morphological and chromosomal evidence. The characters of terminal paniculate to racemose inflorescences and spotted immature berries are synapomorphic for *Maianthemum* (LaFrankie, 1986a,b; Judd, 2003). They also share a constant haploid chromosome number of 18 (Therman, 1956; Kawano et al., 1967; Sen, 1974; Meng et al., 2005). The unique trimodal karyotype (one long, nine medium-length, and eight small chromosomes) is common in most species in the genus (Kawano and Iltis, 1966; Judd, 2003; Meng et al., 2005), though a few of them are not typical (e.g., a bimodal karyotype in *M. tatsienense* and unimodal in *M. dahuricum* and *M. atropurpurem*; Meng et al., 2005).

Based on chloroplast *trnK* data, Kim and Lee (2007) supported the close relationship of the two traditionally recognized genera with a relatively broad sampling (but few samples from two important regions of SW China and Central America). With more extensive sampling from all its distribution areas and more molecular markers, our analysis suggests that species of *Maianthemum sensu stricto* with dimerous flowers (clade D in Figs. 2 and 3) are nested within the trimerous *Smilacina* (Fig. 2). The phylogenetic results are consistent with the hypothesis that taxa with dimerous flowers were derived from the trimerous group (LaFrankie, 1986a). Anatomical evidence showed that the dimerous members of *Maianthemum* had a vestigial whorl of vascular traces in the mature flowers corresponding to the two lost parts in their flowers (Utech and Kawano, 1976).

The merge of *Maianthemum sensu stricto* and *Smilacina* is strongly supported by our molecular results (Figs. 2 and 3) as well as morphological data (LaFrankie, 1986a,b). Without the inclusion of *Maianthemum sensu stricto*, *Smilacina* is paraphyletic (Judd, 2003). The three species of *Maianthemum* with two-merous flowers are usually shorter than 40 cm tall and have a reduced number (usually two) of leaves on the floral stem. However, *M. trifolium* and *M. lichiangense* (with trimerous flowers belonging to the *Smilacina* group) also have only two to four leaves and rarely reach 30 cm in height. A similar variational pattern is also seen in the morphology of the initiation of the adventitious root and the growth habit (LaFrankie, 1986a; Judd, 2003). These similarities among the three two-merous species and trimerous *M. trifolium* and *M. lichiangense* are probably synapomorphic (Judd, 2003) and thus support a close relationship between the two traditionally recognized genera.

4.2. Phylogeny within the genus

Thirteen species have been reported as restricted to SW China to the eastern Himalayan region (Li, 1990; Chen and Kawano, 2000b). In our analyses, all species from this region have been sampled and are shown to form a strongly supported clade (clade A, BS = 100%, PP = 100, in Figs. 2 and 3), except for *Maianthemum tatsienense* and *M. stenolobum*. The latter two species form another clade (clade B in Figs. 2 and 3). Species of clade A are mainly re-

Table 2
The statistics from analyses of the chloroplast and nuclear data sets for parsimony analysis

	Aligned positions	No. informative sites	No. MPTs	Tree length	CI	CI (excluding uninformative characters)	RI	RC
trnL-F	959	35	1890	65	0.86	0.80	0.92	0.79
rps16	908	27	>100,000	57	0.89	0.82	0.94	0.84
rpl16	1094	40	>100,000	90	0.84	0.75	0.91	0.77
psbA-trnH	645	17	>100,000	32	0.78	0.71	0.88	0.69
rbcL	1445	23	>100,000	51	0.74	0.65	0.82	0.61
trnK	1710	51	>100,000	138	0.74	0.59	0.81	0.60
ndhF	1979	50	>100,000	90	0.86	0.81	0.92	0.80
Combined chloroplast data matrix	8747	243	93	551	0.74	0.64	0.88	0.66
ITS	688	148	42	414	0.67	0.60	0.80	0.54
All combined data matrix	9435	391	2	974	0.72	0.63	0.82	0.60

MPTs, most parsimonious trees; CI, consistency index; RI, retention index; RC, rescaled consistency index.

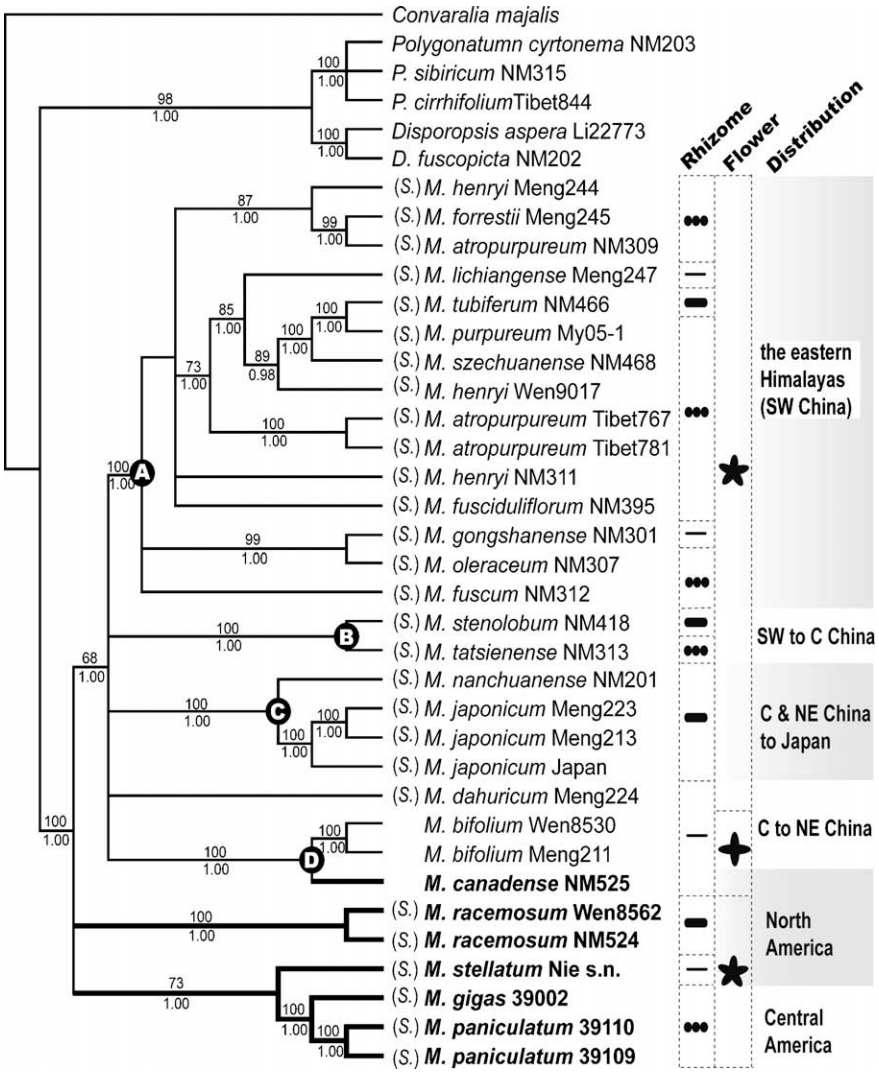

Fig. 2. Strict consensus tree of *Maianthemum* based on the combined nuclear ITS and chloroplast sequences. (tree length = 974 steps, CI = 0.72, and RI = 0.82). The bootstrap values in 1000 replicates are shown above the lines and the Bayesian Markov chain Monte Carlo (MCMC) posterior probabilities higher than 95% are indicated under the lines. Samples belong to the traditional *Smilacina* are shown with (*S.*) before the taxon name and the New World species are indicated with bold lines and letters.

stricted to this region, with the exception of *M. henryi* and *M. purpureum*, which extend to central China (e.g., Hunan, Hubei, and Shaanxi provinces of China). Morphologically, clade A seems to be derived in having large inflorescences and flowers with conspicuous petals. Their petal color ranges from dark purple, light purple, to white. The pubescent leaves and stems are distinctive and may also be a synapomorphy. The two species of *M. tatsienense* and *M. stenolobum* (clade B) are morphologically distinct in having green flowers with narrower petals than those in clade A, and shining leaves and stems, perhaps representing synapomorphies of this clade.

Clade C includes species ranging from central and NE China to Japan (Figs. 2 and 3). Two species were included in our analysis, with three individuals of *M. japonicum* from NE China and Japan, and one *M. nanchuanense* from central China. Species in this clade share the synapomorphic characters of 4–9 ovate to elliptic leaves and white, linear petals. Clade D (Figs. 2 and 3) corresponds to the traditionally recognized *Maianthemum sensu stricto*, characterized by dimerous flowers with linear petal and small herbs with only 2–4 cordiform leaves. It contains only three species disjunct between North America and Eurasia. Two of the three species were sampled from both continents and are sister to each other. The two species have identical sequences in six of the seven chloroplast markers (except for *ndhF*).

Species in the north temperate regions of eastern Asia are not monophyletic, and their relationships are not well resolved (Fig. 2 and 3). They fall into several small groups and occupy a relatively basal position in the trees (clades C–D in Figs. 2 and 3). The phylogeny is consistent with a possible origin in northern part of Asia for the Old World taxa. Karyomorphological data also show that many species from SW China have a more asymmetrical karyotype of 3B or 3C, while those from the north temperate regions usually possess a 2B (such as in *M. dahuricum*) or 2C (such as in *M. japonicum*) karyotype (Meng et al., 2005; Meng, 2006). We hypothesize that ancestral taxa migrated southward to central and SW China with further diversification in the eastern Himalaya including the Hengduan Mountains. Morphological evidence also supports this hypothesis. As discussed above, species from northern parts of eastern Asia (clades C and D) are usually small herbs with a simple racemose inflorescence and small white flowers, whereas those from SW and central China (clades A and B) are morphologically diversified and advanced (small to large herbs, with large inflorescences and colorful flowers).

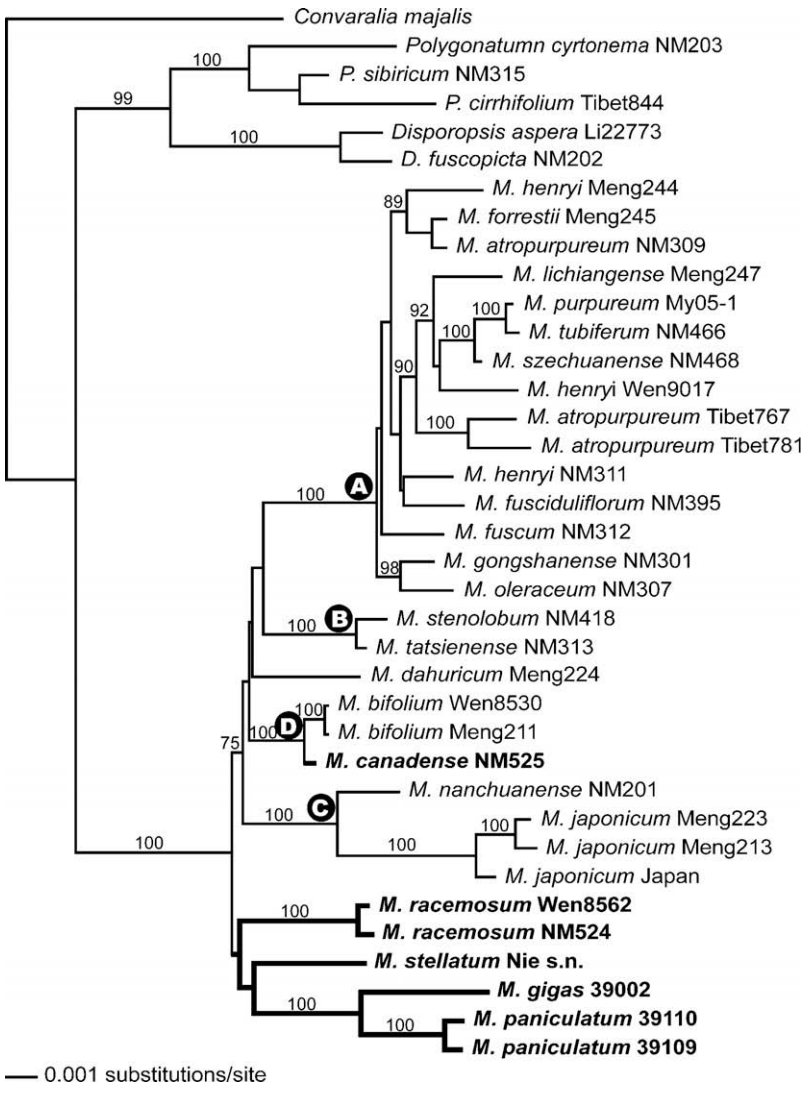

Fig. 3. The Maximum likelihood tree of *Maianthemum* based on the combined chloroplast sequences. The bootstrap values in 100 replicates are shown above the lines. Samples from the New World are indicated with bold lines and letters.

The two species sampled from Central America form a clade sister to the North American *Maianthemum stellatum* with moderate support (BS = 73%, PP = 1.00). Another North American species, *M. racemosum*, is sister to a group including *M. stellatum* and the two Central American samples in the ML tree (Fig. 3), but this clade collapses in the strict consensus tree (Fig. 2). Species from Central America do not have a direct connection with taxa from SW China, although they are morphologically similar (LaFrankie, 1986b). The diversification of the Central American *Maianthemum* is still unclear. Their relationship to the North American species is not well resolved, perhaps due to the present limited sampling in Central America, and/or the possible long isolation of the Central American taxa in the early evolutionary history of the genus.

Although the relationships among deeper clades within *Maianthemum* are not well resolved, phylogenetic results are inconsistent with the morphology-based classifications (Fig. 2). Rhizome shape is considered as the most important character in the evolution of *Maianthemum* because of its ecological importance for perennial plants for nutrient and water storage, and as the source of renewal buds (LaFrankie, 1986a; Hara, 1987; Li, 1990). Li (1990) split the genus into two subgenera based on rhizome morphology. Our results do not support her classification. Species with slender rhizomes are scattered in different lineages in our phylogeny (Fig. 2). For example, *M. lichianense* and *M. gongshanense* from SW China grouped with other taxa from the same geographic region which have thick or knotted rhizomes, rather than the species with similar slender rhizomes, such as *M. dahuricum* or *M. bifolium* from northern China. The traditionally recognized *Smilacina* (not including *Mainthemum sensu stricto*) was divided into four sections by Hara (1987) largely based on flower morphology (e.g., corolla shape). But his classification is not supported by our molecular phylogenetic results either. Species with different corolla shapes group together, such as *M. lijiangensis* (corolla lobed nearly to the base and *M. henryi* (corolla with a distinct tube 3–10 mm long) from clade A (Fig. 2).

4.3. Divergence times within Maianthemum and its intercontinental disjunction

The crown of *Maianthemum* was estimated to be 8.30 mya (Clade M1 in Fig. 4). The divergence time of *Maianthemum sensu stricto* between eastern Asia and North America is recent (Clade M3 in Fig. 4: 1.68 mya with 95% HPD interval of 0.03–3.88 mya). The diversification of the SW China group (clade M2 in Fig. 4, corresponding to Clade A in Figs. 2 and 3) was inferred to be 2.04 mya (95% HPD: 0.40–4.11 mya). The estimate for clade M2 is young

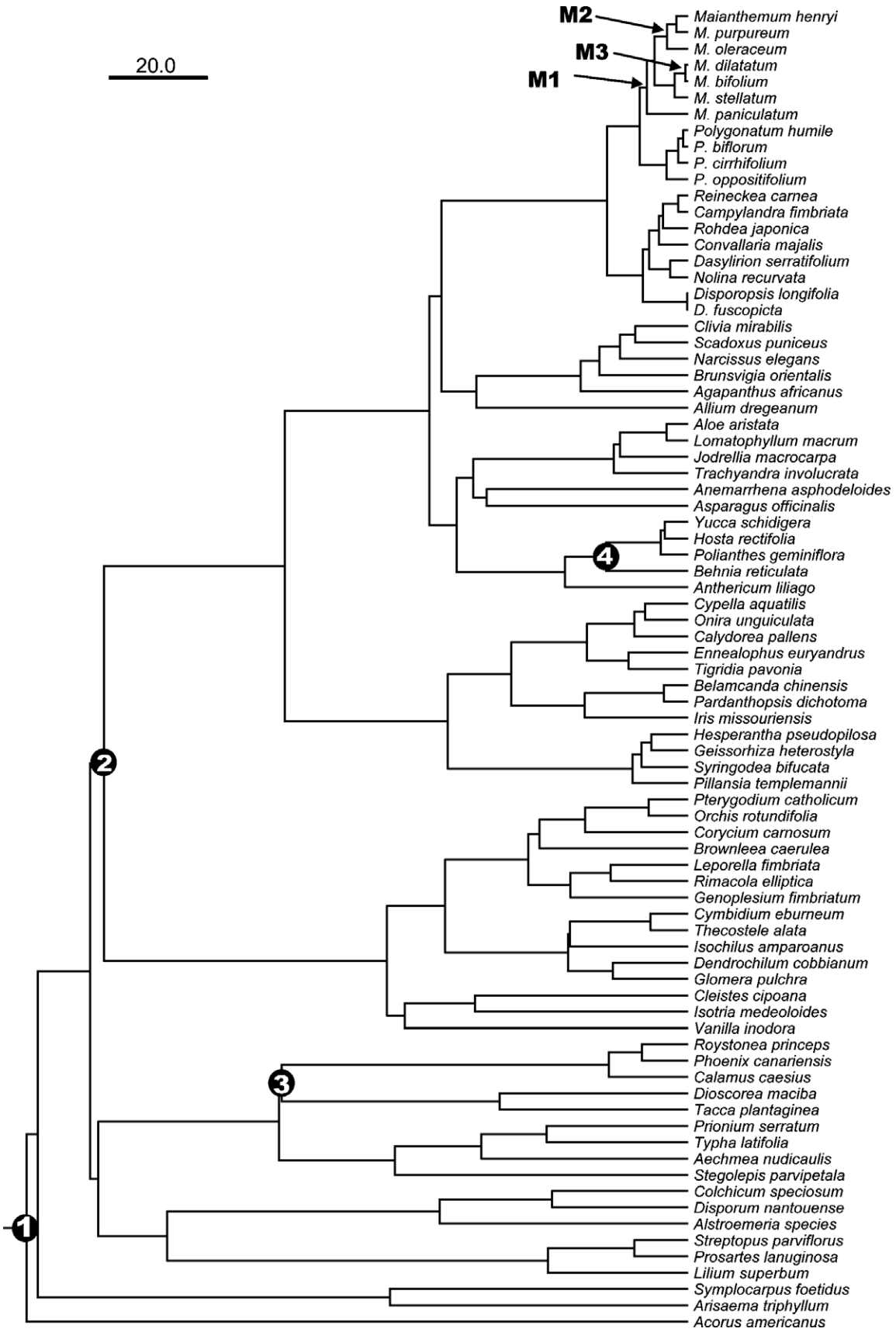

Fig. 4. Chronogram of *Maianthemum* and other related taxa from monocots based on *rbcL* data. Divergence times are shown using the computer program BEAST. The tree was rooted using *Acorus americanus* and calibrated using an estimated age of 134 million years for the age of the root (node 1). The crown group of Asparagales (node 2) and Arecaeae (node 3) was set to be 119 mya and 84 mya, respectively. M1 = *Maianthemum*; M2 = SW China clade; M3 = *Maianthemum sensu stricto*.

when the uplift process of the Himalaya and the Qinghai-Xizang (Tibet) Plateau is taken into consideration.

Multiple fossil-based calibration points and multiple molecular markers are important for the accuracy of divergence time estimates. Our estimates were only based on the *rbcL* genes, but all the other molecular sequences in this study show an overall low variation, which indicates a recent evolutionary history of the *Maianthemum* group. The estimation of the Kimura two-parameter distances within and among selected groups of *Maianthemum* suggested that the genetic distances among some samples were identical, such as in the *Maianthemum sensu stricto* clade for all the chloroplast data except for the *ndhF* gene.

The estimates for the ages of some nodes are usually older than those suggested from the fossil evidence. The oldest Asparagales fossil is 37.5 mya (Couper, 1960; Muller, 1981) and the estimate was 60–69 mya in Good-Avila et al. (2006). In our estimates, however, the Asparagales was treated considerably older and was constrained at 119 mya based on Janssen and Bremer (2004). Similarly, the Liliales has the oldest fossil at about 45.15 mya (Sun and Dilcher, 1988; Herendeen and Crane, 1995), whereas our estimate was 85.70 mya. Bromeliaceae has its oldest fossil at 37.5 mya (Graham, 1987; Herendeen and Crane, 1995), and we estimated its crown age to be 57.19 mya. Node 4 in Asparagales in Fig. 4 has no fossil data and was estimated to be 31.1–32.1 mya in Good-Avila et al. (2006), whereas this node is estimated to be 18.83 mya in this study. If we calibrate this node with the age obtained by Good-Avila et al. (2006), we arrive at similar results for the divergence times for clades in *Maianthemum* (results not shown).

The three species of *Maianthemum sensu stricto* show an intercontinental disjunction between north temperate eastern Asia and North America, with one species in eastern Asia (*M. bifolium*), one in North America (*M. canadense*), and the third (*M. dilatatum*) in areas around the Bering land bridge in both continents (Kawano et al., 1971; LaFrankie, 1986b). The distribution of *M. dilatatum* in northern Pacific coast may directly indicate that the Bering land bridge might have served as the most possible migration route for *Maianthemum sensu stricto* between eastern Asia and North America. Taxa from this group are usually small forest herbs in moist cool temperate areas. This type of ecological conditions is also found near the Bering area.

The divergence time between the Asian–North American taxa in *Maianthemum sensu stricto* was estimated to be 1.68 mya (the Pleistocene). The Beringia was no longer available for direct exchanges of most north temperate plants after about 3.5 mya (Hopkins, 1967; Wen, 1999). It seems that dispersal via long distance possibly across the Beringia is the most likely explanation for the intercontinental disjunction in *Maianthemum*. Recently, more and more biogeographic studies support long distance dispersal as a common pattern for disjunctions in the Northern Hemisphere (Nie et al., 2005a; Milne, 2006). Birds were suggested as dispersing agents for long distance spread (Thompson and Willson, 1979; Howe and Smallwood, 1982). Young fruits of *Maianthemum* are fleshy berries with purple dots and become red at maturity, and the red berries can be easily dispersed via birds (Piper, 1986, 1989; Conran and Tamura, 1998). Additional indirect evidence for bird dispersal is that some species of *Maianthemum* are epiphytes on trees in SW China and Central America because their fruits are consumed by birds and become established on trees.

4.4. Diversification in the eastern Himalayas

The clades in the *Maianthemum* phylogeny strongly correlate with geographical distributions. Almost all species from SW China form a well supported clade (Figs. 2 and 3). Their divergence time is estimated to be in the late Pliocene (2.04 mya), a relatively young age. Geologic uplift of this region began about 50 mya, and the last significant increase in altitudes of the Tibetan plateau was hypothesized at about 10–8 mya ago (Harrison et al., 1992). Although the final major uplift of the Tibet plateau is still controversial (Li and Fang, 1999; Tapponnier et al., 2001), the region likely reached the present elevation no later than about 3.6 mya (Cui et al., 1996; Shi et al., 1998; An et al., 2001). The diversification of *Maianthemum* likely occurred shortly after the uplift and formation of the Himalayas and the Tibet Plateau. It seems that dispersal via birds across the various isolated areas may best explain the diversification of *Maianthemum* in the eastern Himalaya, despite that vicariance has been considered to be the dominant pattern for plant speciation as the response to the Himalayan uplift (He et al., 2001; Peng et al., 2006).

The species relationship within this group is not well resolved (clade A in Figs. 2 and 3). It is strongly correlated to their biogeographic occurrence, but not consistent with morphology (i.e., potential synapomorphies). *Maianthemum henryi* is the only species with long-tubed flowers, but molecular data did not support the monophyly of this species (Figs. 2 and 3). Vicarious species with high level of morphological variation, which are from the same region but different altitudes, are grouped together with the molecular data. For example, *Maianthemum gongshanense*, endemic to Mt. Gongshan above 2500 m, is a small herb with 2–3 leaves, 2–3 flowers, and slender rhizomes. *Maianthemum oleraceum*, from the same area, is a much taller herb 20–80 cm in height with large racemose and thick rhizomes, usually below 2500 m in forests. Molecular data strongly support the close relationship of these two morphologically divergent species (BS = 99%, PP = 1.00 in Fig. 2). The low resolution and homoplasy of molecular sequences appear to be the most possible explanations for the morphological conflicts among tip branches.

However, diversified habitats in this region could also contribute to their morphological divergence. The SW China, including the Hengduan Mountains and eastern Himalayas, is one of the biologically richest temperate regions in the world (Wu, 1988; Sun, 2002). Heterogeneity and complexity of high montane habitats in SW China may explain the high level of morphological divergence and rich biodiversity of its floras (Wu, 1988; Nie et al., 2005b). Similar to the distribution pattern of *Maianthemum* in eastern Asia, the complex of *Spiraea japonica* L. f. is morphologically most diversified in this narrow region of SW China (Zhang et al., 2006). The ITS and biochemical alkaloids data supported two clades corresponding to their distribution in SW and NE China within the complex (Zhang et al., 2006). The authors hypothesized that the uplift of the Himalaya-Tibet plateau and subsequent increase in geographic complexity in SW China has contributed to their morphological diversity. There are numerous taxa (e.g., *Polygonatum*, *Acer*, and *Rodgersia*) with such interesting distribution pattern in eastern Asia (Sun, 2002). More studies are needed, however, especially at the population level, to better understand the evolution of species diversification in the floras of SW China.

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Appendix A. Appendix

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.07.017.

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