Annals of Botany **96**: 413–424, 2005
doi:10.1093/aob/mci188, available online at www.aob.oupjournals.org

Molecular Systematics and Biogeography of *Crawfurdia*, *Metagentiana* and *Tripterospermum* (Gentianaceae) Based on Nuclear Ribosomal and Plastid DNA Sequences

SHENGYUN CHEN[1,2], TAO XIA[1,2], YUJIN WANG[1,2], JIANQUAN LIU[1] and SHILONG CHEN[1,*]

[1]*Laboratory of Qinghai-Tibet Biological Evolution and Adaptation, Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining 810001, P. R. China and* [2]*Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*

Received: 25 November 2004 Returned for revision: 8 March 2005 Accepted: 4 May 2005 Published electronically: 1 July 2005

• *Background and Aims* The systematic position of the genus *Metagentiana* and its phylogenetic relationships with *Crawfurdia*, *Gentiana* and *Tripterospermum* have not been explicitly addressed. These four genera belong to one of two subtribes (Gentianinae) of Gentianeae. The aim of this paper is to examine the systematic position of *Crawfurdia*, *Metagentiana* and *Tripterospermum* and to clarify their phylogenetic affinities more clearly using ITS and *trnL* intron sequences.
• *Methods* Nucleotide sequences from the internal transcribed spacers (ITS) of nuclear ribosomal DNA and the plastid DNA *trnL* (UAA) intron were analysed phylogenetically. Ten of fourteen *Metagentiana* species were sampled, together with 40 species of other genera in the subtribe Gentianinae.
• *Key Results* The data support several previously published conclusions relating to the separation of *Metagentiana* from *Gentiana* and its closer relationships to *Crawfurdia* and *Tripterospermum* based on studies of gross morphology, floral anatomy, chromosomes, palynology, embryology and previous molecular data. The molecular clock hypothesis for the tested sequences in subtribe Gentianinae was not supported by the data ($P < 0{\cdot}05$), so the clock-independent non-parametric rate smoothing method was used to estimate divergence time. This indicates that the separation of *Crawfurdia*, *Metagentiana* and *Tripterospermum* from *Gentiana* occurred about 11·4–21·4 Mya (million years ago), and the current species of these three genera diverged at times ranging from 0·4 to 6·2 Mya.
• *Conclusions* The molecular analyses revealed that *Crawfurdia*, *Metagentiana* and *Tripterospermum* do not merit status as three separate genera, because sampled species of *Crawfurdia* and *Tripterospermum* are embedded within *Metagentiana*. The speciation and rapid radiation of these three genera is likely to have occurred in western China as a result of upthrust of the Himalayas during the late Miocene and the Pleistocene.

Key words: Asia, biogeography, *Crawfurdia*, *Gentiana*, Gentianeae, Gentianaceae, *Metagentiana*, molecular systematics, ITS, *trnL* (UAA) intron, *Tripterospermum*.

INTRODUCTION

Crawfurdia was established by Wallich in 1826 (Wallich, 1826). In the same year, Blume (1826) described a new genus *Tripterospermum* based on *T. trinerve* from Java. These two genera were re-examined by Marquand (1931, 1937), who did not accept *Crawfurdia* and *Tripterospermum* and merged them into *Gentiana*. However, the two have been retained as separate genera by many botanists (Smith, 1965; Ho and Liu, 1990; Struwe *et al*., 2002). *Metagentiana* was separated from *Gentiana* on the basis of observations related to its gross morphology, floral anatomy, chromosomes, palynology, embryology and molecular data (Yuan *et al*., 1996; Ho *et al*., 2002*a*; Struwe *et al*., 2002). It was previously treated as *Gentiana* section *Stenogyne*, established by Franchet (1884) and revised by Kusnezov (1894). Prior to Ho *et al*. (2002*a*), most botanists followed Kusnezov (1894) and considered this group as a section of *Gentiana* (Pringle, 1978; Ho and Liu, 1990; Struwe *et al*., 2002). *Metagentiana* consists of 14 species, two of which are endemic to Thailand and Myanmar, and the rest are concentrated in south-west China. Most species of *Metagentiana* are herbaceous local endemics growing in alpine scrub, meadows and coniferous forests (Ho and Pringle, 1995; Ho *et al*., 2002*a*). The systematic position of *Metagentiana* and its phylogenetic relationships with *Crawfurdia*, *Gentiana* and *Tripterospermum* have not been explicitly addressed. These four genera belong to one of two subtribes (Gentianinae) of Gentianeae (Struwe *et al*., 2002). The other, larger subtribe is *Swertiinae*, which includes *Bartonia*, *Comastoma*, *Frasera*, *Gentianella*, *Gentianopsis*, *Halenia*, *Jaeschkea*, *Latouchea*, *Lomatogonium*, *Megacodon*, *Obolaria*, *Pterygocalyx*, *Swertia* and *Veratrilla* (Struwe *et al*., 2002). Smith (1965) suggested that *Gentiana* section *Stenogyne* (*Metagentiana*) had a closer affinity with *Tripterospermum* and *Crawfurdia* than with any other section of *Gentiana*, and Löve and Löve (1976) recommended that it should be transferred to the genus *Tripterospermum*, tentatively as a subgenus (*Tripterospermum* subgenus *Stenogyne*) based on morphological characters. Halda (1995) treated section *Stenogyne* as a subgenus of *Gentiana.* Karyological studies in *Gentiana* have been reviewed by several authors (Yuan, 1993; Yuan and Küpfer, 1993*a*, *b*; Küpfer and Yuan, 1996). Yuan and Küpfer (1993*a*) reported chromosome numbers and karyotype asymmetry for six species of *Metagentiana* for the first time and suggested that it had a unique and isolated position in the genus

* For correspondence. E-mail chenshil@public.xn.qh.cn

Gentiana because of its higher basic chromosome numbers ($x = 17$, 21 and 23). Ho *et al*. (2002*b*) also reported chromosome numbers for two other species (*M. souliei*, $2n = 46$; *M. serra*, $2n = 34$). Yuan *et al*. (1996) suggested that section *Stenogyne* (*Metagentiana*) should be excluded from *Gentiana* because inclusion of this section in *Gentiana* makes the genus paraphyletic based on a molecular phylogeny reconstructed from nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA; this and the plastid *trnL* (UAA) intron (*trnL*) have been shown to be suitable markers for phylogenetic reconstruction within a genus or closely related genera (Baldwin *et al*., 1995; Wang *et al*., 1999; Bain and Golden, 2000; Liu *et al*., 2002). Moreover, previous studies at the generic level of subtribe Gentianinae and the sectional level of *Gentiana* have shown the phylogenetic utility of ITS and *trnL* sequences (Yuan and Küpfer, 1995; Gielly and Taberlet, 1996; Yuan *et al*., 1996; Yuan and Küpfer, 1997). Phylogenetic trees generated by parsimony analysis of these data are principally congruent with morphological observations, and they have improved or clarified some morphological misinterpretations and conflicts (Yuan and Küpfer, 1995; Gielly and Taberlet, 1996; Yuan *et al*., 1996, 2003; Yuan and Küpfer, 1997). However, only four species representing the three genera *Crawfurdia*, *Metagentiana* and *Tripterospermum* have been studied in previous phylogenetic analyses using ITS sequences (Yuan *et al*., 1996). Although it has been suggested that the section *Stenogyne* should be excluded to maintain the monophyly of *Gentiana*, there were insufficient samples in the cited studies (with regards to *Metagentiana*) to provide deeper insights into the relationships among these genera. Neither divergence dating nor biogeographic analyses of these groups were performed in any previous studies. In addition, major goals of modern biogeography are to reconstruct the phylogenies of genera and evaluate their origin and evolution against the geological and palaeoclimatic histories of their distribution areas (Avise, 2000).

Applying a molecular phylogenetic approach based on ITS and *trnL* sequences, the present study is focused on the new genus *Metagentiana* as well as *Crawfurdia* and *Tripterospermum*, allowing their systematic position to be examined and their phylogenetic affinities clarified. An attempt is also made to infer the divergence time of these three genera using a molecular clock hypothesis and to assess putative correlations between the origin of the three genera and geological events.

MATERIALS AND METHODS

Plant species and material

Sequence data were acquired for 22 species of *Crawfurdia*, *Gentiana*, *Metagentiana* and *Tripterospermum* as indicated by asterisks in Table 1. The voucher specimens were deposited in the Herbarium of the Northwest Plateau Institute of Biology, Xining, Qinghai Province. Sequences for 28 additional species were retrieved from GenBank. The origins of samples, voucher information, GenBank accession numbers and chromosome numbers of species studied are listed in Table 1. Representatives of subtribe Swertiinae, the sister clade of subtribe Gentianinae, *Gentianella moorcroftiana*, *Gentianopsis barbata* (*Gentianopsis crinita* for *trnL*), *Megacodon stylophorus* and *Swertia bimaculata* were used as outgroups according to the results of previous studies (Yuan and Küpfer, 1995; Gielly and Taberlet, 1996; Yuan *et al*., 1996).

DNA extraction and PCR amplification

DNA was extracted from silica-gel-dried leaf material (Chase and Hills, 1991) or from leaf tissue taken from herbarium sheets. Total genomic DNA was extracted using the 2× CTAB procedure of Doyle and Doyle (1987) or the CASsuper Plant Genomic DNA Isolation Kit (CASarray, Shanghai, China). The ITS region was amplified with universal primers 1 and 4 (White *et al*., 1990), and primers 'c' and 'd' were used to amplify the *trnL* intron (Taberlet *et al*., 1991) in 25-μL reactions. PCRs were performed in a Biometra thermal cycler programmed for 4 min at 94 °C, followed by 36 cycles of 94 °C for 50 s, 53 °C (46 °C for *trnL*) for 50 s and 72 °C for 50 s, with a final extension of 72 °C for 7 min.

PCR purification and sequencing

All successfully amplified DNA fragments were purified using a CASpure PCR Purification Kit following the manufacturer's protocol (CASarray) prior to sequencing. The primers used for sequencing were the same as those used for PCR. The sequencing reactions were carried out in a Biometra thermal cycler (Tpersonal 48) using a DYEnamic Dye Terminator Cycle Sequencing Kit (Amersham) following the recommended protocol, but with the reaction volumes scaled down to 10 μL. The cycle sequencing products were cleaned using Autoseq 96 plates (Amersham) and then analysed with a MegaBACE DNA Analysis System (Amersham Biosciences Corp.). Both strands of DNA were sequenced.

Sequence alignment

The ITS and *trnL* sequences were aligned using ClustalX (Thompson *et al*., 1997), with additional minor manual adjustments. Potentially informative and unambiguously assessable indels were scored as binary characters regardless of their length, and added to the sequence data matrix (Simmons and Ochoterena, 2000). The boundaries of the sequences in the studied material were made by comparison with the published sequences of the genera of subtribe Gentianinae retrieved from GenBank (Yuan and Küpfer, 1995; Yuan *et al*., 1996).

Phylogenetic analysis and molecular clock test

Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2003). In maximum parsimony (MP) analysis, characters were equally weighted and unordered (Fitch, 1971), with all gaps treated as missing data. Heuristic searches with 100 random additions of sequence replicates, in combination with ACCTRAN character optimization, MULPARS, tree-bisection-reconnection branch-swapping

TABLE 1. *Origin of samples, voucher information, chromosome numbers and GenBank accession numbers of DNA sequences of* Crawfurdia, Gentiana, Metagentiana *and* Tripterospermum

Genus/section	Species	Locality	Voucher	Chromosome number: $2n$ (n)/x	GenBank accesssion no.	
					ITS or ITS1, ITS2	*trnL* (UAA) intron
Crawfurdia Wall.				$x = 23$		
	C. delavayi Franch.	Mt Cangshan, Yunnan, 3620 m	Chen03140	46 (23)	AY562176*	AY563391*
	C. speciosa Wall.	Cuona, Tibet, 3000 m	751592	46 (23)	AY858675*	AY858682*
	C. tibetica Franch.	Mt Gongga, Sichuan, 2300 m	Chen03097	46 (23)	AY563383*	AY563393*
Gentiana (Tourn.) L.				$x = ?$		
Calathianae Froelich	*G. terglouensis* Hacquet	Hochobir, Austria	–	42, 38 or 40	–	X77897
	G. verna L.	St Cergue, Switzerland	–	28 (14)	–	X75704
Chondrophylla Bunge	*G. aristata* Maxim.	Maqu, Gansu, China; 3500 m	Y92-328	14 (7)	Z48100/Z48116	–
	G. boryi Boissier	Sierra Nevada, Spain; 2300 m	Z93-S1	20 or 26	–	X77874
	G. flexicaulis H. Smith ex Marq.	Mt Taibai, Shaanxi, China; 3400 m	Y92-264	14 (7)	Z71937/Z71938	–
	G. heleonastes H. Smith ex Marq.	Maqu, Gansu, China, 3650 m	G032	12 (6)	Z71939/Z71940	–
	G. piasezkii Maxim.	Mingxian, Gansu, China; 2900 m	Y92-272	36 (18)	Z71955/Z71956	–
	G. squarrosa Ledeb.	Xiahe, Gansu, China; 3000 m	G046	38 (19)	Z71965/Z71966	–
Ciminalis (Adanson) Dumorti	*G. alpine* Villars	Grand Chavalard, Switzerland	Y93-09	36 (18)	–	X77868
	G. angustifolia Villars	Hautes, Franch	Kadereit 95/24	36	–	X75699
	G. clusii Perrier and Songeon	Grand Chavalard, Switzerland	Y93-13	36 (18)	–	X77879
	G. dinarica G. Beck	Abruzzi, Italy	Hungerer	36	–	X77882
	G. ligustica R. de Vilmorin and Chopinet	Alpes Maritimes, France	Merxmuller	36	–	X77886
Cruciata Gaudin	*G. crassicaulis* Duthie ex Burk.	Mangkang, Tibet, 3800 m	Ge015	26 (13)	AY858676*	AY858684*
	G. cruciata L.	Botanic Garden, Geneva, Switzerland	–	52	–	AF102434
	G. macrophylla Pall.	Dangchang, Gansu, China	Y92-271	26 (13)	Z48067/Z48086	–
	G. straminea Maxim.	Maqu, Gansu, China	Y92-313	52 (13)	AF346015	–
Dolichocarpa T. N. Ho	*G. tetrasticha* Marq.	Dangxiong, Tibet, China; 4500 m	Y92-128	24 (12)	Z71967/Z71968	–
Frigida Kusnez.	*G. algida* Pall.	Rocky Mt, Colorado, USA	Y91-S10	24 (12)	Z48142/Z48117	AJ490239
	G. frigida Haenke	Mt Rila, Bulgaria	NEU93-17	24 (12)	–	X77883
Isomeria Kusnez.	*G. depressa* D. Don	Zhangmu, Tibet, China	Y92-118	24 (12)	Z48062/Z48081	–
Gentiana	*G. montserratii* Vivant ex Greuter	Castanesa, Spain	–	–	–	X77887
Monopodiae T. N. Ho	*G. callistantha* Diels et Gilg	Luqu, Gansu, China	Y92-298	26 (13)	Z48095/Z48078	–
	G. futtereri Diels et Gilg	Dingqing, Tibet, China, 4160 m	Liu1056	–	–	AY858685*
	G. veitchiorum Hemsl.	Leiwuqi, Tibet, China, 4280 m	Liu1042	24 (12)	AY858677*	
Microsperma T. N. Ho	*G. atropurpurea* T. N. Ho	Dangxiong, Tibet, China, 4620 m	Liu1095	–	AY858678*	AY858686*
	G. delavayi Franch.	Lijiang, Yunnan, China; 2850 m	Y92-229	26 (13)	Z48099/Z48080	–
Phyllocalyx T. N. Ho	*G. phyllocalyx* C. B. Clarke	Makalu, Nepal	Neu97-30	26 (13)	AJ318537/ AJ410316	AJ315189
Gentianella Moench.						
	G. moorcroftiana (Wall. Ex Griseb.) Airy-Shaw	–	R. McBeath 2093	26	AJ294615/ AJ294675	AJ408007
Gentianopsis Ma	*G. barbata* (FrÖel.) Ma	Mengyuan, Qinghai, China	Liu J.Q.654	26 (13)	AF346007	–
	G. crinita (Froelich) Ma	Mainz Botanic Garden, Germany	–	78	–	AF102433
Megacodon (Hemsl.) H. Smith				$x = 7$		
	M. stylophorus (C. B. Clarke) H. Smith	Lijiang, Yunnan, China	Ge106	$x = ?$	AY858679*	AY858687*
Metagentiana T. N. Ho and S. W. Liu				$x = 17, 21, 23$		
	M. eurycolpa (Marquand) T. N. Ho and S. W. Liu	Luquan, Yunnan, 2600 m	Zhang0274	–	AY858673*	–

TABLE 1. *Continued*

Genus/section	Species	Locality	Voucher	Chromosome number: $2n$ (n)/x	GenBank accesssion no.	
					ITS or ITS1, ITS2	*trnL* (UAA) intron
	M. gentilis (Franchet) T. N. Ho and S. W. Liu	Kunming, Yunnan, 2220 m	Chen03122	42 (21)	AY562177*	AY563386*
	M. leptoclada (I. B. Balfour and Forrest) T. N. Ho and S. W. Liu	Dali, Yunnan, 3500 m	Liu22798	–	AY858674*	AY858681*
	M. primuliflora (Franchet) T. N. Ho and S. W. Liu	Kunming, Yunnan, 2290 m	Chen03124	42 (21)	AY562178*	AY563385*
	M. pterocalyx (Franchet) T. N. Ho and S. W. Liu	Heqing, Yunnan, 3620 m	Chen03158	34 (17)	AY562171*	AY563384*
	M. rhodantha (Franchet) T. N. Ho and S. W. Liu	Kunming, Yunnan, 2220 m	Chen03123	46 (23)	AY562174*	AY563390*
	M. serra (Franchet) T. N. Ho, S. W. Liu and S. L. Chen	Lijiang, Yunnan, 2450 m	Chen03141	34 (17)	AY562175*	AY563387*
	M. souliei (Franchet) T. N. Ho, S. W. Liu and S. L. Chen	Lijiang, Yunnan, 3320 m	Chen03146	46 (23)	AY562170*	AY563388*
	M. striata (Maximowicz) T. N. Ho, S. W. Liu and S. L. Chen	Daofu, Sichuan, 3510 m	Chen03081	46 (23)	AY562173*	AY563389*
	M. villifera (H. W. Li ex T. N. Ho) T. N. Ho and S. W. Liu	Yunlian, Sichuan, 800 m	Chuan0018	–	AY858672*	AY858680*
Swertia L.	*S. bimaculata* (Sieb. Et Zucc.) Hook. f. et Thoms ex C. B. Clarke	Makalu, Nepal	NEU97Z-22	x = 11	AJ318552/AJ410331	AJ315204
Tripterospermum Blume				x = 23 or 10		
Tripterospermum	*T. cordatum* (Marq.) H. Smith	Kunming, Yunnan, 2210 m	Chen03112	46 (23)	AY562172*	AY563392*
	T. filicaule (Hemsl.) H. Smith	Mt Gongga, Sichuan, 1990 m	Chen03098	–	–	AY858683*
	T. volubile (D. Don) Hara	Nielamu, Tibet, 2300 m	1279	–	AY858667*	–
Platyspermum C. J. Wu	*T. chinense* (Migo) H. Smith	Mt Tianmu, Zhejiang, 1506 m	Liu160810	–	AY858668*	–

*Indicates sequences that were registered in the present study (?, not affirmative; –, not available).
Chromosomal numbers reported by Yuan and Küpfer (1993*a*, *b*), Chen *et al*. (1997) and Ho *et al*. (2002*a*, *b*).

and STEEPEST DESCENT on were utilized to search for possible multiple islands of most-parsimonious trees (Maddison, 1991). The relative support for individual clades was evaluated by bootstrap (BS) analysis (Felsenstein, 1985). BS values were calculated using 1000 replicates of heuristic searches, each with 10 random addition sequence replicates using tree-bisection-reconnection and MULPARS on options.

Support for each branch was assessed using both BS and Bayesian analyses. BPP were estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions (Larget and Simon, 1999). Analyses were performed with MrBayes v2.01 (Huelsenbeck and Ronquist, 2001), with GTR + Γ + PINVAR parameters being estimated during the run, and using the default value of four Markov chains. Multiple chains can assist in more easily traversing tree-space and help avoid entrapment in local topological optima. The Monte Carlo Markov chain length was 1 000 000 generations, and the chain was sampled every 100 generations. Log-likelihood values for sampled trees stabilized after approx. 200 000 generations. Therefore, the last 8000 sampled trees were used to estimate BPP, also called Bayesian support values. If >95 % of the sampled trees contained a given clade, it was considered to be significantly supported by the data produced.

A maximum likelihood (ML) analysis was also conducted, using PAUP* 4.0b10. For the ML analyses, among-site rate variations were modelled using a gamma distribution and the shortest trees from the MP analyses as starting points for ML estimation of transitions : transversion (ti/tv) ratios and the alpha parameter of the gamma distribution for among-site rate variation. Then an iterative procedure as described in Swofford *et al*. (1996), in which the most likely tree from each heuristic search was used to re-estimate the ti/tv ratio and alpha parameter, was followed. This procedure was repeated until essentially no change occurred in the likelihood estimate between iterations. The program Modeltest, Version 3.06 (Posada and Crandall, 1998) was used to find the model of sequence evolution that best fits the data set according to hierarchical likelihood ratio (LR) tests ($P = 0{\cdot}05$). The GTR + I + G model (GTR = general time reversible; I = proportion of invariable sites; G = gamma distributed among-site rate variation) and TVM + G model (TVM = transversion model) in the ITS and *trnL* data, respectively, and parameter settings (gamma shape, base frequencies) were selected through the Hierarchical Likelihood Ratio Tests procedure implemented in Modeltest. The LR test statistic is distributed as χ^2 with degrees of freedom equal to the number of free parameters between the two models (Goldman, 1993) when the models of sequence evolution are nested. A molecular clock hypothesis was also tested using the LR test, based on twice the difference between the log-likelihoods for trees generated from clock and clock-free analyses (Muse and Weir, 1992; Baldwin and Sanderson, 1998; Wang *et al*., 2000), using a χ^2 distribution with $N - 2$ degrees of freedom (where N is the number of terminal taxa in the tree). When the LR test rejected a molecular clock hypothesis, ML trees based on the ITS and *trnL* intron sequences were subjected to non-parametric rate smoothing (NPRS) (Sanderson, 1997) using the default settings in TreeEdit v.1.0a8 (Rambaut and Charleston, 2000; Richardson *et al*., 2001) to estimate divergence times.

RESULTS

The lengths of the unaligned ITS and *trnL* sequences varied from 689 to 692 base pairs (bp) and from 252 to 271 bp, respectively, among species of *Crawfurdia*, *Metagentiana* and *Tripterospermum*. The aligned ITS and *trnL* sequences were 699 bp and 327 bp long, respectively. Mean pairwise distances ranged from 0·96 % (*M. euryolapa vs. M. leptoclada*) to 7·72 % (*M. pterocalyx vs. M. rhodantha*) for ITS and from 0·37 % (*M. gentiles vs. M. serra* and *M. leptoclada vs. M. serra*) to 7·91 % (*M. pterocalyx vs. M. primuliflora*) for *trnL* within *Metagentiana*. The highest pairwise distance found between *Metagentiana* and other genera in subtribe Gentianinae was 17·37 % (*G. aristata vs. M. rhodantha*) and 15·23 % (*G. boryi vs. M. primuliflora*) for ITS and *trnL*, respectively (distance matrix not shown).

When gaps were excluded, the ITS sequences used for analyses contained 193 potentially informative changes. The strict consensus tree was generated from 387 most-parsimonious trees in one island each with 740 steps, a consistency index of 0·57 and a retention index of 0·72 (not shown). The Bayesian majority rule consensus tree pooled from the Bayesian trees is shown in Fig. 1. Many of the nodes along the spine of this tree have strong or moderate BPP and BS support. One of the ML trees is also shown (Fig. 2A). The topology of the ML trees is similar to that of the Bayesian majority rule consensus tree with only minor differences between them. The present analysis strongly supports the placement of *Crawfurdia*, *Metagentiana* and *Tripterospermum* (grey frame), as sister to the *Gentiana* clade (BPP = 100 %; BS = 94 %), as previously reported by Yuan *et al*. (1996). In the *Crawfurdia*, *Metagentiana* and *Tripterospermum* clade (Fig. 1), *M. eurycolpa* and *M. leptoclada* form a clade (BPP = 64 %; BS = 85 %) sister to the rest. Five species of *Metagentiana* (*M. gentiles*, *M. primuliflora*, *M. rhodantha*, *M. serra* and *M. villifera*) form a second clade (BPP = 93 %; BS = 93 %). Three species of *Metagentiana* (*M. pterocalyx*, *M. striata* and *M. souliei*) form a third clade placed with species of *Crawfurdia* and *Tripteropermum*. In this third clade, *Crawfurdia tibetica* forms a clade with *Tripterospermum cordatum* and *T. volubile*; thus the recognition of *Metagentiana*, *Tripterospermum* and *Crawfurdia* as three distinct genera is not supported.

The aligned *trnL* data included 75 potentially informative characters when gaps were excluded. The Bayesian majority rule consensus tree is shown in Fig. 3. Parsimony analysis identified 3321 most parsimonious trees with 212 steps, a consistency index of 0·72 and a retention index of 0·85. The ML analysis resulted in a tree with a likelihood score of –ln 1598·70548 (Fig. 2B). Because the *trnL* sequence of *M. eurycolpa* was not obtained, this species was omitted in the phylogenetic analyses of *trnL*. These analyses also support the placement of *Crawfurdia*, *Metagentiana* and *Tripterospermum* (grey frame) together

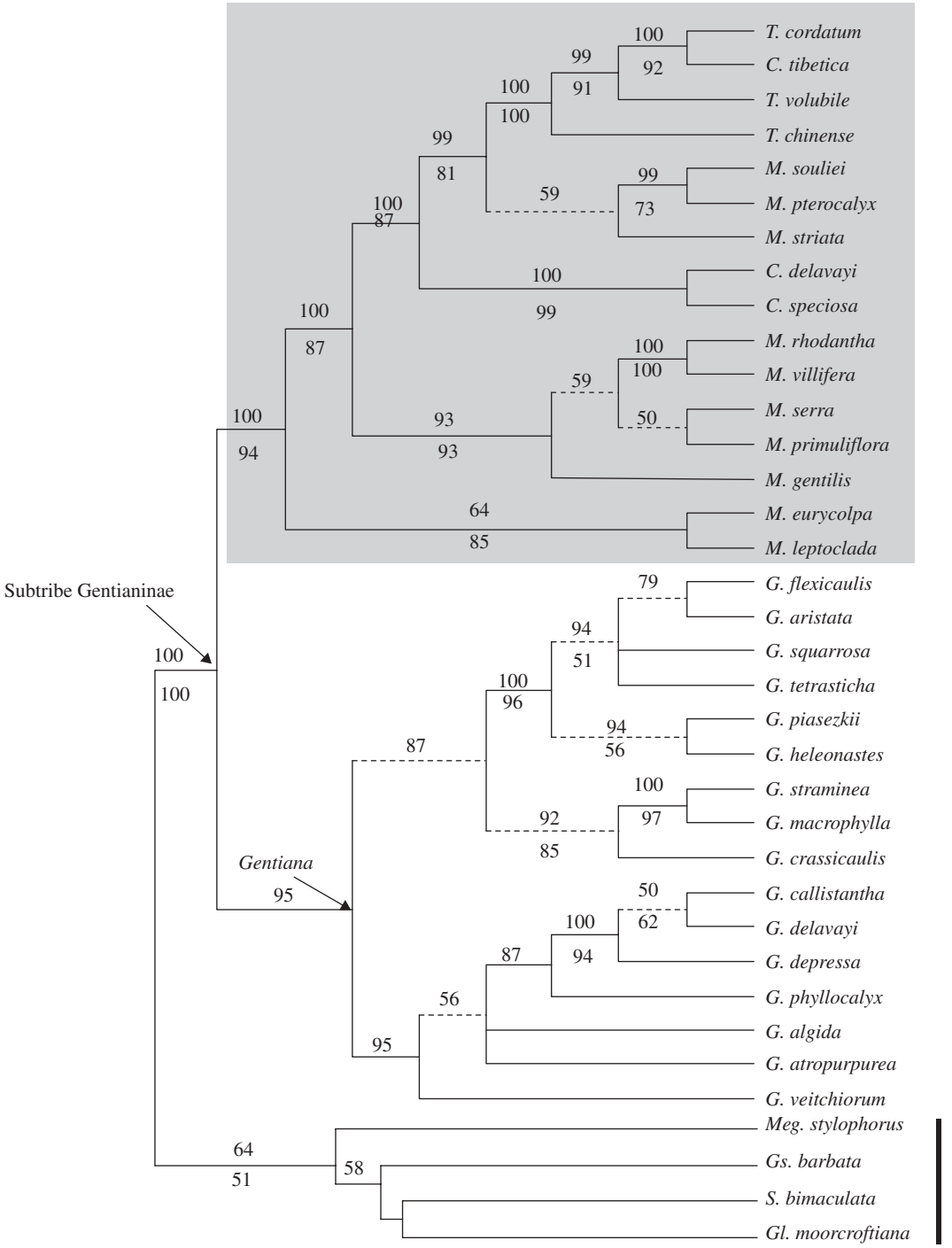

FIG. 1. Fifty per cent Bayesian majority rule consensus tree based on ITS sequence data. Numbers above and below the branches indicate the Bayesian posterior probabilities and the MP bootstrap values based on 1000 replicates, respectively. Dashed lines denote branches that collapse in the strict consensus tree from the parsimony analysis. The species of *Crawfurdia*, *Metagentiana* and *Tripterospermum* are indicated by grey shading. The thick black line indicates the four outgroups.

as sister to the *Gentiana* clade (BPP = 100 %; BS = 93 %). In agreement with the results based on ITS data, *Crawfurdia*, *Metagentiana* and *Tripterospermum* were polyphyletic.

The LR test rejected a molecular clock hypothesis based on their models and correlative parameters for both ITS and *trnL* ($P < 0{\cdot}01$). The ML trees based on ITS and *trnL* were subjected to clock-independent NPRS to obtain homogenized rates. There is no fossil record for the tribe Gentianeae to calibrate the molecular phylogeny of *Crawfurdia*, *Metagentiana* and *Tripterospermum.* The lowest rate of $4{\cdot}48 \times 10^{-9}$ substitutions per site per year (s/s/y) and the highest rate of $8{\cdot}41 \times 10^{-9}$ s/s/y previously reported for ITS

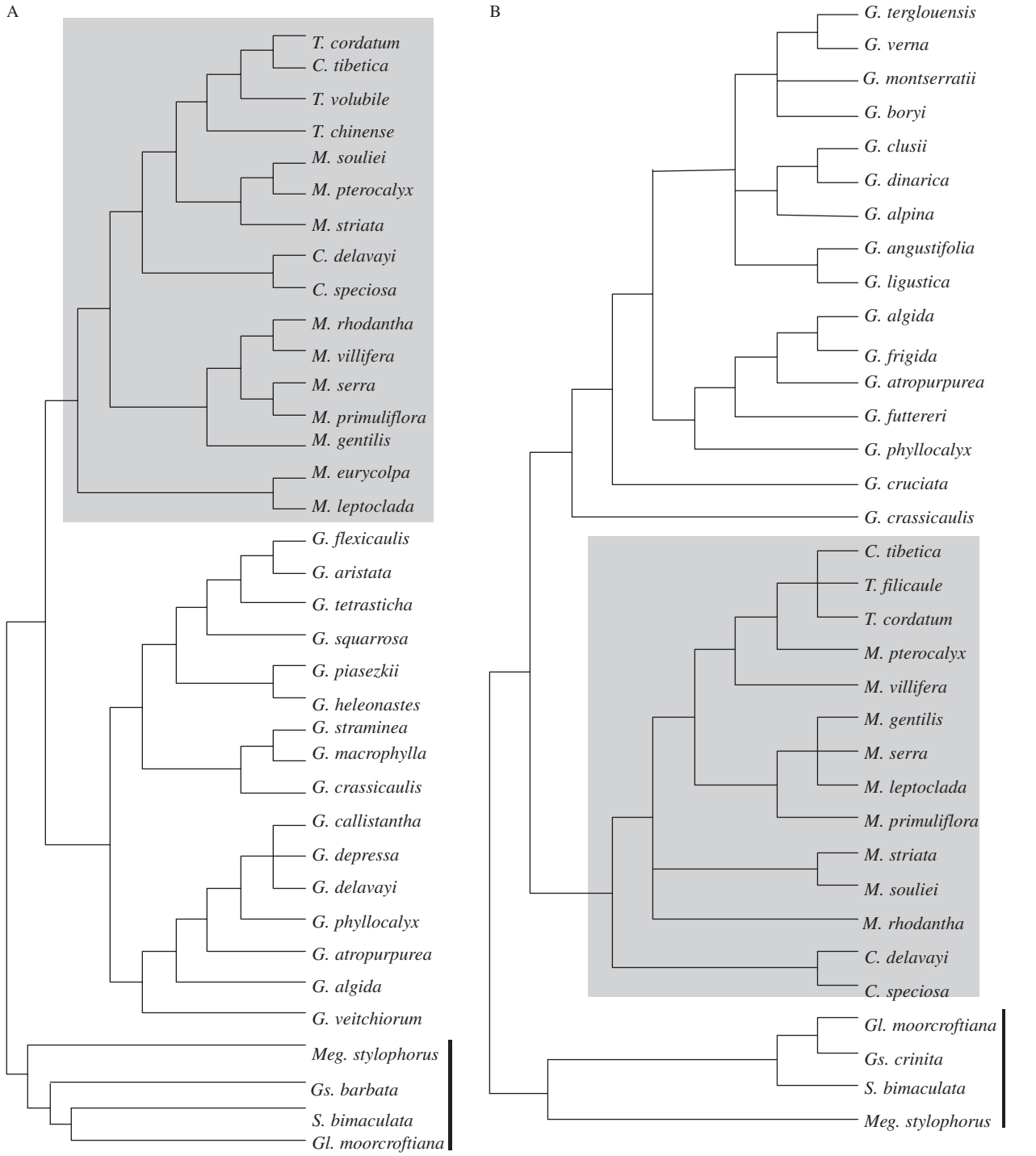

FIG. 2. (A) The best maximum likelihood tree based on the ITS matrix (likelihood score, –ln 4997·54138). (B) The the best maximum likelihood tree based on the *trnL* matrix (likelihood score, –ln 1598·70584). The species of *Crawfurdia*, *Metagentiana* and *Tripterospermum* are indicated by grey shading. The thick black line indicates the four outgroups.

data of *Gentianella* (Hagen and Kadereit, 2001) were used to estimate divergence times within the clade consisting of *Crawfurdia*, *Metagentiana* and *Tripterospermum*. The inferred divergence times were plotted on the ITS phylogenetic tree obtained with ML (Fig. 4). According to the rates and ITS sequence divergence, the clade formed by *Crawfurdia*, *Metagentiana* and *Tripterospermum* diverged from *Gentiana* about 11·4–21·4 Mya (million years ago). The divergence times between extant species range from 0·4 to 6·2 Mya.

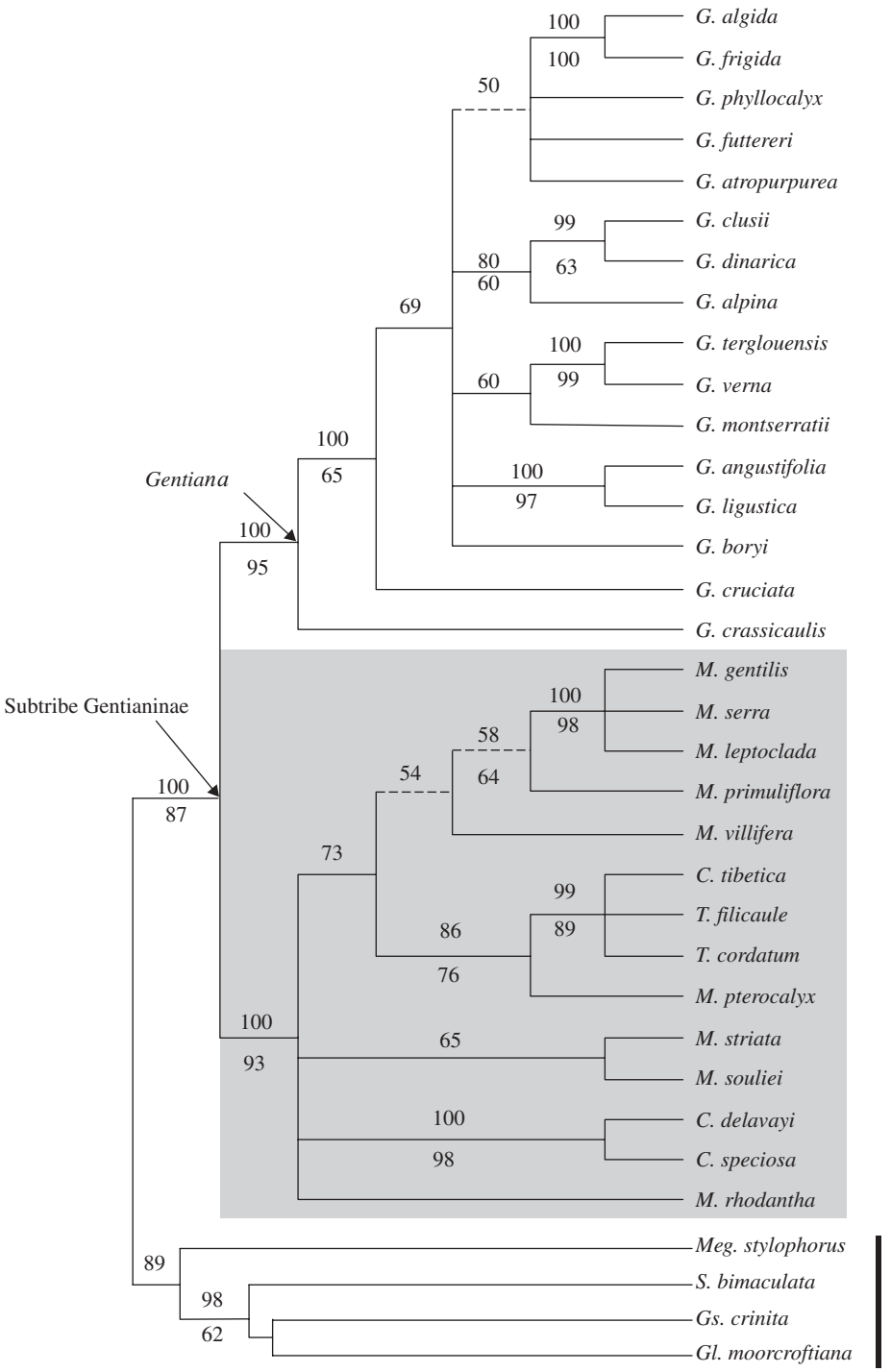

FIG. 3. Fifty per cent Bayesian majority rule consensus tree based on *trnL* sequence data. See Fig. 1 for further details.

DISCUSSION

Polyphyly of Crawfurdia, Metagentiana *and* Tripterospermum

Crawfurdia, *Metagentiana* and *Tripterospermum* are not monophyletic according to the molecular data presented here. In the phylogenetic tree based on ITS, ten species of *Metagentiana* fell mainly in two clades: *M. pterocalyx*, *M. striata* and *M. souliei* grouped with the six sampled species of *Crawfurdia* and *Tripteropsermum*, whereas *M. gentiles*, *M. primuliflora*, *M. rhodantha*, *M. serra* and *M. villifera* grouped together as a strongly supported clade (Figs 1, 2A). With *trnL*, nine species of *Metagentiana* formed four clades in the Bayesian majority rule consensus

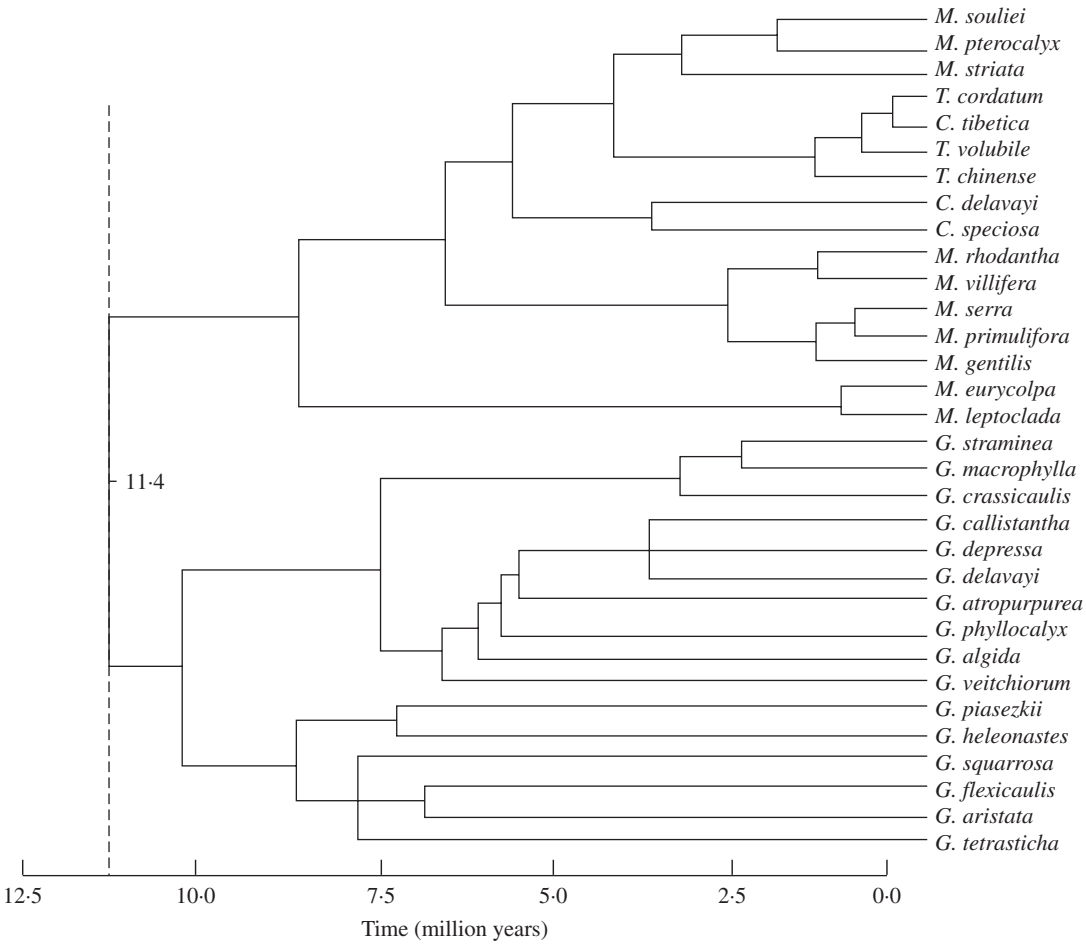

FIG. 4. The maximum likelihood tree with estimated ages based on NPRS of ITS ($8{\cdot}41 \times 10^{-9}$ s/s/y of the evolutionary rates). The dashed line denotes the divergence time of *Crawfurdia*, *Metagentiana* and *Tripterospermum* from *Gentiana*.

tree (Fig. 3) and three clades in the ML tree (Fig. 2B). *Metagentiana villifera* formed a clade with *M. gentiles*, *M. leptoclada*, *M. primuliflora* and *M. serra* in the Bayesian majority rule consensus tree and grouped with *M. pterocalyx* and the three sampled species of *Crawfurdia* and *Tripterospermum* as a clade in the ML tree. These findings indicate that *Crawfurdia*, *Metagentiana* and *Tripterospermum* should not be treated as three distinct genera. However, the present study sampled only six species (out of about 41 defined by Ho and Pringle, 1995) of *Crawfurdia* and *Tripterospermum*. Analysis including more species of *Crawfurdia* and *Tripterospermum* is necessary to clarify the phylogenetic and taxonomic status of the three genera.

Relationships of Crawfurdia, Metagentiana *and* Tripterospermum

Phylogenetic trees for *Crawfurdia*, *Metagentiana* and *Tripterospermum* based on the ITS and *trnL* in this study agree to a certain extent with the phylogenetic hypothesis based on analyses of gross morphology, floral anatomy, chromosomes, palynology, embryology and other molecular data (Smith, 1965; Nilsson, 1967; Löve and Löve, 1976; Yuan *et al*., 1993*a*, *b*, 1996; Chen *et al*., 1999*a*, *b*, 2000; Ho *et al*., 2000, 2002*b*). The genus *Metagentiana* is easily recognized by its solitary, sessile flowers, sessile, broad-ovate to ovate-triangular leaves and large leaf-like bracts, which make this genus more similar to *Crawfurdia* and *Tripterospermum* than to *Gentiana* (Ho *et al*. 2002*a*). The stems of *Gentiana* and *Metagentiana* are erect and branched, whereas in *Crawfurdia* and *Tripterospermum* they are twining. The midveins of the calyx lobes are keeled and winged into the calyx tube in *Crawfurdia*, *Metagentiana* and *Tripterospermum* but are not keeled in *Gentiana.* The style is filiform and about as long as the ovary in these three genera, but is linear to cylindrical and shorter than the ovary in *Gentiana* (Ho *et al*., 2002*a*). Pollen morphology of subtribe *Gentianinae* was studied by Nilsson (1967), who suggested that there were close relationships between *Crawfurdia*, *Metagentiana* and *Tripterospermum*. Chromosome numbers have been reported for eight species of *Metagentiana* (Yuan and Küpfer, 1993*a*; Ho *et al*., 2002*b*). Their basic chromosomal numbers are $x = 17$, 21 and 23. The karyotypes of *Metagentiana* are 3A and 3B according to Stebbin's classification (Stebbins, 1971). The higher and apparently secondary basic numbers and asymmetrical karyotypes suggested that *Metagentiana*

had an isolated position in the genus *Gentiana* (Yuan and Küpfer, 1993*a*; Table 1). *Metagentiana* is more similar to *Crawfurdia* and *Tripterospermum* than to *Gentiana* in karyological characters because *Crawfurdia* and *Tripterospermum* also have high basic numbers ($x = 23$) and asymmetrical karyotypes (S. L. Chen, unpubl. res.). In embryological characters, *Metagentiana* has a unitary original tapetum, uninucleate tapetal cells which do not protrude into the anther locule, one-celled middle layers, a typical parietal placenta, a hypertropous ovule type and ovules often arranged in four columns (Ho *et al*., 2000). *Gentiana* has a dual original tapetum, binucleate tapetal cells which elongate and protrude into the anther locule to form 'trabeculae' and 'placentoids', two-celled middle layers, a superficial parietal placenta, an anatropous ovule and ovules arranged in 10–30 columns (Ho and Liu, 1999). *Crawfurdia* and *Tripterospermum* share the same unitary original tapetal and typical parietal placenta with *Metagentiana* (Chen *et al*., 2000; Ho *et al*., 2000). Thus previous evidence indicates that *Metagentiana* has a closer relationship with *Crawfurdia* and *Tripterospermum* than with *Gentiana*. The molecular data in this study indicate that these three genera form a monophyletic group as sister group to *Gentiana* and that all three genera are polyphyletic as currently circumscribed.

Biogeographic considerations

Tribe Gentianeae is widely distributed, with the highest diversity occurring in the Old World. Of the two major clades of Gentianeae, subtribe Gentianinae is clearly centred in the Old World (Struwe *et al*., 2002). All members of *Crawfurdia*, *Metagentiana* and *Tripterospermum* grow in central to east Asia. Within *Gentiana*, some sections are primarily European, with a few species in north-west Africa, north-east North America and central Asia (Meusel *et al*., 1978). The widespread sections are generally most diverse in eastern Asia (Ho and Liu, 1990). An exception to this is *Gentiana* sect. *Pneumonanthe* which is most diverse in eastern North America. In the context of this distributional pattern and the inferred phylogenetic relationships, it seems most likely that the ancestor of Gentianinae occupied an alpine temperate range in the Old World, and that the New World and southern hemisphere were colonized secondarily (Yuan *et al*., 1996). The distribution of generic and specific diversity would suggest an eastern Asian origin for Gentianinae (Struwe *et al*., 2002). Virtually nothing is known about the timescale of this diversification, except that the European *Gentiana* sect. *Ciminalis* may have begun to radiate 2 Mya according to Hungerer and Kadereit (1998). However, geological and paleobotanical studies in south-east Asia, especially in south-western China, provide a good framework to develop a scenario regarding the divergence and radiation of *Crawfurdia*, *Metagentiana* and *Tripterospermum.* South-east Asia has a relatively high proportion of Tertiary relicts of vascular plants (Wu, 1980; Tiffney, 1985*a*, *b*; Qian *et al*., 2003). During the early Tertiary, a relatively uniform, warm climate covered the northern Hemisphere (Tiffney, 1985*a*). During this time, a relatively continuous, homogeneous flora with many tropical and subtropical elements, called 'the boreotropical flora' (Wolfe, 1975), spanned most of the current Arctic area (Latham and Ricklefs, 1993). This boreotropical flora was gradually shaped into a mixed mesic forest and became fragmented as cooling climates in the middle and late Tertiary towards the Pleistocene forced the flora southward (Wolfe, 1975; Tiffney, 1985*a*). During the climate cooling, cold-intolerant taxa at higher latitudes either migrated to lower latitudes or went extinct, giving way to cool-adapted taxa derived from the boreotropical flora or which evolved during the climate cooling (Leopold and MacGinitie, 1972; Wolfe, 1975; Tiffney, 1985*a*; Xiang and Soltis, 2001). The present data from the ITS analysis suggest that the three genera diverged from *Gentiana* about 11·4–21·4 Mya. This estimated time correlates well with the climate cooling in the Miocene (5·3–23 Mya). During the Miocene, south-western China was apparently occupied by mesic mixed deciduous hardwood forest with numerous broad-leaved evergreens (Hu and Chaney, 1940). Especially during the late Miocene, the drier climate had spread extensively in the western Himalayan region including south-western China, where evergreen forest was replaced gradually by semi-deciduous and dry deciduous forest with a rapid expansion of grasslands (Quade *et al*., 1989, 1995). A major reason is that the uplift of several thousand metres of the Himalayas with perhaps 2300–3000 m increase since the middle Miocene had resulted in drastic changes in the regional biota and dry climates in the Himalayan region. *Gentiana*, *Primula* and *Rhododendron* are among many genera that radiated widely in the mountains of China and underwent rapid radiation, probably driven by the Himalayan uplift since the late Miocene (Axelrod *et al*., 1996). Therefore, it is possible that the ancestors of these three genera grew in south-western China during the Miocene and produced more species and occupied a wider distribution in the subsequent radiation. The current species of these three genera are mainly distributed in western China and grow in coniferous forest, alpine shrub and alpine meadow habitats. The divergence time of the current species of three genera (about from 0·4 and 6·2 Mya) estimated from ITS sequences corresponds well with this hypothesis.

ACKNOWLEDGEMENTS

The authors thank the decision editor Dr Mike Fay and anonymous reviewers for critical comments, Dr Yuan Yongming for constructive comments on the manuscript, and Professor Ho Tingnong for identifying specimens. This work was supported by the key innovation project of the Chinese Academy of Sciences (KSCX2-SW-106) and the National Science Foundation of China (30170066).

LITERATURE CITED

Avise JC. 2000. *Phylogeography: the history and formation of species.* Cambridge, MA: Harvard University Press.

Axelrod DI, Al-Shehbaz I, Raven PH. 1996. History of the modern flora of China. In: Zhang A, Wu SG, eds. *Floristic characteristics and diversity of East Asian plants*. Beijing: China Higher Education Press, 43–55.

Bain JF, Golden JL. 2000. A phylogeny of *Packera* (Senecioneae; Asteraceae) based on internal transcribed spacer region sequence data and a

broad sampling of outgroups. *Molecular Phylogenetics and Evolution* **16**: 331–338.

Baldwin BG, Sanderson MJ. 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). *Proceedings of the National Academy of Sciences of the USA* **95**: 9402–9406.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**: 247–277.

Blume CL. 1826. *Tripterospermum.* In: *Bijdrangen tot de Flora van Nederlandsch Indië.* Batavia, 849.

Chase MW, Hills HH. 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon* **40**: 215–220.

Chen SL, Ho TN, Liu JQ. 1997. The chromosome number of 8 species in *Gentiana* (Gentianaceae) from the alpine mountains of the western China. *Acta Botanica Boreali-Occidentalia Sinica* **17**: 547–550.

Chen SL, Ho TN, Liu JQ, Hong DY. 1999*a*. Mega-microsporogeneis and female-melegametagenesis of *Tripterospermum chinense* (Gentianaceae). *Acta Biologica Plateau Sinina* **14**: 26–34.

Chen SL, Ho TN, Liu JQ, Hong DY. 1999*b*. Floral anatomy of *Crawfurdia* and its related taxa (Gentianaceae). *Acta Biologica Plateau Sinina* **14**: 35–46.

Chen SL, Ho TN, Liu JQ, Hong DY. 2000. Embryology of *Tripterospermum cordatum* (Gentianaceae). *Acta Botanica Yunnanica* **22**: 53–58.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemical Bulletin* **19**: 11–15.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fitch WM. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology* **20**: 406–416.

Franchet A. 1884. Description de quelques especes de Gentiana de Yunnan. *Bulletin de la Société Botanique de France* **31**: 373–378.

Gielly L, Taberlet P. 1996. A phylogeny of the European gentianas inferred from plastid *trnL* (UAA) intron sequences. *Botanical Journal of Linnean Society* **120**: 57–75.

Goldman N. 1993. Statistical tests of models of DNA substitution. *Journal of Molecular Evolution* **36**: 182–198.

Hagen KB, Kadereit JW. 2001. The phylogeny of *Gentianella* (Gentianaceae) and its colonization of the southern hemisphere as revealed by nuclear and plastid DNA sequence variation. *Organisms Diversity and Evolution* **1**: 61–79

Halda JJ. 1995. Synopsis of the new system of the *Gentiana. Acta Musei Richnoviensis Section Nature* **3**: 25–29.

Ho TN, Liu JQ. 1999. Embryology of *Gentiana lawrencei* var. *farreri*. *Acta Botanica Boreali-Occidentalia Sinica* **19**: 234–240.

Ho TN, Liu SW. 1990. The infrageneric classification of *Gentiana* (Gentianaceae). *Bulletin of the British Museum (Natural History) Botany* **20:** 169–192.

Ho TN, Pringle JS. 1995. Gentianaceae. In: Wu ZY, Raven PH, eds. *Flora of China*, *Vol. 16*. Beijing/St Louis, MO: Science Press/Missouri Botanical Garden, 1–140.

Ho TN, Chen SL, Liu JQ, Hong DY. 2000. Embryology of *Gentiana striata* (Gentianaceae). *Acta Botanica Boreali-Occidentalia Sinica* **20**: 960–967.

Ho TN, Chen SL, Liu SW. 2002*a*. *Metagentiana*, a new genus of Gentianaceae. *Botanical Bulletin of Academia Sinica* **43**: 83–91.

Ho TN, Liu JQ, Chen SL. 2002*b*. Contribution to the karyomorphology of seven species in *Gentiana* (Gentianaceae). *Acta Biologica Plateau Sinina* **15**: 67–73.

Hu HH, Chaney RW. 1940. A Miocene flora from Shantung Province, China. In: Whyte RO, ed. *The evolution of East Asian environments*. *Vol. 2. Palaeobotany, palaeozoology and palaeoanthropology,* Publications of the Carnegie Institution of Washington, 507: 1. Hong Kong: Center of Asian Studies, 472–481.

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

Hungerer KB, Kadereit JW. 1998. The phylogeny and biogeography of *Gentiana* L. sect. *Ciminalis* (Adans.) Dumort.: a historical interpretation of distribution ranges in the European high mountains. *Perspectives in Plant Ecology, Evolution and Systematics* **1**: 121–135.

Küpfer P, Yuan YM. 1996. Karyological studies on *Gentiana* sect. *Chondrophyllae* (Gentianaceae) from China. *Plant Systematics and Evolution* **200**: 161–176.

Kusnezov NI. 1894. Subgenus *Eugentiana* of genus *Gentiana*. *Trudy S.-Peterburghskago Obščestvah. Estesttvoispytatelej, Otde lenie Botaniki* **24**: 1–507.

Larget B, Simon DL. 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**: 750–759.

Latham RE, Ricklefs RE. 1993. Global patterns of tree species richness in moist forests: energy-diversity theory does not account for variation in species richness. *Oikos* **67**: 325–333.

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

Liu JQ, Gao TG, Chen ZD, Lu AM. 2002. Molecular phylogeny and biogeography of the Qinghai-Tibet Plateau endemic Nannoglottis (Asteraceae). *Molecular Phylogenetics and Evolution* **23**: 307–325.

Löve A, Löve D. 1976. The natural genera of Gentianaceae. In: Kachroo P, ed. *Recent advances in botany*. Delhi: Dehra Dun, 205–222.

Maddison DR. 1991. The discovery and importance of multiple islands of most-parsimonious trees. *Systematic Zoology* **40**: 315–318.

Marquand CVB. 1931. New Asiatic gentianas: II. *Bulletin of Miscellaneous Information, Royal Gardens, Kew* **1931**: 68–88.

Marquand CVB. 1937. The gentianas of China. *Bulletin of Miscellaneous Information, Royal Gardens, Kew* **1937**: 134–180.

Meusel H, Jäger EJ, Rauschert S, Weinert E. 1978. Vergleichende chorologie der zentraleuropäischen Flora I-II. Jena: Fischer Verlag.

Muse SV, Weir BS. 1992. Testing for equality of evolutionary rates. *Genetics* **132**: 269–276.

Nilsson S. 1967. Pollen morphological studies in the Gentianaceae-Gentianinae. *Grana Palynologica* **7**: 46–143

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Pringle JS. 1978. Sectional and subgeneric names in *Gentiana* (Gentianaceae). *Sida* **7**: 232–247.

Qian H, Song JS, Krestov P, Guo QF, Wu ZM, Shen XS, Guo XS. 2003. Large-scale phytogeographical patterns in East Asia in relation to latitudinal and climatic gradients. *Journal of Biogeography* **30**: 129–141.

Quade J, Cerling TE, Bowman JR. 1989. Development of Asian monsoon revealed by marked ecological shift during the latest Miocene in northern Pakistan. *Nature* **342**: 163–165.

Quade JJ, Cater ML, Ojha TP, Adam J, Harrison TM. 1995. Late Miocene environmental change in Nepal and the northern Indian subcontinent: stable isotopic evidence from paleosols. *Geological Society of America Bulletin* **107**: 1381–1397.

Rambaut A, Charleston M. 2000. TreeEdit: Phylogenetic Tree Editor. Oxford: University of Oxford.

Richardson JE, Weitz FM, Fay MF, Cronk QC, Linder HP, Reeves G, Chase MW. 2001. Rapid and recent origin of species richness in the Cape flora of South Africa. *Nature* **412**: 181–183.

Sanderson MJ. 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. *Molecular Biology and Evoution* **14**: 1218–1231.

Simmons MP, Ochoterena H. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* **49**: 369–381.

Smith H. 1965. Notes on *Gentianaceae*. *Notes from the Royal Botanic Garden, Edinburgh* **26**: 237–258.

Stebbins GL. 1971. *Chromosomal evolution in higher plants*. London: Edward Arnold.

Struwe L, Kadereit JW, Klackenberg J, Nilsson S, Thiv M, von Hagen KB, *et al*. 2002. Systematics, character evolution, and biogeography of Gentianaceae, including a new tribal and subtribal classification. In: Struwe L, Albert VA, eds. *Gentianaceae—systematics and natural history*. Cambridge: Cambridge University Press, 21–309.

Swofford DL. 2003. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4.0b10. Sunderland, MA: Sinauer Associates.

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM. 1996. Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics.* Sunderland, MA: Sinauer, 407–514.

Taberlet P, Gielly L, Patou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of plastid DNA. *Plant Molecular Biology* **17**: 1105–1109.

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

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

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

Wallich N. 1826. *Tentamen Florae Napalensis Illustratae, Part 2*. Calcutta, 37–64.

Wang JB, Zhang WJ, Chen JK. 1999. Application of ITS sequences of nuclear DNA in phylogenetic and evolutionary studies of angiosperms. *Acta Phytotaxonomica Sinica* **37**: 407–416.

Wang XQ, David CT, Sang T. 2000. Phylogeny and divergence times in Pinaceae: evidence from three genomes. *Molecular Biology and Evoution* **17**: 773–781.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and application.* San Diego, CA: Academic Press, 315–322.

Wolfe JA. 1975. Some aspects of plant geography of the Northern Hemisphere during the Late Cretaceous and Tertiary. *Annals of the Missouri Botanical Garden* **62**: 264–279.

Wu CY. 1980. *The vegetation of China*. Beijing: Science Press.

Xiang QY, Soltis DE. 2001. Dispersal-vicariance analyses of intercontinental disjuncts: historical biogeographical implications for angiosperms in the Northern Hemisphere. *International Journal of Plant Sciences* **162**: S26–S39.

Yuan YM. 1993. Karyological studies on Gentiana sect. Cruciata (Gentianaceae) from China. *Cytologia* **46**: 99–114.

Yuan YM, Küpfer P. 1993*a*. Karyological studies on *Gentiana* sect. *Frigida senso lato* and sect. *Stenogyne* (Gentianaceae) from China. *Bulletin de la Société entomologique de France* **116**: 65–78.

Yuan YM, Küpfer P. 1993*b*. Karyological studies of *Gentianopsis* Ma and some related genera of Gentianaceae from China. *Cytologia* **58**: 115–123.

Yuan YM, Küpfer P. 1995. Molecular phylogenetics of the subtribe Gentianinae (Gentianaceae) inferred from the sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Plant Systematics and Evolution* **196**: 207–226.

Yuan YM, Küpfer P. 1997. The monophyly and rapid evolution of *Gentiana* sect. *Chodronphyllae* Bunge s.l. (Gentianaceae): evidence from the nucleotide sequences of the internal transcribed spacers of nuclear ribosomal DNA. *Botanical Journal of the Linnean Society* **123**: 25–43.

Yuan YM, Küpfer P, Doyle JJ. 1996. Infrageneric phylogeny of the Genus *Gentiana* (Gentianaceae) inferred from nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. *American Journal of Botany* **83**: 641–652.

Yuan YM, Wohlhauser S, Möller M, Chassot P, Mansion G, Grant J, *et al*. 2003. Monophyly and relationships of the tribe *Exaceae* (Gentianaceae) inferred from nuclear ribosomal and plastid DNA sequences. *Molecular Phylogenetics and Evolution* **28**: 500–517.