American Journal of Botany 93(5): 762–769. 2006.

ALKALOID POLYMORPHISM AND ITS SEQUENCE VARIATION IN THE *SPIRAEA JAPONICA* COMPLEX (ROSACEAE) IN CHINA: TRACES OF THE BIOLOGICAL EFFECTS OF THE HIMALAYA-TIBET PLATEAU UPLIFT[1]

Zhaoyang Zhang, Liming Fan, Junbo Yang, Xiaojiang Hao, and Zhijian Gu[2]

Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204, China

Spiraea japonica, a diverse, perennial shrubby species complex widespread across E Asia, was a useful model in a first attempt to link specific phylogeny, floristic evolution, and historical environmental changes in E Asia. DNA-based phylogenetic reconstruction served as a framework to investigate whether the patterns of variation in alkaloids and nrITS from varieties of *S. japonica* reflect the geological history of China and the uplifting of the Himalaya-Tibet plateau, the most significant geological event in E Asia since the late Paleocene. The high geographical structure found in the phylogenetic trees was corroborated by the distribution of alkaloids. All populations from E China, with mainly hetisine-type alkaloids, formed a single clade, sister to a clade of all populations from SW China, with mainly atisine-type alkaloids. The distribution boundary between the two clades roughly matched the floristic division between the Sino-Japanese Forest and the Sino-Himalayan Forest subkingdoms, as well as the environmental division between SW and E China, suggesting a close link between specific phylogeny, floristic evolution, and geographical changes in E Asia. The divergence between lineages at variety and population level within the eastern clade was slightly older than those within the southwestern clade, supporting the hypothesis of a northeast–southwest migration of *Spiraea* since the Eocene. The uplift of the Himalaya-Tibet plateau and subsequent increase in geographical complexity in SW China, could facilitate divergence maintenance, thus accelerating the evolutionary rate.

Key words: biological effects; East Asia; environmental heterogeneity; Himalaya-Tibet Plateau uplift; Rosaceae; *Spiraea japonica*.

Spiraea japonica L. f. is a perennial shrubby species complex widespread across East Asia that has received considerable attention in China for its potential medicinal uses. Morphologically, the complex is very diverse with nine intraspecific varieties, discriminated by differences in leaf size and shape (Rehder, 1913; Yu, 1974; Wu and Raven, 2003; Table 1, Fig. 3b). With respect to variation and distribution patterns, the complex seems to reflect some floristic characteristics of the plant flora in the East Asiatic kingdom (Wu and Wu, 1998). The complex is morphologically most diversified in southwest China, with the majority of varieties occurring in this extremely narrow region. By contrast, only three varieties occupy central and eastern China, Japan, and south Korea, a combined area that is much larger than southwest China (Fig. 1c). From the perspective of dispersal and variation, the *S. japonica* complex represents a useful model to investigate the correlation between biodiversity patterns and environmental heterogeneity on a large, regional scale.

One of the most significant geographical features of East Asia is the sharp increase in altitude from east to west due to the uplift of the Himalayas and the Tibetan Plateau. In the east are low plains with elevations ranging from 0–500 m a.s.l., while to the west, the Himalayas and Tibetan Plateau attain elevations of 4000–5000 m a.s.l. In between these regions is a narrow transitional belt comprising a mosaic of plateau, mountains, basins, and gorges with wide ranging elevations from 500-4000 m a.s.l. (Committee of Chinese Academy of Sciences for Physical Geography of China, 1985; Fig. 1a). The physical environments in the western mountainous plateau and the eastern plains are relatively homogenous, compared with the complex mix of habitats in the transitional belt—these habitats are especially diverse in southwest China. This complexity of habitat types is mirrored by climatic complexity: the eastern plains are affected by the southeastern Pacific monsoon, the western mountainous plateau by the southwestern monsoon from the Indian Ocean, while the transitional belt (especially in southwest China) encounters both monsoons (Committee of Chinese Academy of Sciences for Physical Geography of China, 1985; Wang, 1994; Fig. 1a).

Botanical diversity is greatest in the eastern plains and the transitional belt, with fewer plant species in the high western regions. Moreover, bearing almost half of the total plant species in China, the transitional belt, especially the southwest China is an important center for biodiversity, with more than a third of its species being endemic (Wang, 1961; Wu, 1980; Wu and Wu, 1998). Floristic studies (Wu, 1979; Wu and Wu, 1998) suggested that the forest area in the East Asiatic Kingdom could be divided into two subkingdoms, namely the Sino-Japanese Forest subkingdom and the Sino-Himalayan Forest subkingdom (Fig. 1b). The formation of these spatiotemporal patterns of biodiversity has long been suspected of correlating with physical environmental changes resulting from the Himalaya-Tibet Plateau uplifting (Chang, 1983; Tanai, 1992; Axelrod et al., 1998). However, the nature and extent of this correlation remains a question. Intensive

---

[1] Manuscript received 19 August 2005; revision accepted 13 February 2006.

The authors thank M. Hollingsworth and two anonymous reviewers for critical comments that greatly improved the manuscript, X. Gong for kindly providing resources to keep the cultivated plants, Y. Y. Chen and L. M. Gao for experimental help, and D. Melick for improving the English. This study was financially supported by the National Natural Science Foundation of China Project 30270120, the Xibuzhiguang Project of CAS, and the Provincial Natural Science Foundation of Yunnan Project 2004C0021G.

[2] Author for correspondence (e-mail: zhangzy@mail.kib.ac.cn)

TABLE 1. List of plant material resources, vouchers, ploidy levels, types of molecular skeletons of alkaloids produced, GenBank accession numbers and floristic areas in which varieties or populations are distributed. Floristic divisions follow Wu and Wu (1998); missing information is indicated by a question mark (?). Detailed information for ploidy level is given in Zhang et al. (2002).

Taxon	Locality	Voucher	Ploidy level	Type of alkaloids produced	GenBank accession no.	Floristic division
Spiraea japonica var. *glabra* Koidz.	Linhai,Zhejiang	Zzy008	2x	Hetisine	AY742230	Sino-Japanese Forest Subkingdom
	Hangzhou, Zhejiang	Zzy051	2x	Hetisine	AY742229	
Spiraea japonica var. *fortunei* Rehd.	Nanchuan, Sichuan	Zzy005	4x	Hetisine	AY742233	
	Longshan, Hunan	Zzy013	4x	Hetisine	AY742232	
	Guiyang, Guizhou	Zzy101		Hetisine	AY742234	
Spiraea japonica var. *japonica* Schneid.	Kyushu, Japan	Zzy040	2x	?	AY742231	
Spiraea japonica var. *acuminata* Franch	Chengkou, Sichuan	Zzy501	2x	Atisine	AY742240	Sino-Himalayan Forest Subkingdom
	Kunming, Yunnan	Zzy001		Atisine	AY742239	
Spiraea japonica var. *stellaris* Rehd.	Yangbi,Yunnan	Zzy113	2x	Atisine	AY742238	
	Chuxiong, Yunnan	Zzy018		Atisine	AY742237	
Spiraea japonica var. *ovalifolia* Franch.	Wuding, Yunnan	Zzy016	2x	Atisine	AY742236	
	Kunming, Yunan	Zzy030		Atisine	AY742235	
Spiraea japonica var. *acuta* Yu	Dali, Yunnan	Zzy105	2x	Atisine	AY742242	
Spiraea japonica var. *incisa* Yu	Weixi, Yunnan	Zzy022	2x	Atisine	AY742241	
Spiraea japonica var. *pinnatifida* Yu	Motuo, Xizang		?	?		
Spiraea cantonensis					AF318722	
Sorbaria sorbifolia					AF318758	
Rubus glabratus					AY083369	
Rosa sempervirens					AB048595	

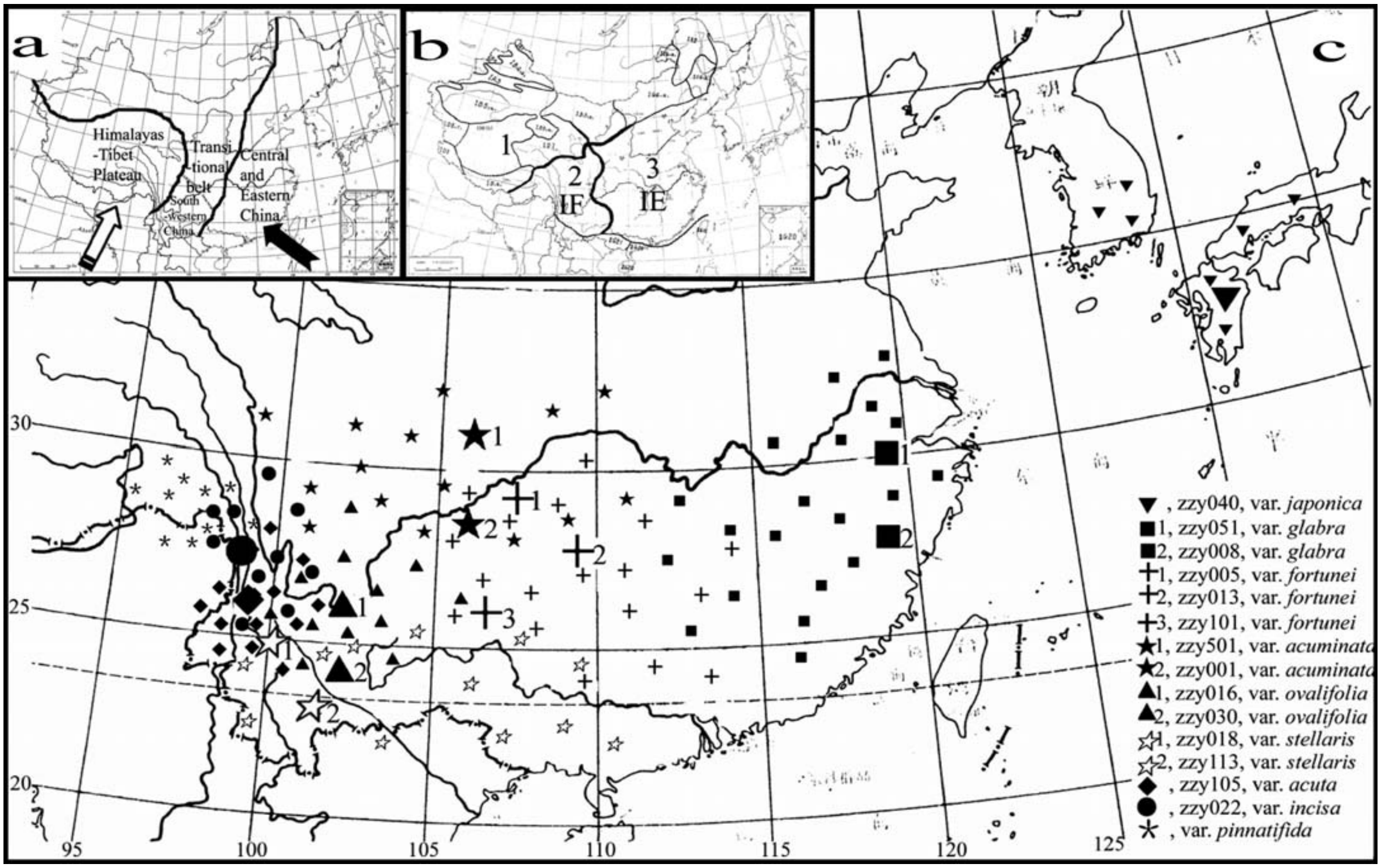

Fig. 1. Dispersal of the *Spiraea japonica* complex and the environmental background in East Asia. (a) Demarcations of general physical areas. Arrows represent directions of the monsoons. (b) Floristic divisions of East Asia and the three environmental gradients. Symbols: 1, Himalaya-Tibet Plateau area; 2, Southwest China in the transitional belt; 3, Plains in east China; IF, Sino-Himalayan Forest Subkingdom; IE, Sino-Japanese Forest Subkingdom [see Wu (1979) and Wu and Wu (1998) for detailed information of the floristic division of East Asia]. (c) Distribution of varieties in the *Spiraea japonica* complex across East Asia. Enlarged symbols are sampling sites for the corresponding varieties.

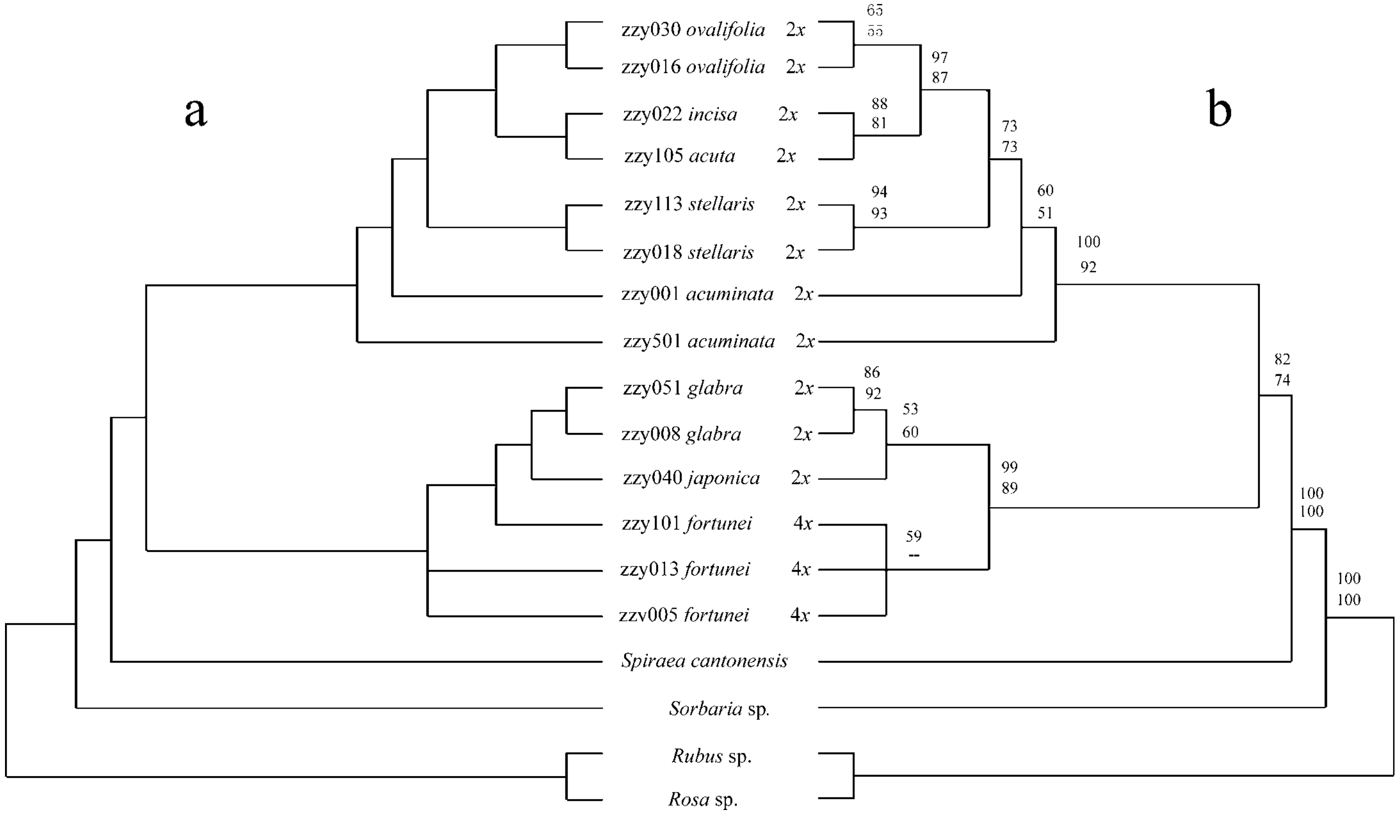

Fig. 2. Topology of the phylogenetic trees based on ITS sequences. (a) Maximum likelihood tree topology. –lnL = 2562.66024; each accession number is followed by a corresponding variety epithet and a ploidy level indicator. (b) The 50% majority rule consensus tree of the seven MP trees. Tree length = 358 steps; CI = 0.897; RI = 0.908; RC = 0.814; numbers above branch are parsimony bootstrap support (upper value) and maximum likelihood bootstrap support (lower value), '–' indicates support value of <50; the clade containing populations of the *S. japonica* var. *fortunei* will collapse under the strict consensus rule.

biological observations of widespread species at population levels may provide insights into the biogeography of this region. In this paper, we use DNA-based phylogenetic reconstruction as a framework to look at alkaloid variation in the *S. japonica* complex and to look for a correlation between the variation in molecular data (alkaloids, nrITS) from taxonomic varieties of *S. japonica* and the geological history of China and the Himalaya-Tibet plateau.

MATERIALS AND METHODS

Taxa and sampling—New sequences for *Spiraea japonica* were produced in present study, while all other sequences were from the databank in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The material resources are listed in Table 1 and distribution of the samples in Fig. 1c. All vouchers were deposited in the Herbarium of Kunming Institute of Botany, CAS [KUN]. A total of 14 populations representing eight varieties within the *S. japonica* complex and *S. cantonensis* Lour. were analyzed for ITS sequence variation. A total of 13 populations representing seven varieties within the *S. japonica* complex were analyzed for alkaloid polymorphisms. The sampling strategy was to allow both detection of variation at the intraspecific level and an estimation of variation at the interspecific level.

Producing a chronology of divergence events is essential to make any biogeographical inference in relation to historical events. However, phylogenetic calibrations of the rate of evolution within species have not been possible because of the difficulty in assigning fossils to specific lineages at the species level. As a result, most estimates of molecular evolution rates have been at the level of genera or higher (Drummond et al., 2003). To provide calibration points in a divergence time analysis (explained later), we reconstructed a more inclusive phylogeny. A species of the closely related genera *Sorbaria* [*S. sorbifolia* (L.) A. Br.] was included in the phylogenetic reconstruction, and one species of each of the two genera *Rosa* L. and *Rubus* L. were used as distant outgroups. The inclusion of *Sorbaria sorbifolia* was based on reviews (Yu, 1984; Lu, 1996) of the subfamily Spiraeoideae, and the outgroup choice was based on phylogenetic placements in a *rbcL* analysis (Morgan et al., 1994), as well as allowing inclusion of fossil information of *Spiraea* (Heer, 1869; Knowlton, 1894; Hollick, 1936; Committee of Chinese Academy of Sciences for Plant of Cenozoic China, 1978) and *Sorbaria* (Tao and Xiong, 1986; Tao, 2000) for data calibration in the divergence time analysis (described later).

Alkaloid extraction—Procedures for alkaloid extraction and isolation from 13 populations representing seven varieties within the *Spiraea japonica* complex followed Fan et al. (2004). Dried powder of whole plants was extracted with ethanol by reflux. The ethanol extracts were suspended in 5% hydrochloric acid and centrifuged. The supernatants were neutralized with aqueous ammonia solution to pH 10, and extracted with chloroform. After repeated column chromatography over silica gel (200–300 mesh, eluted with petroleum-ether–acetone–diethylamine [100 : 5 : 1, 100 : 10 : 2, 85 : 15 : 5, v/v/v]) and petroleum-ether–chloroform–diethylamine (100 : 3 : 1, 90 : 10 : 3, 80 : 20 : 5, 75 : 25 : 5, 60 : 40 : 5, v/v/v) and over Sephadex LH-20 reversed-phase C-18 silica gel, alkaloids were recrystallized from petroleum-ether–acetone. Structures of extracted alkaloids were determined by comparing spectroscopic data (mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance) with those previously reported.

DNA extraction and sequencing—Total DNA was extracted from silica-gel-dried leaf tissue according to a modified CTAB procedure (Doyle and Doyle 1987). Double-stranded DNA was directly amplified by symmetric PCR amplification with the ITS5 and ITS4 primers (White et al., 1990). Reaction volumes were 20 μL and contained 1.5 units AmpliTaq (TaKaRa Bio-

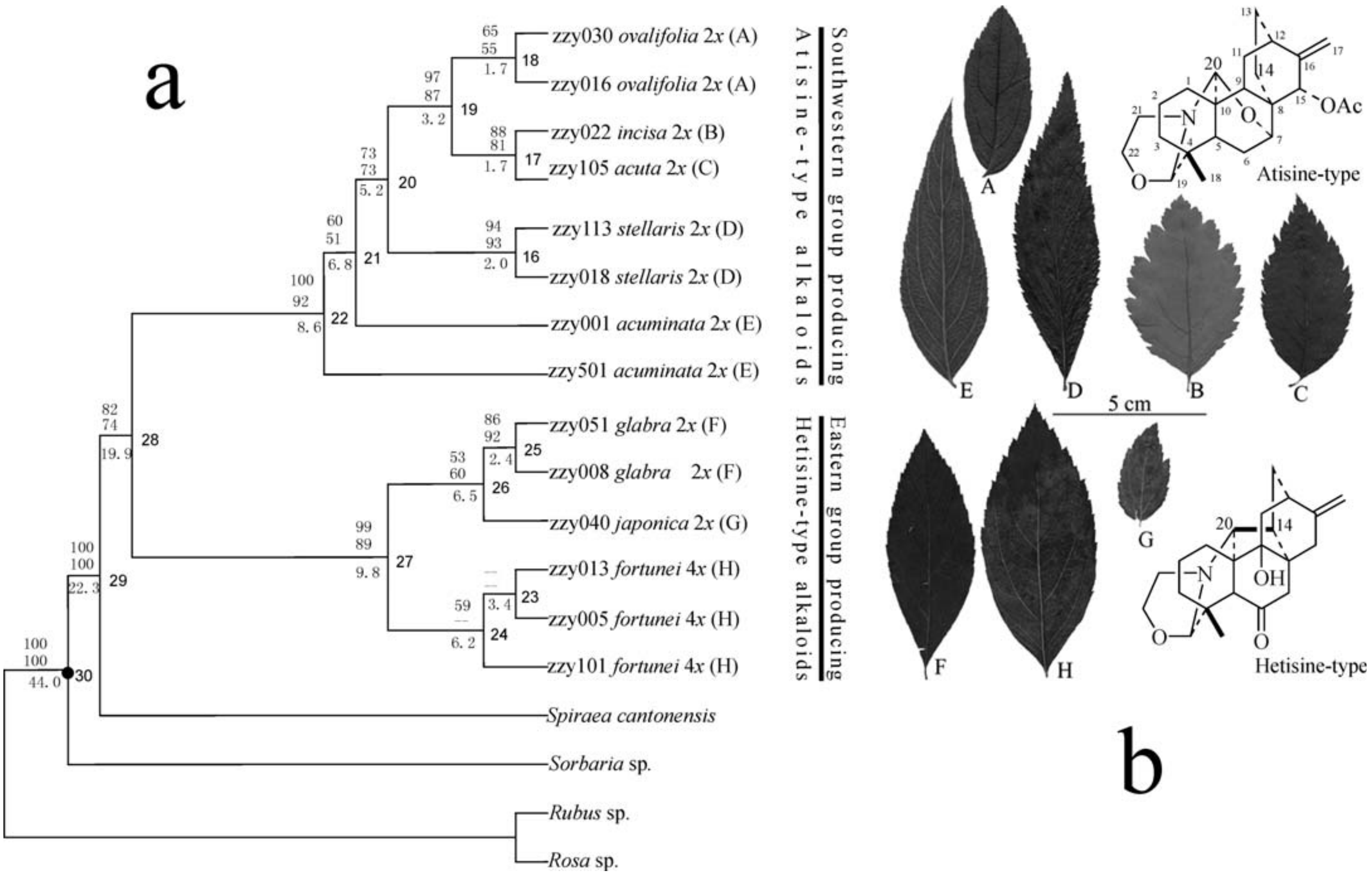

Fig. 3. (a) The chosen maximum parsimony tree topology and divergence time estimation. Tree length = 358 steps; CI = 0.897; RI = 0.908; RC = 0.814; node numbers were placed to the right of the corresponding node; numbers above branch are parsimony bootstrap support (upper value) and maximum likelihood bootstrap support (lower value), '–' indicates support value of <50; numbers below branches and adjacent to nodes indicate estimates of divergence time; each accession number is followed by a corresponding variety epithet, a ploidy level indicator. The letter in parentheses is a variety code that corresponds to the letters below the leaves in panel b. (b) Representative leaves of the *Spiraea japonica* complex and the two types of molecular skeletons of the alkaloids. Letters below leaves correspond to varieties in panel a.

technology, Dalian, China) DNA polymerase, Replitherm buffer (TaKaRa Biotecnology), 1.5 mmol/L $MgCl_2$, 0.4 mmol/L dNTPs, 0.1 μmol/L primer, 5% dimethyl sulfoxide, 25–60 ng sample DNA. PCR was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Applied Biosystems, Foster City, California, USA) and consisted of 30 cycles of 1.5 min at 94°C for template denaturation, 2 min at 55°C for primer annealing, 1 min at 72°C for primer extension, followed by a final extension of 7 min at 72°C. PCR products were purified with a purification kit (Promega, Madison, Wisconsin, USA) prior to being sequenced. Double-stranded purified PCR products were sequenced by the dideoxy chain termination method with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem, Foster City, California, USA) with AmpliTaq DNA polymerase FS (Perkin-Elmer). Reactions and programs were chosen according to the recommendations of the manufacturer's handbook (Perkin-Elmer, Applied Biosystem). Samples were electrophoresed in an ABI310 automated sequencer.

Phylogenetic analysis—The DNA sequences were edited in Seqman (Lasergene, DNASTAR Inc., Madison, Wisconsin, USA). The ITS region limit was determined by referring to the published sequence of *S. cantoniensis*. Sequences were aligned in Megalign (DNASTAR Package) with minor manual adjustments where necessary.

The phylogenetic reconstruction was performed with PAUP* (Swofford, 2002) using maximum-parsimony (MP) and maximum likelihood methods. For MP analysis, characters were weighted equally and gaps treated as missing data; a heuristic search strategy with stepwise data addition (1000 random replications) and tree-bisection-reconnection (TBR) branch swapping was applied to search trees; parsimony trees with equal length were summarized using the 50% majority rule as well as strict rule consensus method; bootstrap values were calculated from 1000 pseudoreplicate analyses with the same heuristic search strategy to assess the relative support for each clade. For ML analyses, the best-fitting model of nucleotide substitution (TrN + G) was selected based on likelihood ratio tests (LRTs) implemented in Modeltest version 3.06 (Posada and Crandall, 1998). ML analyses were performed with PAUP* specifying the optimal model as reflected by the LRTs implemented in Modeltest v3.06. A heuristic search strategy with stepwise data addition (1000 random replications) and TBR branch swapping was used for searching trees, and bootstrap values were calculated from 1000 pseudoreplicate analyses applying a heuristic search strategy with stepwise data addition and TBR branch swapping.

Estimation of divergence time—Bayesian approaches are a valuable method for divergence time estimation without assuming a constant molecular mutation rate, albeit with methodologies still being refined (Kishino et al., 2001; Thorne and Kishino, 2002). Divergence times in the present study were estimated with Bayesian approaches using the software Multidivtime (Thorne and Kishino, 2002) and PAML (Yang, 1997). A chosen MP tree and the sequence data were input into the PAML package to estimate the maximum likelihood of the branch lengths of the tree and the variance–covariance matrix. The prior and posterior distribution of substitution rates and divergence times were approximated by performing a Bayesian Markov Chain Monte Carlo (MCMC) analysis in the Multidivtime package.

Specification of prior distributions for parameters is indispensable to the Bayesian method. The prior mean and standard deviation for gamma-distributed time of the ingroup root node (rttime and rttimesd) was set to 43 million years. The value was an estimate based on the split of *Spiraea* and *Sorbaria*. Fossil

TABLE 2. Sequence characteristics of the ITS region of the *Spiraea japonica* complex.

Sequence character	ITS
Length variation within *S. japonica*	632 bp to 639 bp
Mean base frequency for G+C	64%
Total aligned positions	671
Mean sequence divergence within eastern group	0.2–2.1% (1.1%)
Mean sequence divergence within western group	0.3–2.2% (1.3%)
Sequence divergence within *S. japonica*	0.2–7.1% (3.9%)
Sequence divergence between *S. japonica* and *S. cantonensis*	5.9– 8.8% (7.5%)
Sequence divergence between *Spiraea* and *Sorbaria*	17.5–18.5% (18.0%)
Sequence divergence between outgroup and ingroup	21.4–26.5% (25.1%)

reports of *Spiraea* are much more abundant than that of *Sorbaria*, although fossil records are available for both genera. Over a long period, various authors (Heer, 1869; Knowlton, 1894; Hollick, 1936) had identified oldest fossil *Spiraea* from within different Eocene sediments (53–33.7 myr). In contrast, essentially only one report (Tao and Xiong, 1986; Tao, 2000) indicated the existence of fossil *Sorbaria* in late Cretaceous sediment (96–65 myr). In addition, Tao and Xiong (1986) pointed out that the analogues of the leaf fossils used as evidence of the existence of *Sorbaria* were very few; only two were found. Normally, the reliability of fossil information is dependent upon the number of available fossils and sediments to corroborate its existence and how many authors have identified these fossils. In this case, the minimum age of the split between *Spiraea* and *Sorbaria* was conservatively dated back to the Eocene (53–33.7 myr, with mean value c. 43 myr). Other data-dependent settings were also applied in the multidivtime control file according to recommendations in the manual: (1) Length, sampling frequency, and burn-in period of the Markov chain were set to 100 000 trees sampled every 100th generation, with a burn-in of 100 000 trees. (2) The priori rate at the root node (rtrate) was set to 0.005, calculated by dividing the median distance between the ingroup root and the ingroup tips obtained from estbranches by the time unit. (3) The prior for the Brownian motion parameter nu (brownmean), which determines the permitted rate change between ancestral and descendant nodes, was set to 0.023, following the manual's recommendation that the time units between root and tips to the power of nu be about 1; the standard deviation on nu was also set to 0.023. In addition, as stated previously, it is difficult to get appropriate calibration points in divergence time estimation at intra-specific level, although the Bayesian approach has the advantage of allowing multiple calibrations. We used only one calibration point in this study: the split of *Spiraea* and *Sorbaria*, which was calibrated at 53–33.7 myr.

RESULTS

Alkaloid polymorphism within the Spiraea japonica complex—The alkaloid survey indicated that the alkaloids were good indicators for geographical varieties or populations of the *S. japonica* complex. Two types of fundamental molecular skeletons (Fig. 3b) recognizable for the alkaloids (the atisine and hetisine types) were highly homologous. Although both comprised a skeleton of 20 C atoms C20 was connected to C7 by an *O*-bridge in the atisine type, while C20 was connected to C14 in the hetisine type. Accordingly, the alkaloids could be classified into two groups: alkaloids produced by populations from the southwest China were mainly the atisine type, while those by populations from the east China were mainly of hetisine type (Table 1, Fig. 3b).

Sequence characteristics and phylogenetic reconstruction—Characteristics of the ITS region of the *S. japonica* complex are shown in Table 2. The seven most parsimonious trees were recovered from the parsimony analysis with a length of 358 steps, a CI of 0.897, an RI of 0.908, an RC of 0.814, and a single ML tree (Fig. 2a) was recovered from the likelihood analysis with a –lnL being 2562.66024. The MP trees and the ML tree were similar. Both kinds of tree topology were highly geographically structured and matched the alkaloid distribution pattern (Figs. 2, 3). In the MP tree (Fig. 3a), which was most fully resolved and used in the Bayesian analysis, all populations (varieties) from the east formed a clade with 99% bootstrap support (for convenience, only bootstrap values under parsimony criterion are presented here), while all populations (varieties) from the southwest China formed another clade, also with high bootstrap support of 100%. However, phylogenetic structure within the two clades was ill-defined, with variable levels of bootstrap support. In a clade consisting of the western populations, the monophyletic nature of var. *stellaris* was supported with a bootstrap value of 94%. The close relationship of var. *incisa* and var. *acuta* was also supported, with the two varieties being sister to each other within a subclade with 88% bootstrap support. The monophyletic nature of var. *ovalifolia* was also weakly supported with a bootstrap value of 65%. Accessions of var. *acuminata* were unresolved at the base of this clade. In the clade consisting of the eastern populations, the monophyletic nature of var. *glabra* was supported with a bootstrap value of 86%. A clade containing populations of var. *fortunei*, the only tetraploid variety, had a lower bootstrap value (59%), and was sister to a weakly supported clade bearing the diploid varieties from the east (var. *glabra* and var. *japonica*).

Estimation of divergence time—Posterior distribution of substitution rates and divergence times at each node as well as their standard deviations are summarized in Table 3 and Fig. 3a. Divergence time of the southwest clade from the eastern clade (node 28) was dated to 19.87 (8.26–36.10) myr. Divergence times between varieties within the eastern clade ranged from 6.54 (0.98–17.28) to 9.82 (2.11–22.22) myr with an average of 8.18 myr, slightly older than that for the southwestern clade, which ranged from 3.19 (0.44–9.39) to 6.78 (1.53–16.84) myr with an average of 5.06 myr. Similarly, divergence times of lineages at population level within the eastern clade ranged from 2.42 (0.07–9.39) to 6.16 (0.85–16.06) myr with an average of 4.00 myr, were also slightly older than that for the southwestern clade that ranged from 1.66 (0.05–6.05) to 1.95 (0.07–7.08) myr with an average of 1.76 myr. Moreover, relative evolutionary rates at internal nodes within the southwestern clade ranged from 0.00115 (0.00009–0.00385) to 0.00143 (0.00026–0.00401) with an average of 0.001254, greater than those for the eastern clade that ranged from 0.00075 (0.00005–0.00285) to 0.00094 (0.00018–0.00286) with an average of 0.000814.

DISCUSSION

Biodiversity pattern displayed by the S. japonica complex—Four aspects of the revealed biogeographical patterns of the *S. japonica* complex deserve attention. First, the *S. japonica* complex is morphologically most diversified in southwest China, with the majority of the varieties occurring in this extremely narrow region. By contrast, only three varieties occupy the eastern part, which is much larger than the southwest region (Fig. 1). Second, the phylogeny of the *S. japonica* complex reconstructed by ITS sequences was corroborated by the distribution pattern of alkaloids (Table 1; Fig. 3), and the distribution boundary between the two major clades roughly matched the division line between the Sino-

Table 3. The prior and posterior distribution of divergence times estimated by Bayesian molecular dating. The following abbreviations were applied: A.D. = mean for divergence times; Pop. = at population level; Var. = at variety level; A.R. = mean for evolutionary rates.

Clades	Node no.	Prior mean for node times and the 95% credibility interval (mya)	Posterior mean for divergence times and the 95% credibility interval (mya)	Posterior mean of evolutionary rates and the 95% credibility interval (changes/myR)	A.D. Pop.	A.D. Var.	A.R.
Southwestern clade	16	8.22 (0.34–23.34)	1.95 (0.07–7.08)	0.00115 (0.00009–0.00385)	1.76 (nodes 16–18)	5.06 (nodes 19–21)	0.001254 (nodes 16–22)
	17	5.46 (0.17–17.84)	1.67 (0.05–6.07)	0.00129 (0.0001–0.00418)			
	18	5.43 (0.16–18.17)	1.66 (0.05–6.05)	0.00124 (0.0001–0.00412)			
	19	10.94 (1.50–25.90)	3.19 (0.44–9.39)	0.00127 (0.00013–0.00402)			
	20	16.37 (4.41–31.80)	5.22 (1.05–13.61)	0.00122 (0.00018–0.00375)			
	21	21.76 (7.89–37.46)	6.78 (1.53–16.84)	0.00118 (0.0002–0.00363)			
	22	27.24 (12.46–42.33)	8.63 (2.07–20.08)	0.00143 (0.00026–0.00401)			
Eastern clade	23	8.28 (0.28–25.44)	.42 (0.15–11.48)	0.00075 (0.00005–0.00285)	4.00 (nodes 23–25)	8.18 (nodes 26–27)	0.000814 (nodes 23–27)
	24	16.42 (2.88–34.49)	6.16 (0.85–16.06)	0.00078 (0.00009–0.00278)			
	25	8.24 (0.25–25.03)	2.42 (0.07–9.39)	0.00077 (0.00005–0.00293)			
	26	16.43 (2.83–34.53)	6.54 (0.98–17.28)	0.00083 (0.0001–0.00286)			
	27	24.62 (8.64–40.99)	9.82 (2.11–22.22)	0.00094 (0.00018–0.00286)			
	28	32.56 (17.99–46.32)	19.87 (8.26–36.10)	0.00315 (0.00115–0.00599)			
	29	37.71 (24.49–49.97)	22.34 (10.54–39.64)	0.00357 (0.00151–0.00581)			
	30	42.67 (34.10–52.36)	43.99 (34.32–52.56)	0.00357 (0.00151–0.00581)			

Japanese Forest Subkingdom and the Sino-Himalayan Forest Subkingdom, as well as the division of environmental heterogeneity between the east and the transitional belt (Table 1, Figs. 1, 3). Third, the only tetraploid variant (*S. japonica* var. *fortunei*) occurs around this division line suggesting a possible correlation between environmental changes and polyploidization (Fig. 1). Fourth, the average sequence divergence among populations within the southwestern group is slightly higher than that within the eastern group (Table 2), while divergence times estimated for the southwestern group are younger than that for the eastern clade (Table 3), suggesting a difference in the evolutionary rate between the east and the southwest.

General known picture of the natural history and presumed migration and dispersal of Spiraea in East Asia—During the early Tertiary, East Asia was rather even in geography, with the altitude gradually decreasing from the east to the west, while the continent was climatically dominated by a planetary wind belt (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984; Wang, 1984; Liu and Ding, 1984). Since the late Palaeocene (about 55myr), East Asia has changed greatly topographically and climatically; the western regions has undergone phased uplift with the development of the Himalaya, while the east has remained comparatively stable (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984; Sharma, 1984; Harrison et al., 1992; Molnar et al., 1993). By the Miocene, the topological configuration of East Asia had been radically reshaped. The formerly lower western region had been uplifted higher than the east (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984; Chung et al., 1998) and underwent further uplift (Sharma, 1984; Molnar et al., 1993). By the Early Quaternary, the present topography of East Asia had largely taken shape, with the three distinguishable regions of environmental heterogeneity (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984; Committee of Chinese Academy of Sciences for Collection of Articles on Tibet Plateau, 1999; Fig. 1a, b). Meanwhile, this geographic evolution in East Asia was accompanied by climatic changes, including global cooling (Collinson et al., 1981), and the formation of a monsoon system in East Asia (Quade et al., 1989). The global cooling led to the southward movement of climate belts, and the development of different monsoon systems affected sites regionally (Guo, 1981; Liu and Ding, 1984). Presumably, this history of environmental changes has influenced floristic evolution in East Asia.

A hypothesis for the migration and dispersal of *Spiraea* in East Asia since the Eocene can be developed, given that fossils of *Spiraea* were found in Eocene, Oligocene, and Miocene sediments in Alaska, Europe, and East Asia, respectively (Heer, 1869; Knowlton, 1894; Hollick, 1936; Committee of Chinese Academy of Sciences for Plant of Cenozoic China, 1978). The origin of *Spiraea* can be dated back at least to the Eocene based on the fossil records. During the Eocene, the temperate zone was still located at higher latitudes (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984; Wang, 1984; Liu and Ding, 1984; Tanai, 1992). At that time, therefore, occurrence of *Spiraea* as a temperate plant would be limited to the higher latitudes; this is corroborated by the fact that fossils of *Spiraea* have so far only been found at higher latitudes. With global cooling in the Cenozoic and the southwards movement of the climate belts, it is conceivable that ancestral *Spiraea* also migrated southward to reach its present occurrence latitude. During the southward migration of *Spiraea,* the development of an arid belt from northwest to southeast in central East Asia since the Paleogene (Wang, 1988) would have prevented *Spiraea* from reaching southwest China directly from the north. Therefore, *Spiraea* in southwest China today may have migrated from the northeast China after the gradual retreat of the arid belt (Wang, 1988).

Biogeography implications—The biodiversity patterns of the *S. japonica* complex and the approximate estimates of divergence times resulting from the Bayesian molecular dating are compatible with the environmental and floristic history of East Asia. The finding of two main clades in the ITS phylogeny and the agreement of the distribution pattern of alkaloidsin the *S. japonica* complex with the two clades suggest a divergence early in the evolution of *S. japonica*. This divergence between the two main lineages was dated to 19.87 mya, corresponding to the second stage of Himalayan movements. Thus, the divergence may be correlated with the hypsography transformation during this period.

During the Miocene, environmental conditions were suitable for temperate plants in southwest China (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984; Wang, 1984; Liu and Ding, 1984 ; Tanai, 1992), and it was possible for an ancestral form of *S. japonica* to have migrated here from the northeast. It is conceivable that the two clades of *S. japonica* diverged from their common ancestor during this migration, adapting to the great environmental changes caused by the second phase of uplift of the Himalaya (Chung et al., 1998). Since then, the two clades have been exposed to different environmental conditions, and divergence between varieties within the two major clades are estimated to have occurred within the last 10 myr. Although the eastern part of East Asia has been affected by geological events, the physical environment has remained relatively stable and homogenous (Committee of Chinese Academy of Sciences for Physical Geography of China, 1985). Arguably, fewer geographical barriers, weaker divergent selection pressures, higher gene flow, and fewer bottleneck events could be expected for populations growing in this region. This is supported by the comparatively low morphological and genetic differentiation among populations for the eastern *S. japonica* group (Table 2). In contrast, a different evolutionary scenario can be envisaged for the southwestern clade. Southwest China is located at the boundary belt between Eurasia, the Indian subcontinent, and the Burma-Malaya Geoblock, with the interface being directly impacted by continental collision (Zhang et al., 1984; Sengor and Natal'in, 1996). With further development of the Himalaya movement, physical environmental heterogeneity in this region has increased continuously, with plateaus, mountains, basins, and gorges compressed within a narrow geographical mosaic (Committee of Chinese Academy of Sciences for Physical Geography of China, 1984). This was intensified by the third phase of Himalayan movements during the early Quaternary, with extreme environments developing (Committee of Chinese Academy of Sciences for Physical Geography of China, 1985). This increased environmental complexity could have resulted in more geographical barriers, higher selection pressures, greater isolation, and less gene flow between populations. All these factors might explain the higher divergence and diversification among *S. japonica* populations within the southwestern group. Moreover, the estimations of evolutionary rates at internal nodes within the southwestern clade are slightly higher than those for the eastern clade (Table 3). This suggests that higher evolutionary rates might have contributed to the diversification of the southwestern group and that the rate increase might be correlated with a large number of factors such as environmental stress, reduced population sizes, and strong, changing selection pressures.

It is a general principle that the higher the genetic divergence between two phylogenetic lines, the earlier the two lines diverged. This has been widely applied in the interpretation of biogeographical history using sequence data (for example Richardson et al., 2001). Our observation of the *S. japonica* complex suggests caution in the use of this principle. As we stated earlier, a migration of *S. japonica* from the northeast to the southwest China is one hypothesis as to how *S. japonica* reached southwest China, and diversification of the southwest group could be related to the environmental heterogeneity associated with the relatively recent phase of the Himalayan movements. Therefore, we presume that the divergence of phylogenetic lines from each other in the eastern group might be prior to that in the southwest group. This point is corroborated by the Bayesian molecular dating (Table 3). However, the detected divergence among the supposedly younger phylogenetic lines in the southwest group was slightly higher than that for the eastern group (Table 2). A possible explanation might be that although molecular mutation of closely related taxa typically occurs at a similar rate, different areas with different environments will vary in mutation accumulation or divergence maintenance and consequently differ in detectable evolutionary rate. In effect, the increased environmental complexity in southwest China could facilitate different divergence maintenance compared with the homogenous environment in eastern China and hence contribute toward the observed patterns.

Summary—In terms of the distribution of East Asian flora, two of the most notable features are the rich biodiversity center of southwest China, and the east–west axial floristic divergence (Wu, 1979; Wu and Wu, 1998). In this paper, we conclude that the specific phylogeny of *S. japonica* is closely linked to the general floristic evolution and historical environmental changes in East Asia. More specifically, physical environmental changes stemming from the Himalaya-Tibet plateau uplifting might have played a key role in specific phylogeny and origination of polyploid variants. More generally, in the floristic evolution in the region, the increased environmental complexity that facilitated divergence maintenance might be a key reason for the high biodiversity in southwest China. However, further studies are required to assess the extent to which the Himalaya-Tibet uplifting is responsible for the formation of the present biodiversity patterns in East Asia.

LITERATURE CITED

AXELROD, D. I., I. AL-SHEHBAZ, AND P. H. RAVEN. 1998. History of the modern flora of China. *In* A. L. Zhang and S. G. Wu [eds.], Proceedings of the First International Symposium on Floristic Characteristics and Diversity of East Asian Plants, 43–45. China Higher Education Press, Beijing, China.

CHANG, D. H. S. 1983. The Tibetan Plateau in relation to the vegetation of China. *Annals of Missouri Botanical Garden* 70: 564–570.

CHUNG, S. L., C. H. LO, T. Y. LEE, Y. Q. ZHANG, Y. W. XIE, X. H. LI, K. L. WANG, AND P. L. WANG. 1998. Diachronous uplift of the Tibetan Plateau starting 40 Myr ago. *Nature* 394: 769–773.

COLLINSON, M. E., K. FOWLER, AND M. E. BOULTER. 1981. Floristic changes indicate a cooling climate in the Eocene of southern England. *Nature* 291: 315–317.

COMMITTEE OF CHINESE ACADEMY OF SCIENCES FOR COLLECTION OF ARTICLES ON TIBET PLATEAU. 1999. Impact of Tibet uplift on physical environment. Ganxing Press, Lanzhou, China.

COMMITTEE OF CHINESE ACADEMY OF SCIENCES FOR PHYSICAL GEOGRAPHY OF CHINA. 1984. Physical geography of China: palaeogeography. Science Press, Beijing, China.

COMMITTEE OF CHINESE ACADEMY OF SCIENCES FOR PHYSICAL GEOGRAPHY OF CHINA. 1985. Physical geography of China: pandect. Science Press, Beijing, China.

COMMITTEE OF CHINESE ACADEMY OF SCIENCES FOR PLANT OF CENOZOIC CHINA. 1978. Fossil plant from China (III): plants of Cenozoic China. Science Press, Beijing, China.

DOYLE, J. J., AND J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemistry Bulletin* 19: 11–15.

DRUMMOND, A. J., O. G. PYBUS, A. RAMBAUT, R. FORSBERG, AND A. G. RODRIGO. 2003. Measurably evolving populations. *Trends in Ecology and Evolution* 18: 481–488.

FAN, L. M., Z. Y. ZHANG, Y. M. SHEN, AND X. J. HAO. 2004. Five diterpene alkaloids from *Spiraea japonica* (Rosaceae). *Biochemical Systematics and Ecology* 32: 75–78.

GUO, S. Z. 1981. On the elevation and climate changes of the Qinghai-Xizang Plateau based on fossil angiosperms. Proceedings of the symposium on Qinghai-Xizang (Tibet) Plateau, 201–205. Science Press, Beijing, China.

HARRISON, T. M., P. COPELAND, W. S. F. KIDD, AND A. YIN. 1992. Raising Tibet. *Science* 255: 1663–1670.

HEER, O. 1869. Flora fossilis Arctica, vol. 2. Friedrich Schulthess, Zurich, Switzerland.

HOLLICK, C. A. 1936. The Tertiary floras of Alaska: U.S. Geological Survey Professional Paper 182. United States Government Printing Office, Washington, D.C., USA.

KISHINO, H., J. L. THORNE, AND W. J. BRUNO. 2001. Performance of a divergence time estimation method under a probabilistic model of rate evolution. *Molecular Biology and Evolution* 18: 352–361.

KNOWLTON, F. H. 1894. A review of the fossil flora of Alaska with descriptions of new species. *Proceedings of the United States National Museum* 17: 207–240.

LIU, D. S., AND M. L. DING. 1984. The characteristics and evolution of palaeoenvironment of China since the late Tertiary. *In* R. O. Whyte [ed.], The evolution of East Asian environment, vol. 1: geology and palaeoclimatology, 11–40. Center of Asian Studies, Hong Kong.

LU, L. T. 1996. The evolution and distribution of subfam. Spiraeoideae (Rosaceae) of China, with special reference to distribution of the subfamily in the world. *Acta Phytotaxonomica Sinica* 34: 361–375.

MOLNAR, P., P. ENGLAND, AND J. MARTIOD. 1993. Mantle dynamics, uplift of the Tibetan Plateau and the Indian monsoon development. *Reviews of Geophysics* 34: 357–396.

MORGAN, D. R., D. E. SOLTIS, AND K. R. ROBERTSON. 1994. Systematic and evolutionary implications of *rbcL* sequence variation in Rosaceae. *American Journal of Botany* 81: 890–903.

POSADA, D., AND K. A. CRANDALL. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

QUADE, J., T. E. CERLING, AND J. R. BOWMAN. 1989. Development of Asian monsoon revealed by marked ecological shift during the latest Miocene in northern Pakistan. *Nature* 342: 163–165.

REHDER, A. 1913. Rosaceae. *In* C. S. Sargent [ed.], Plantae Wilsonianae, 451–453. Arnold Arboretum, Boston, Massachusetts, USA.

RICHARDSON, J. E., R. T. PENNINGTON, T. D. PENNINGTON, AND P. M. HOLLINGSWORTH. 2001. Rapid diversification of a species-rich genus of neotropical rain forest trees. *Science* 293: 2242–2245.

SENGOR, A. M. C., AND B. A. NATAL'IN. 1996. Palaeotectonics of Asia: fragments of a synthesis. *In* A. Yin and M. Harrison [eds.], The tectonic evolution of Asia, Rubey Colloquium, 486–640. Cambridge University Press, Cambridge, UK.

SHARMA, K. W. 1984. The sequence of phased uplift of the Himalayas. *In* R. O. Whyte [ed.], The evolution of East Asian environment, vol. 1: geology and palaeoclimatology, 56–70. Center of Asian Studies, Hong Kong.

SWOFFORD, D. L. 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods). Sinauer, Sunderland, Massachusetts, USA.

TANAI, T. 1992. Tertiary vegetation history of East Asia. *Mizunami Fossil Museum Bulletin* 19: 125–163.

TAO, J. R. 2000. The evolution of the late Cretaceous-Cenozoic floras in China. Science Press, Beijing, China.

TAO, J. R., AND X. Z. XIONG. 1986. The latest cretaceous flora of Helongjiang province and the floristic relationship between east Asia and North America (cont.). *Acta Phytotaxonomica Sinica* 24: 121–135.

THORNE, J. L., AND H. KISHINO. 2002. Divergence time and evolutionary rate estimation with multilocus data. *Systematic Biology* 51: 689–702.

WANG, C. W. 1961. The forests of China with a survey of grassland and desert vegetation. Maria Moores Cabot Foundation Publication, series 5, 313. Harvard University, Cambridge, Massachusetts, USA.

WANG, P. X. 1984. Progress in late Cenozoic palaeoclimatology of China: a brief review. Proceedings of the First Conference on the Palaeoenvironment of East Asia, 165–187. Beijing, China.

WANG, P. X. 1988. Late Cenozoic palaeoclimatology in China: progress and problems. Proceedings of the Second Conference on the Palaeoenvironment of East Asia From the Mid-Tertiary: geology, sea level changes, palaeoclimatology and palaeobotany, 377–392. Beijing, China.

WANG, W. M. 1994. Paleofloristic and paleoclimatic implications of Neogene palynofloras in China. *Review of Palaeobotany and Palynology* 82: 239–250.

WHITE, T. J., Y. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal RNA genes for phylogenetics. *In* M. Innis, D. Gelfand, J. Sninsky, and T. White [eds.], PCR protocols: a guide to methods and amplifications, 315–322. Academic Press, San Diego, California, USA.

WU, C. Y. 1979. The regionalization of Chinese flora. *Acta Botanica Yunnanica* 1: 1–22.

WU, Z. Y. 1980. China's vegetation. Science Press, Beijing, China.

WU, Z. Y., AND P. H. RAVEN. 2003. Flora of China. Science Press and Missouri Botanical Garden Press, Beijing, China and St. Louis, Missouri, USA.

WU, Z. Y., AND S. G. WU. 1998. A proposal for a new floristic kingdom (realm): the E. Asiatic kingdom, its delimitation and characteristics. *In* A. L. Zhang and S. G. Wu [eds.], Proceedings of the First International Symposium on Floristic Characteristics and Diversity of East Asian Plants, 3–42. China Higher Education Press, Beijing, China.

YANG, Z. H. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Bioinformatics* 13: 555–556.

YU, T. T. 1974. Rosaceae. *In* Delectis florae reipublicae popularis sinicae agendae academiae sinicae edita [ed.], Flora Reipublicae Popularis Sinicae, 1–20. Science Press, Beijing.

YU, T. T. 1984. Origin and evolution of Rosaceae. *Acta Phytotaxonomica Sinica* 6: 431–444.

ZHANG, Z. M., J. G. LIOU, AND R. G. COLEMAN. 1984. An outline of the plate tectonics of China. *Geological Society of America Bulletin* 95: 295–312.

ZHANG, Z.-Y., H. SUN, AND Z.-J. GU. 2002. Karyomorphological study of the *Spiraea japonica* complex (Rosaceae). *Brittonia* 54: 168–174.