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Placing Biebersteiniaceae, a herbaceous clade of Sapindales, in a temporal and geographic context

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Abstract. Biebersteiniaceae comprise a single genus with four species of perennial herbs occurring from central Asia to Greece. A previous molecular phylogenetic study placed one of the species in an isolated position in Sapindales, while morphological studies had placed *Biebersteinia* in or near Geraniaceae, albeit doubtfully. We tested the monophyly and placement of the family with data from the chloroplast genes *rbc*L and *atp*B obtained for all four species, other major clades of Sapindales and outgroups for a total of up to 114 taxa. Parsimony, Bayesian, and likelihood analyses place *Biebersteinia* in Sapindales, possibly as sister to the other eight families. Strict and relaxed molecular clocks constrained with fossils of *Biebersteinia* and up to eight other Sapindales suggest that the *Biebersteinia* crown group diversified in the Oligocene and Miocene, while the stem lineage dates back to the Late Paleocene. Ages for other sapindalean families are earlier than those obtained in more sparsely sampled analyses, although estimates for Burseraceae agree surprisingly well. Ancestral area analyses suggest that *Biebersteinia* expanded from the eastern part of its range (i.e. Tibet and Inner Mongolia) to the west, although analyses are hampered by the unclear sister group relationships.

Key words: Bayesian relaxed clock, *Biebersteinia*, biogeography, fossil constraints, molecular clock, phylogenetics, Sapindales.

Introduction

Biebersteinia comprises four species of glandular-hairy herbs that occur in temperate mountainous regions from central Asia to Greece. Three of the species are adapted to arid or semi-arid environments, while the natural habitat of *B. orphanidis* are open patches in mid-altitude *Abies* and *Cedrus* forests in Greece and Turkey. All species have medicinal properties and are therefore utilized by local communities (Zhang et al. 1995, Farsam et al. 2000, Vassiliades and Yannitsaros 2000, Miceli et al. 2005).

When describing *Biebersteinia odora*, Stephan (1806) placed his new genus between *Grielum* (Neuradaceae) and *Suriana* (Simaroubaceae). Subsequent authors suggested affinities with Zygophyllaceae, Rosaceae, Geraniaceae, and Rutaceae (see Bakker et al.

1998 for the taxonomic history of *Biebersteinia*). Boissier, in his *Flora Orientalis* (1867), placed *Biebersteinia* in the Geraniaceae, and other workers then followed this option. *Biebersteinia*, however, never fit well in Geraniaceae in having only one ovule per locule, in its pollen morphology, and in having a gynophore, and Takhtajan (1997) therefore recognized it as a separate order and family (as first suggested by Endlicher 1841). Phylogenetic analyses based on the plastid DNA markers *rbc*L and *atp*B revealed that *B. orphanidis* belongs in the Sapindales, albeit in an isolated position (Bakker et al. 1998), and this result is reflected in the classifications of the Angiosperm Phylogeny Group, which rank *Biebersteinia* as one of the nine families of the Sapindales (APG 1998, APG II 2003).

We here test the monophyly of *Biebersteinia* by analyzing DNA sequences from all four species, and we also place the family in a phylogenetic, temporal, and geographic context by including a dense sample of representatives of Sapindales and information from fossils of *Biebersteinia* and its relatives. Specifically, we address the following questions: (1) is *Biebersteinia* monophyletic; (2) who are the closest relatives of *Biebersteinia*; and (3) what is the likely time of divergence of the *Biebersteinia* crown group? This last question involved performing molecular clock analyses for a large sample of Sapindales so as to include multiple fossils from as many lineages as possible.

Materials and methods

Taxon sampling. Material of *Biebersteinia* was collected in the field or taken from cultivated plants. *Rbc*L sequences of 84 other taxa were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) and 27 were available from the first author's previous work on Sapindales (Muellner et al. 2003, 2006). For the *atp*B dataset, 61 sequences were downloaded from GenBank. Besides the four species of Biebersteiniaceae, our *rbc*L matrices include representatives of the other eight families of Sapindales (Anacardiaceae, Burseraceae, Kirkiaceae, Meliaceae, Nitrariaceae (including Peganaceae and Tetradiclidaceae), Rutaceae, Sapindaceae, and Simaroubaceae) plus representatives of the related orders Malvales, Brassicales, Oxalidales, Geraniales, and Saxifragales (cf. Bakker et al. 1998, APG II 2003). Voucher information is given in Wikström et al. (2001) and Muellner et al. (2003, 2006). Vouchers of *Biebersteinia* are deposited in the following herbaria: *B. heterostemon* at Harvard (A; Boufford et al. 31189, Xizang, Changdu Xian, 2004); *B. multifida* and *B. odora* in the Herbarium Senckenbergianum (FR; Vassiliades 165, Rasjera, 2000, and Holubec s.n., Chuiskie Belkie, Altai Mts., 2005, respectively); *B. orphanidis* in the herbarium of the University of Reading (RNG; Vassiliades s.n., Peloponnisos, Mt. Saitas, 1997). Sample designation and GenBank accession numbers are listed in Table 1. A fifth named entity, *Biebersteinia leiosepala* Jaub. and Spach (Paris herbarium, P, seen by DV), is usually considered a synonym of *B. multifida* (Knuth 1912, Schönbeck-Temesy 1970) and was not included in our study.

Isolation of DNA, amplification and sequencing. Leaf fragments were dried in silica gel prior to DNA extraction or taken from herbarium vouchers. Total DNA was extracted following the cetyltrimethyl-ammonium bromide (CTAB) procedure of Doyle and Doyle (1987), except that after precipitation with isopropanol and subsequent centrifugation, the DNA pellet was washed with 70% ethanol, dried at 37°C, and resuspended in TRIS-EDTA (TE) buffer. Samples were purified on caesium chloride/ethidium bromide gradients. For *B. heterostemon*, DNA was extracted using a NucleoSpin® Plant kit (Macherey-Nagel, Dueren, Germany). *Rbc*L was amplied with the primers 1F, 636F, 724R, and 1326R (Olmstead et al. 1992, Fay et al. 1998) and *atp*B with the primers 2F, 611F, 766R, and 1494R (Hoot et al. 1995). A 50-µl reaction mix contained 45 µl ReddyMix polymerase chain reaction (PCR) mastermix at 2.5 mM $MgCl_2$ concentration (ABgene, Epsom, Surrey, UK), 1µl of two primers each (10pmol), 1µl template DNA, and 2 µl bovine serum albumin (BSA; 0.4%). Initial denaturation was 3 min at 95°C, followed by one cycle of denaturation for 1.5 min at 95°C, annealing for 1 min at 45°C and extension for 1 min at 72°C, followed by 36 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 48°C, and extension for 1 min at 72°C;

Table 1. Taxa and classification for the material used in this study. Family circumscription follows APG II (2003). All sequences are available from GenBank; new sequences have the accession numbers DQ408665–DQ408667 and EF431913–EF431915 (http://www.ncbi.nlm.nih.gov/)

Order/Family	Species	GenBank no. *rbc*L	GenBank no. *atp*B
Sapindales			
Meliaceae	*Aglaia elaeagnoidea* Benth.	AY128209	
	Aphanamixis polystachya (Wall.) R.N. Parker	AY128213	
	Azadirachta indica A. Juss.	AY128215	
	Capuronianthus mahafalensis Leroy	AY128218	
	Carapa guianensis Aubl.	AY128219	
	Cedrela odorata L.	AY128220	
	Chisocheton macrophyllus King	AY128221	
	Chukrasia tabularis A. Juss.	AY128223	
	Cipadessa baccifera Miq.	AY128224	
	Dysoxylum gaudichaudianum (A. Juss.) Miq.	AY128227	
	Ekebergia capensis Sparrm.	AY128228	
	Guarea glabra Vahl	AY128229	
	Khaya anthotheca C.DC.	AY128231	
	Lansium domesticum Correa	AY128232	
	Lovoa swynnertonii E.G. Baker	AY128233	
	Melia azedarach L.	AY128234	
	Munronia pinnata (Wall.) Theob.	AY128236	
	Naregamia alata Wight & Arn.	DQ238059	
	Nymania capensis Lindb.	AY128238	AF066855
	Quivisianthe papinae Baill.	AY128239	
	Ruagea pubescens Karst.	DQ238057	
	Sandoricum cf. koetjape (Burm. f.) Merr.	DQ238068	
	Swietenia macrophylla King	AY128241	AJ235616
	Toona sp.	AY128243	
	Trichilia emetica Vahl	AY128244	AJ235629
	Turraea sericea Sm.	AY128245	
	Vavaea amicorum Benth.	DQ238066/67	
Simaroubaceae	*Ailanthus altissima* (Mill.) Swingle	AY128247	AF035895
	Quassia amara L.	AY128250	
	Simarouba glauca DC.	AY128252	AF235602
	Soulamea soulameoides (A. Gray) Noot.	U38923	
Rutaceae	*Adenandra uniflora* Willd.	AF066803	AF066832
	Aegle marmelos (L.) Correa ex Roxb.	AF066811	AF066839
	Atalantia ceylanica (Arn.) Oliver	AF066812	AF066840
	Calodendrum capensis Thunb.	AF066805	AF066834
	Casimiroa edulis La Llave	AF066808	AF066837
	Chloroxylon swietenia DC.	AF066802	AF066831
	Choisya mollis Standley	AF066800	AF066829
	Chorilaena quercifolia Endl.	AF066810	AF066838
	Citrus glauca (Lindl.) I.H. Burkill	AF066819	
	Citrus japonica Swingle	AF066799	
	Citrus trifoliata L.	AJ235806	

Table 1. (Continued)

Order/Family	Species	GenBank no. *rbc*L	GenBank no. *atp*B
	Clausena excavata Burm. f.	AF066813	AF066841
	Cneorum pulverulentum Vent.	U38858	AF066828
	Correa pulchella J.B. Mackay ex Sweet	AF066816	AF066844
	Dictamnus sp.	AF066801	AF066830
	Diplolaena dampieri Desf.	AF066807	AF066836
	Eriostemon brevifolius A. Cunn. ex Endl.	AF156882	AF156882
	Flindersia australis R.Br.	U38861	
	Glycosmis pentaphylla (Retz.) DC.	AF066820	
	Harrisonia perforata (Blanco) Merr.	U38863	
	Lunasia amara Blanco	AF066814	AF066842
	Melicope ternata J.R. Forst. & G. Forst.	AF116271	AF066826
	Phebalium woombye (Bailey) Domin	AF066822	AF066852
	Phellodendron amurense Rupr.	AF066804	AF066833
	Pilocarpus pennatifolius Lem.	AF066809	AF066825
	Pleiospermium alatum Wight & Arn.	AF066821	AF066850
	Ptaeroxylon obliquum (Thunb.) Radlk.	AF123276	AF066848
	Ruta graveolens L.	AY128251	AF035913
	Sarcomelicope simplicifolia (Endl.) T.G. Hartley	AF066817	AF066845
	Severinia buxifolia (Poir.) Tenore	AF066806	AF066835
	Skimmia anquetilia N.P. Taylor & H.K. Airy Shaw	AF066818	AF066846
	Spathelia excelsa Krause	AF066798	AF066854
	Zanthoxylum monophyllum (Lam.) P. Wilson	ZMU39282	
	Zanthoxylum sp.		AF066843
Burseraceae	*Bursera inaguensis* Britton	L01890	AF035899
	Canarium ovatum Engl.	U38856	
Anacardiaceae	*Blepharocarya depauperata* Specht	U38928	
	Buchanania latifolia Roxb.	U39275	
	Cyrtocarpa procera Knuth	U39272	
	Mangifera indica L.	U39269	
	Pistacia vera L.	AJ235786	AJ132282
	Rhus copallina L.	U00440	AF035912
	Schinus molle L.	U39270	AF035914
	Spondias cytherea Sonn.	U39274	
	Tapirira mexicana Marchand	U39273	
Kirkiaceae	*Kirkia wilmsii* Engl.	U38857	
Sapindaceae	*Acer saccharum* L.	L01881	AF035893
	Aesculus pavia Castigl.	U39277	AF035894
	Allophyllus cobbe (L.) Reusch	AY128248	
	Cupaniopsis anacardioides (A. Rich.) Radlk.	L13182	AF035903
	Harpullia arborea (Blanco) Radlk.	AY128249	
	Koelreuteria paniculata Laxm.	U39283	AJ235513
Biebersteiniaceae	*Biebersteinia heterostemon* Maxim.	DQ408667	EF431915

Table 1. (Continued)

	Biebersteinia multifida DC.	DQ408665	EF431913
	Biebersteinia odora Stephan	DQ408666	EF431914
	Biebersteinia orphanidis Boiss.	AF035920	AF035921
Nitrariaceae	*Malacocarpus crithmifolius* (Retz.) Fisch. & C.A. Mey.	U39280	
	Peganum harmala L.	U39279	
	Tetradiclis tenella Litwinow	AJ403009	
	Nitraria retusa (Forssk.) Asch	U39278	
Malvales			
Bixaceae	*Bixa orellana* L.	Y15139	AF035897
Malvaceae	*Bombax buonopozense* P. Beauv.	AF022118	AJ233051
	Gossypium hirsutum Cav.	M77700	AJ233063
	Ochroma pyramidale (Cav. ex Lam.) Urb.	AJ233118	AF035910
	Thespesia populnea Sol. ex Correa	L01961	
Cistaceae	*Cistus revolii* H.J.Coste & Soulie	Y15140	AF035902
	Helianthemum grandiflorum DC.	Y15141	AF035907
Dipterocarpaceae	*Anisoptera marginata* Korth.	Y15144	AF035918
Muntingiaceae	*Muntingera calabra* L.	Y15146	AJ233068
Brassicales			
Brassicaceae	*Capparis spinosa* L.	AY167985	AF035900
Tovariaceae	*Tovaria pendula* Ruiz & Pav.	M95758	
Limnanthaceae	*Floerkea prsoerpinicoides* Willd.	L12679	AF035904
Bataceae	*Batis maritima* L.	L22438	AF209538
Caricaceae	*Carica papaya* L.	M95671	AF035901
Tropaeolaceae	*Tropaeolum majus* L.	L14706	AF035917
Oxalidales			
Cunoniaceae	*Eucryphia lucida* (Labill.) Baill.	L01918	AF209584
Geraniales			
Geraniaceae	*Erodium variabile* A.C. Leslie	L14694	
	Geranium cinereum Boiss. & Reut. Ex Willk. & Lange	L14695	AF093373
	Hypseocharis sp.	L14699	
	Monsonia emarginata L'Hér.	L14701	AF209632
	Pelargonium exstipulatum L'Hér.	L14704	
	Pelargonium cotyledonis L'Hér.		AF035911
Melianthaceae	*Greyia radlkoferi* Szyszyl.	L11185	AF209594
Saxifragales	*Heuchera micrantha* Douglas	L01925	AF093399
(outgroup)	*Itea virginia* L.	L11188	AF093383

final extension for 10 min at 72°C. Alternatively, the *rbc*L gene was amplified in two overlapping fragments, with an initial denaturation for 2 min at 94°C, followed by 28 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 48°C and extension for 1 min at 72°C; final extension for 7 min at 72°C. PCR products were cleaned using a NucleoSpin® Extract II kit (Macherey-Nagel, Dueren, Germany). Sequencing reactions were run on an ABI 3100 capillary sequencer or a CEQ™ 8800 Genetic Analysis System (Beckman Coulter, Krefeld, Germany), following the manufacturer's protocols.

Sequence editing and alignment. Editing and assembly of the complementary strands were carried out with Autoassembler version 1.4.0 (ABI), SeqMan™ II version 5.07 (Lasergene, DNASTAR, Inc., Madison, WI, USA), and DNA STRIDER version 1.2 (Christian Marck, CEA - Commissariat à L'énergie atomique/Saclay, France). Alignment of *rbc*L and *atp*B sequences was performed by eye. Matrices and trees have been deposited in TreeBASE (Sanderson et al. 1994) and new sequences in GenBank (http://www.ncbi.nlm.nih.gov/).

Phylogenetic analyses. Individual and combined maximum parsimony (MP) analyses of the *rbc*L and *atp*B datasets were performed using PAUP*4.0b10 (Swofford 2002). Substitutions at each nucleotide position were treated as independent, unordered, multi-state characters of equal weight (Fitch parsimony; Fitch 1971). Heuristic searches were performed using 1000 random additions of taxa, DELTRAN character state optimization, tree bisection-reconnection (TBR) branch swapping, and the option MulTrees (keeping multiple, shortest trees). For tree searches from *rbc*L, we held only 10 trees per replicate (Salamin et al. 2003). Following the 1000 replicates, we used the shortest trees found as starting trees for a swapping-to-completion search (but with a tree limit of 10000). Robustness of clades was estimated using bootstrapping (Felsenstein 1985) with 1000 replicates, using simple sequence addition, TBR branch swapping, and MulTrees, again holding 10 trees per replicate. We consider 75–84% bootstrap values moderate support and 85–100% strong support.

Maximum likelihood (ML) analyses were performed with RAxML version 2.2.1 (Stamatakis 2006; http://icwww.epfl.ch/~stamatak/index-Dateien/Page443.htm) and Bayesian analyses with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003; http://mrbayes.csit.fsu.edu/). The substitution models employed in these analyses were found using Modeltest version 3.06 (Posada and Crandall 1998; http://darwin.uvigo.es/software/modeltest.html), which indicated the general time reversible model as best fitting our data with a proportion of invariable sites and a gamma shape parameter alpha to model rate heterogeneity (GTR + I + G). For the Bayesian analyses, model parameters were estimated directly during runs, using four simultaneous chains and three million cycles (*rbc*L) or one million cycles (*atp*B, *rbc*L/*atp*B), sampling one tree every 100 generations. Trees that preceded the stabilization of the likelihood value were excluded, and the remaining trees were used to calculate posterior probabilities via the construction of a majority rule consensus tree in PAUP. For the ML searches we employed the GTR + G model, using 25 rate categories (instead of four as used in the Bayesian analyses), this being the only model implemented in RAxML.

Divergence time estimation. A likelihood-ratio test (LRT) rejected the null hypothesis of rate constancy for *rbc*L, and we therefore employed non-parametric rate smoothing (NPRS; Sanderson 1997) as implemented in TreeEdit version 1.0-a4.61 (Rambaut and Charleston 2000; http://evolve.zoo.ox.ac.uk/software.html?id=TreeEdit) or a relaxed Bayesian clock approach as implemented in the *multidivtime* program of Thorne and Kishino (2002; http://statgen.ncsu.edu/thorne/). Standard deviations on the NPRS estimates were calculated by reapplying NPRS to 100 bootstrapped matrices.

The input topology for the Bayesian time estimation was the *rbc*L ML tree for 80 representative Sapindales obtained with RAxML. The only substitution model implemented in *multidivtime* is the F84 + G model, and parameter values under this model were estimated with PAML's baseml version 3.14 (Yang 1997; http://abacus.gene.ucl.ac.uk/software/paml.html). Thorne's program *estbranches* was then used to calculate branch lengths and their variance, given the sequence data (80 *rbc*L sequences of a length of 1387 nt), the model parameter values from PAML, and the specified rooted topology. Branch lengths from *estbranches* became the priors for mcmc searches in *multidivtime* that sought to find the most likely model of rate change (with rate change assumed to be

log-normally distributed), given the topology, time constraints on nodes (below), and a Brownian motion parameter (v) that controls the magnitude of autocorrelation per million years (my) along the descending branches of the tree. Prior gamma distributions on parameters of the relaxed clock model were as follows: the mean and SD of the prior distribution for the root age were set to 100 my, based on fossils (below). The mean and SD of the prior distribution for the ingroup root rate were set to 0.0003 substitutions/site/my by dividing the median of the distances between the ingroup root and the tips by 100 my. The prior and SD for v were set to 0.01, following Thorne's manual's recommendation that the time between root and tips multiplied by v be about 1. Markov chains in *multidivtime* were run for 1 million generations, sampling every 100th generation for a total of 10,000 trees, with a burn-in of 10,000 generations before the first sampling of the Markov chain. To check for convergence, we ran two analyses of different chain lengths. We also tested the effect of the root rate by running one analysis with a rate of 0.0004, one with a rate of 0.0003.

Likelihood ratio tests are very sensitive to rate variation and may overreject the clock (Sanderson 1998). We therefore evaluated the estimates obtained from NPRS and the Bayesian relaxed clock against a strict clock model. Branch lengths were calculated in PAUP under GTR + I + G with the clock assumption enforced, using the same ML topology as used for the relaxed clock.

Constraints and calibrations. Absolute time estimates in the Bayesian approach were obtained by simultaneously constraining nine nodes (numbered 1–9 below), for the NPRS clock and the strict clock we alternatively constrained two nodes (numbers 3 and 4 below; Table 2).

1) The root node of our dataset (i.e. the most recent common ancestor of Geraniales and Oxalidales) was constrained to maximally 137 my old, based on the onset of angiosperm radiation (Hughes 1994, Brenner 1996).
2) The divergence between *Acer* and *Aesculus* was constrained to minimally 63 my old, based on fossil fruits of the sister group of *Acer*, *Dipteronia brownii* from the Late Paleocene of Wyoming (McClain and Manchester 2001; the fruits of *Dipteronia* and *Acer* are distinct among angiosperms and different from each other).
3) The crown group of *Biebersteinia* was constrained to minimally 54.8 my old, based on fossil pollen from the Neomugen Formation of Inner Mongolia (Late Paleocene, 57.0–54.8 million years). This pollen has been assigned to *B. heterostemon* on the basis of the structure of its colpi and pores (Song et al. 2004; W.-M. Wang, Chinese Academy of Sciences, personal communication, 2007).
4) The crown group of Simaroubaceae were constrained to minimally 52 my old, based on fossil fruits of *Ailanthus*, which are distinct in their samaroid mericarps with a centrally placed seed. According to molecular phylogenetic evidence, *Ailanthus* is sister to the rest of Simaroubaceae (Fernando et al. 1995, Chase et al. 1999). The fossil *A. confucii* is thought to be related to the extant *A. altissima* (Corbett and Manchester 2004), which is the species included in our DNA analyses.
5) The crown group of Cedreleae were constrained to minimally 49 my old, based on fruit and seed fossils of *Toona* from the London Clay (Reid and Chandler 1933, DeVore et al. 2005). Since these specimens share morphological features of both modern *Toona* and *Cedrela* (T. D. Pennington, RBGK, personal communication, 2005), we alternatively used these fossils to constrain the minimum age of the stem of Cedreleae.
6) The divergence between *Allophyllus* and *Cupaniopsis* was constrained to minimally 33.7 my old, based on leaves of *Allophyllus* from the Eocene Green River Formation of Colorado (MacGinitie 1969; leaf matching in *Allophyllus* is problematic, however; M. Harrington, personal communication, 2007).
7) The crown group of Trichilieae plus Turraeeae was constrained to minimally 33.7 my old, based on fossil leaves of *Trichilia* from the Eocene Florissant Flora of Colorado (MacGinitie 1953, however, these fossils require further study, S. Manchester, personal communication, 2007).
8) The crown group comprising Guareae and Aglaieae was constrained to minimally 23.8 my old, based on fossil pollen of *Guarea* from the Oligocene San Sebastian Formation in northern Puerto Rico (Graham and Jarzen 1969).
9) The clade uniting *Chisocheton* and *Aglaia* was constrained to minimally 5.3 my old, based on Miocene fossil wood of *Chisocheton*

Table 2. Age estimates for key events in the history of Sapindales i) based on the branch lengths observed in the NPRS tree under the GTR + I + G model using two calibration points: calibration 1 (based on 54.8 my old pollen of *Biebersteinia*), and calibration 2 (based on 52.0 my old fruits of *Ailanthus*); ii) based on the Bayesian approach; and iii) under the assumption of a strict molecular clock using the *Ailanthus* calibration; c.i. = confidence interval; s.d. = standard deviation. Data are compared to estimates by Muellner et al. (2006) and Wikström et al. (2001). "Direct" age estimates are NPRS estimates computed directly from the tree by using the original dataset rather than the bootstrapped matrices. Approach i) is based on 114 ingroup and outgroup sequences, while approaches ii) and iii) are based on a reduced size data set of 80 sequences

Node	i) NPRS (calibration 1)		i) NPRS (calibration 2)		ii) Bayesian	iii) strict clock	Muellner et al. (2006)		Wikström et al. (2001)
	direct	mean (s.d.)	direct	mean (s.d.)	mean (c.i.)		direct	mean (s.d.)	mean (s.d.)
1. Melioideae	79.4	72.2 (15.2)	81.9	81.1 (11.9)	67.3 (53.7–80.0)	77.9	80.9	77.4 (9.7)	-
2. Meliaceae	85.4	77.2 (15.5)	88.1	86.1 (12.5)	73.0 (61.4–83.9)	84.6	87.2	84.6 (11.0)	36 (4.0)
3. Swietenioideae	73.5	66.8 (13.5)	75.8	74.8 (12.5)	63.9 (52.5–76.8)	52.7	77.5	75.1 (11.1)	-
4. *Cedrela*/*Toona*	49.7	47.7 (11.6)	51.2	53.6 (12.5)	39.5 (14.7–57.1)	26.8	53.8	55.5 (11.0)	-
5. Simaroubaceae	50.4	47.5 (11.9)	52	52	61.1 (52.3–76.6)	52	52	52	44 (4.0)
6. Rutaceae	90.5	82.1 (17.3)	93.3	91.3 (12.3)	72.9 (56.5–86.0)	97.3	90.1	90.8 (11.7)	38 (4.0)
7. Burseraceae	50.1	43.3 (11.2)	51.7	48.1 (9.3)	41.4 (20.2–65.2)	56.3	-	-	52 (5.0)
8. Anacardiaceae	74.2	65.2 (14.7)	76.6	72.7 (12.3)	54.8 (36.4–73.6)	86.3	-	-	-
9. Burseraceae/ Anacardiaceae/ Kirkiaceae clade	95.3	83.6 (16.2)	98.3	93.6 (14.7)	74.1 (58.7–86.7)	108.3	-	-	-
10. Burseraceae/ Anacardiaceae/ Kirkiaceae/ Sapindaceae clade	105.4	93.3 (18.4)	108.7	104.2 (14.9)	82.7 (73.1–91.5)	122.2	-	-	-
11. Sapindaceae	66.7	58.8 (12.8)	68.8	65.8 (12.9)	75.5 (65.7–86.3)	76.4	-	-	39 (4.0)
12. Sapindales	119.0	104.9 (20.1)	122.8	117.4 (18.0)	90.5 (82.0–96.9)	132.6	-	-	62 (4.0)
13. *Biebersteinia* clade 1	25.2	30.8 (7.8)	26.0	36.1 (14.0)	32.6 (12.8–55.6)	15.3	-	-	-
14. *Biebersteinia* clade 2	5.0	8.2 (6.6)	5.2	9.6 (9.2)	12.6 (0.6–33.7)	1.9	-	-	-
15. *Biebersteinia* crown group	54.8	54.8	56.5	63.3 (15.0)	61.5 (55.0–76.4)	35.7	-	-	-
16. Nitrariaceae	94.8	86.1 (17.0)	97.8	96.5 (16.6)	57.7 (34.6–82.5)	76.4	-	-	-

(Chisochetonoxylon) from the Tertiary beds of the Birbhum District in West Bengal (Ghosh and Roy 1979).

For absolute ages we relied on the geologic time scale of Palmer and Geissman (1999).

Ancestral area analyses. Besides considering tree topology and the distribution of fossils, we inferred the ancestral area of Biebersteiniaceae with an area-based biogeographic approach (ancestral area analysis; Bremer 1992) and an event-based approach (dispersal vicariance analysis, DIVA; http://www.ebc.uu.se/systzoo/research/diva/diva.html; Ronquist 1996, 1997). Based on the geological history of the relevant regions and the extant distribution of *Biebersteinia*, we defined eight areas (Fig. 1): (A) Eastern Tibetan plateau including the Hengduan Mountains (EAST TIB HENG); (B) Eastern Himalaya (EAST HIM); (C) Western Himalaya, Karakoram, Pamir, Tien Shan, Alatau, and Altai mountain ranges (WEST HIM); (D) Dzungaria and western Tien Shan (DZUN); (E) Semi-arid mountainous regions to the west, including the northern Baluchistan, Turkestanian, part of Turanian and Armeno-Iranian floristic provinces of Takhtajan (1986) (SEM-AR MOUN); (F) Mountains near the southeastern Mediterranean coast (SE MED COAST); (G) Taurus mountains in Asia minor (TAUR MOUN); and (H) Mountains of Peloponnisos in the southern Balkan peninsula (SOUTH BALK PEN).

Results

Phylogeny estimation. Parsimony statistics for all three datasets (*rbc*L, *atp*B, *rbc*L/*atp*B) are summarized in Table 3. To the extent that taxon sampling overlapped, topologies of trees obtained with parsimony, Bayesian inference, and Maximum Likelihood were nearly identical (compare Figs. 2–5). Figure 2 shows the Bayesian tree obtained from *rbc*L sequences of 112 ingroup and two outgroup taxa, based on 30000 total trees and a burn-in of 2880 trees Figure 5 shows the Bayesian tree obtained from combined *rbc*L and *atp*B sequences of 62 ingroup and two outgroup taxa, based on 10000 total trees and a burn-in of 1060 trees.

Divergence time estimation. Table 2 summarizes all divergence time estimates (and their errors or confidence intervals) obtained with the different clock approaches, and Figs. 3 and 4 show NPRS and Bayesian chronograms. NPRS analyses using a 54.8-my minimum age

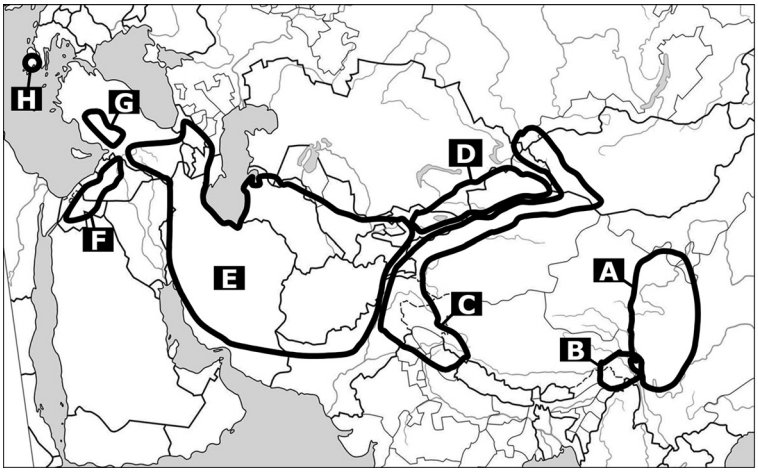


Fig. 1. Areas defined for the ancestral area analyses, based on the geological history of the area of occurrence and the extant distribution of the four species of *Biebersteinia*. The areas are: *A* Eastern Tibetan plateau including the Hengduan Mountains; *B* Eastern Himalaya; *C* Western Himalaya, Karakoram, Pamir, Tien Shan, Alatau, and Altai mountain ranges; *D* Dzungaria and western Tien Shan; *E* Semi-arid mountainous regions to the west, including the northern Baluchistan, Turkestanian, part of Turanian and Armeno-Iranian floristic provinces of Takhtajan (1986); *F* Mountains near the southeastern Mediterranean coast; *G* Taurus mountains in Asia minor; *H* Mountains of Peloponnisos in the southern Balkan peninsula

Table 3. Statistics for the parsimony analyses

Data set	*rbc*L	*atp*B	*rbc*L/*atp*B
Number of taxa	114	64	64
Characters included	1387	1491	2878
Variable characters	557	545	1030
Parsimony-informative characters	391	362	703
Length of shortest tree (no. of steps)	2180	1507	3059
Shortest trees	>10000	510	122
Consistency index	0.31	0.42	0.39
Retention index	0.70	0.67	0.67

for the *Biebersteinia* crown group (Fig. 3) and a 52-my minimum age for *Ailanthus* yielded estimates for Meliaceae and Rutaceae similar to previous estimates based on a smaller set of *rbcL* sequences (Muellner et al. 2006). Age estimates by Wikström et al. (2001) for these families are much lower (Table 2). Bayesian and strict clock approaches gave similar results to those obtained with NPRS, except for a few cases, such as the age of Sapindales and Nitrariaceae (see Table 2). With *Ailanthus* used for calibration, the NPRS-based age estimate for the *Biebersteinia* crown group (56.5 my; Table 2) was slightly older than the oldest *Biebersteinia* pollen (54.8 my); with *Biebersteinia* used for calibration, *Ailanthus* was estimated as slightly younger (50.4 my) than its oldest fossils (52 my). The Bayesian clock yielded an even older estimate (61.5 my) for the *Biebersteinia* crown group, while the strict clock yielded a younger estimate (35.7 my; Table 2).

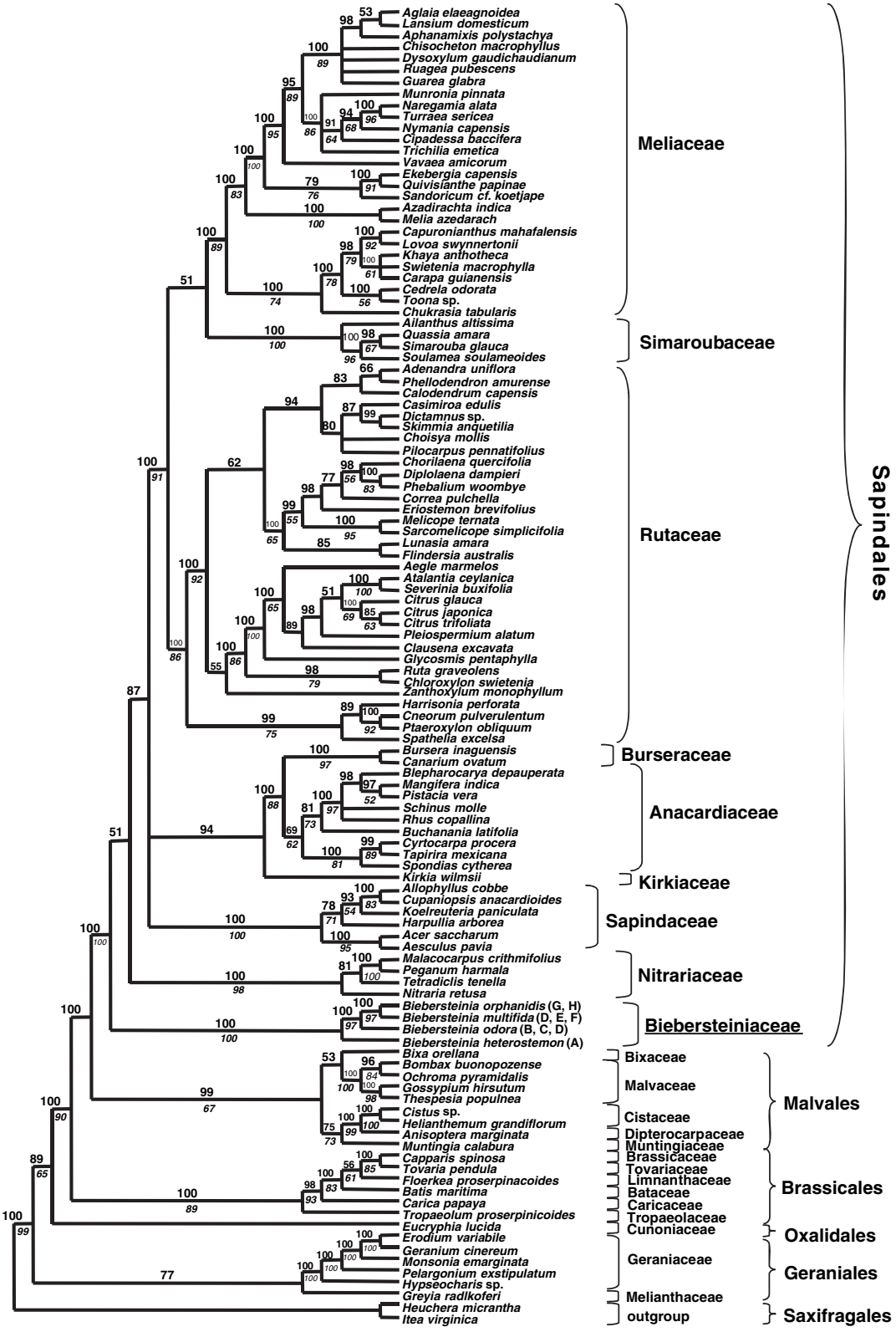
Ancestral area analyses. Table 4 summarizes the results of the ancestral area analysis that used the approach of Bremer (1992). A high gain/loss (G/L) ratio indicates a high probability that a geographic region was part of the ancestral area. Based on this measure, the eastern Tibetan plateau, the Hengduan Mountains (area A), Dzungaria, and western (fide legend) Tien Shan (area D) likely were part of the ancestral area of Biebersteiniaceae. In DIVA, we used the "maxareas" option to impose a constraint on the number of regions allowed in ancestral distributions. With this constraint, the ancestral region inferred was the same as with the Bremer analysis.

Discussion

Our study (1) confirms that *Biebersteinia* is a member of Sapindales; (2) provides evidence for the monophyly of *Biebersteinia*; (3) suggests that the family crown group diversified in the Oligocene and Miocene; and (4) makes it likely that an area comprising the eastern Tibetan plateau, including the Hengduan Mountains, Dzungaria, Tien Shan, and eastern and western Himalayan and Altai mountain ranges as well as Inner Mongolia was the ancestral area of *Biebersteinia*. (5) In addition, our age estimates for Sapindales suggest that previous studies may have underestimated ages for some of the family crown groups.

***Biebersteinia* – phylogenetic position among Eurosid II orders.** Although the molecular data of Bakker et al. (1998) provided evidence of a sapindalean rather than geranialean affinity of *Biebersteinia*, their analysis included

Fig. 2. Bayesian tree obtained from *rbc*L sequences of 112 ingroup and two outgroup taxa. Capital letters A-H after *Biebersteinia* species names refer to the areas defined in the biogeographical analysis and shown in Fig. 1. Numbers above branches are Bayesian posterior probabilities, numbers below branches are bootstrap values (1000 replicates; italicized). Families and orders after APG II (2003)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Chisocheton macrophyllus
Dysoxylum gaudichaudianum
Ruagea pubescens
Guarea glabra
Munronia pinnata
Naregamia alata
Turraea sericea
Nymania capensis
Cipadessa baccifera
Trichilia emetica
Vavaea amicorum
Ekebergia capensis
Quivisianthe papinae
Sandoricum cf. koetjape
Azadirachta indica
Melia azedarach
Capuronianthus mahafalensis
Lovoa swynnertonii
Khaya anthotheca
Swietenia macrophylla
Carapa guianensis
Cedrela odorata
Toona sp.
Chukrasia tabularis
Meliaceae
Ailanthus altissima
Quassia amara
Simarouba glauca
Soulamea soulameoides
Simaroubaceae
Adenandra uniflora
Phellodendron amurense
Calodendrum capensis
Casimiroa edulis
Dictamnus sp.
Skimmia anquetilia
Choisya mollis
Pilocarpus pennatifolius
Chorilaena quercifolia
Diplolaena dampieri
Phebalium woombye
Correa pulchella
Eriostemon brevifolius
Melicope ternata
Sarcomelicope simplicifolia
Lunasia amara
Flindersia australis
Aegle marmelos
Atalantia ceylanica
Severinia buxifolia
Citrus glauca
Citrus japonica
Citrus trifoliata
Pleiospermium alatum
Clausena excavata
Glycosmis pentaphylla
Ruta graveolens
Chloroxylon swietenia
Zanthoxylum monophyllum
Harrisonia perforata
Cneorum pulverulentum
Ptaeroxylon obliquum
Spathelia excelsa
Rutaceae
Bursera inaguensis
Canarium ovatum
Burseraceae
Blepharocarya depauperata
Mangifera indica
Pistacia vera
Schinus molle
Rhus copallina
Buchanania latifolia
Cyrtocarpa procera
Tapirira mexicana
Spondias cytherea
Anacardiaceae
Kirkia wilmsii
Kirkiaceae
Allophyllus cobbe
Cupaniopsis anacardioides
Koelreuteria paniculata
Harpullia arborea
Acer saccharum
Aesculus pavia
Sapindaceae
Malacocarpus crithmifolius
Peganum harmala
Tetradiclis tenella
Nitraria retusa
Nitrariaceae
Biebersteinia orphanidis (G, H)
Biebersteinia multifida (D, E, F)
Biebersteinia odora (B, C, D)
Biebersteinia heterostemon (A)
Biebersteiniaceae
Sapindales
Bixa orellana
Bixaceae
Bombax buonopozense
Ochroma pyramidalis
Gossypium hirsutum
Thespesia populnea
Malvaceae
Cistus sp.
Helianthemum grandiflorum
Cistaceae
Anisoptera marginata
Dipterocarpaceae
Muntingia calabura
Muntingiaceae
Malvales
Capparis spinosa
Brassicaceae
Tovaria pendula
Tovariaceae
Floerkea proserpinacoides
Limnanthaceae
Batis maritima
Bataceae
Carica papaya
Caricaceae
Tropaeolum proserpinicoides
Tropaeolaceae
Brassicales
Eucryphia lucida
Cunoniaceae
Oxalidales
Erodium variabile
Geranium cinereum
Monsonia emarginata
Pelargonium exstipulatum
Geraniaceae
Geraniales
Hypseocharis sp.
Greyia radlkoferi
Melianthaceae
Heuchera micrantha
Itea virginica
outgroup
Saxifragales

Fig. 3. NPRS chronogram based on a parsimony tree obtained from the *rbcL* data set used in Fig. 2 and calibrated with the *Biebersteinia* crown group set to 54.8 my. Nodes labelled 1–16 refer to locations in the tree for which age estimates are summarized in Table 2. **A**, **B** Fruits of *Ailanthus confucii*, Fossil Butte, Wyoming, USA, Green River Formation, Early Eocene, c. 52 my (Corbett and Manchester 2004). **C**, **D** Pollen of *Biebersteinia heterostemon*, Neomugen Formation, Inner Mongolia, China, Late Paleocene, c. 54.8 my (Song et al. 2004). Numbers along the bars indicate million years. *J* = Jurassic, *C* = Cretaceous, *Pa* = Paleocene, *E* = Eocene, *O* = Oligocene, *M* = Miocene, *P* = Pliocene to present

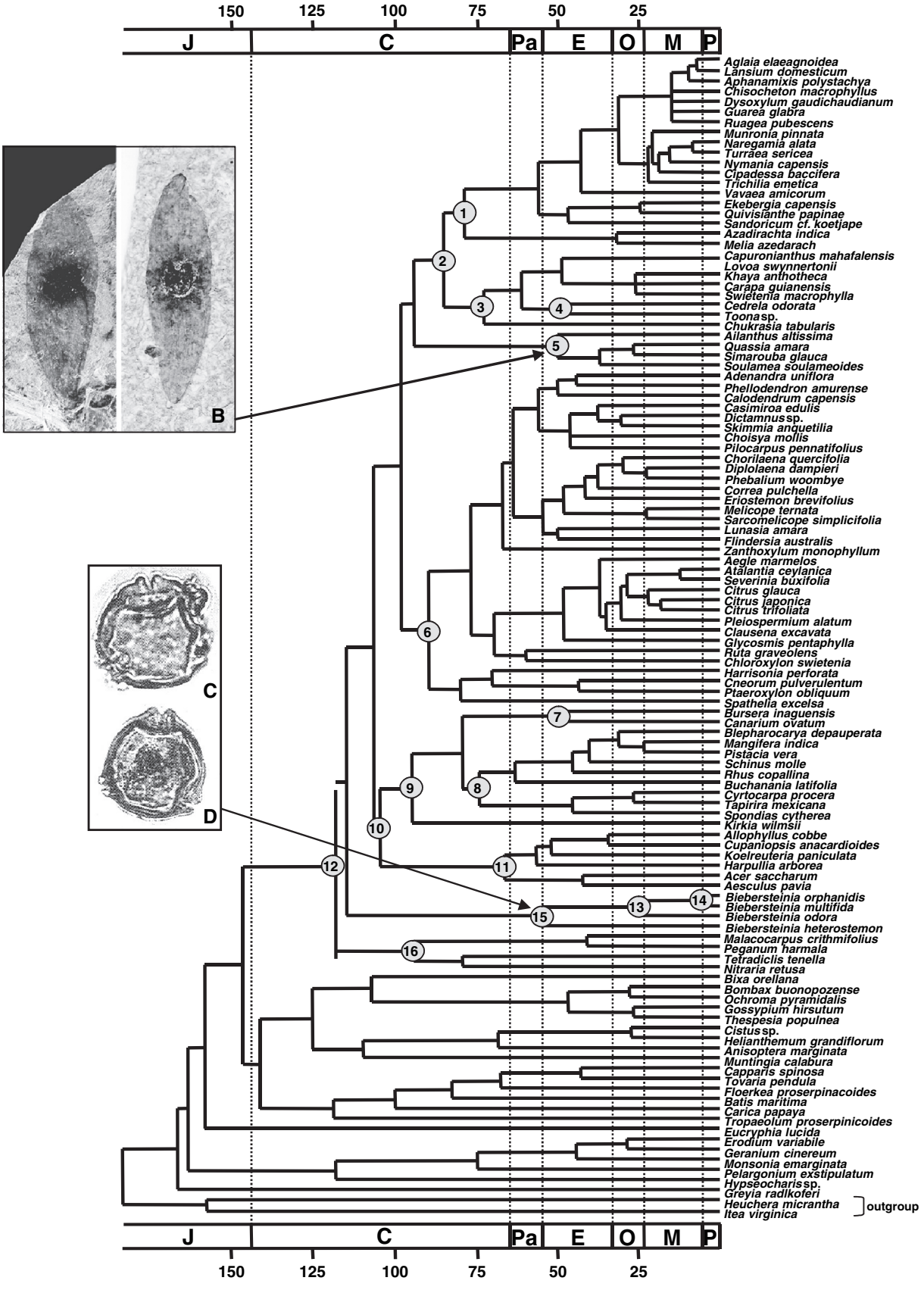
Table 4. Estimation of the ancestral areas for *Biebersteinia* (Sapindales) applying Bremer's (1992) ancestral area analysis. *G* = number of implied gains under forward Camin-Sokal parsimony. *L* = number of implied losses under reverse Camin-Sokal parsimony

Areas	*G*	*L*	*G*/*L*
Area A: EAST TIB HENG	1	1	1.00
Area D: DZUN	2	2	1.00
Area B: EAST HIM	1	2	0.50
Area C: WEST HIM	1	2	0.50
Area E: SEM-AR MOUN	1	3	0.33
Area F: SE MED COAST	1	3	0.33
Area G: TAUR MOUN	1	3	0.33
Area H: SOUTH BALK PEN	1	3	0.33

only *B. orphanidis* (and 27 genera of Sapindales compared to 85 included here). Our study now provides strong support for the monophyly of *Biebersteinia* and its inclusion in Sapindales (with generally 100 bootstrap support and posterior probability, PP; Figs. 2 and 5). Based on the combined two genes, Bayesian and ML analyses weakly suggest that *Biebersteinia* may be sister to the remainder of Sapindales (97 PP, Fig. 5).

The placement of *Biebersteinia* in Sapindales, rather than Geraniales, agrees with the flavonoid composition of *B. orphanidis*, which resembles that of Rutaceae in certain uncommon methylated flavones, including sudachitin (Greenham et al. 2001). The flavonoid profile of *B. orphanidis* differs from the profiles of the central Asian species *B. odora* and *B. heterostemon* in containing six closely related flavone methyl ethers, with dihydroxy, dimethoxy A-ring substitution (Greenham et al. 2001). Also, fatty acids in *B. orphanidis* leaves are of the $C_{18:3}$ type, a type not found in Geraniacae, but present in Sapindaceae (Tzakou et al. 2001). An investigation of the flower structure of *Biebersteinia*, in particular the type of nectaries, an important distinguishing feature of Sapindales, is currently in progress (Smets et al., in preparation).

Biogeography of Biebersteiniaceae. Our results imply that the eastern Tibetan plateau including the Hengduan Mountains, Dzungaria and western Tien Shan were part of the ancestral area of Biebersteiniaceae, and that the family diversified in the Oligocene and Miocene (Figs. 3 and 4, Tables 2 and 4). Both ancestral area analyses and the topologies of our trees (Figs. 2–5) suggest that early-branching *Biebersteinia* clades occurred in the eastern and western Himalaya, Karakoram, Pamir, Tien Shan, Alatau, and Altai mountain ranges. The Late Paleocene pollen fossils of *B. heterostemon* in the Neomugen Formation may indicate that Inner Mongolia was occupied even earlier. *Biebersteinia* then seems to have extended its range westwards to the mountains of Peloponnisos in the southern Balkan Peninsula. The sister species *B. multifida* and *B. orphanidis* have adjacent geographic ranges that meet where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Aglaia elaeagnoidea
Lansium domesticum
Aphanamixis polystachya
Chisocheton macrophyllus
Dysoxylum gaudichaudianum
Guarea glabra
Ruagea pubescens
Munronia pinnata
Naregamia alata
Turraea sericea
Nymania capensis
Cipadessa baccifera
Trichilia emetica
Vavaea amicorum
Ekebergia capensis
Quivisianthe papinae
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Azadirachta indica
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Capuronianthus mahafalensis
Lovoa swynnertonii
Khaya anthotheca
Carapa guianensis
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Atalantia ceylanica
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Citrus trifoliata
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Ruta graveolens
Chloroxylon swietenia
Harrisonia perforata
Cneorum pulverulentum
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Pistacia vera
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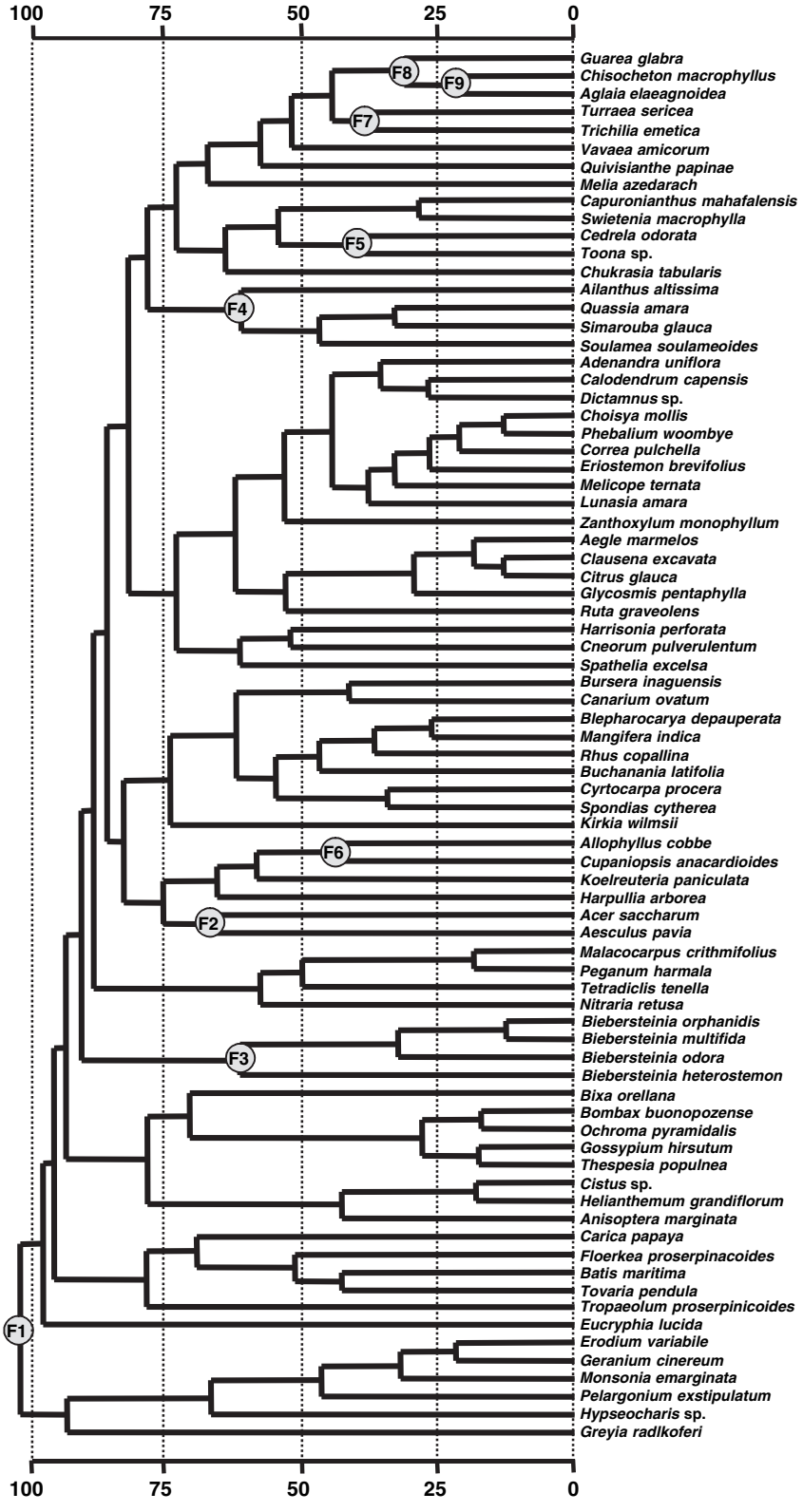
Rhus copallina
Buchanania latifolia
Cyrtocarpa procera
Tapirira mexicana
Spondias cytherea
Kirkia wilmsii
Allophyllus cobbe
Cupaniopsis anacardioides
Koelreuteria paniculata
Harpullia arborea
Acer saccharum
Aesculus pavia
Biebersteinia orphanidis
Biebersteinia multifida
Biebersteinia odora
Biebersteinia heterostemon
Malacocarpus crithmifolius
Peganum harmala
Tetradiclis tenella
Nitraria retusa
Bixa orellana
Bombax buonopozense
Ochroma pyramidalis
Gossypium hirsutum
Thespesia populnea
Cistus sp.
Helianthemum grandiflorum
Anisoptera marginata
Muntingia calabura
Capparis spinosa
Tovaria pendula
Floerkea proserpinacoides
Batis maritima
Carica papaya
Tropaeolum proserpinicoides
Eucryphia lucida
Erodium variabile
Geranium cinereum
Monsonia emarginata
Pelargonium exstipulatum
Hypseocharis sp.
Greyia radlkoferi
Heuchera micrantha
Itea virginica
outgroup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Chisocheton macrophyllus
Aglaia elaeagnoidea
Turraea sericea
Trichilia emetica
Vavaea amicorum
Quivisianthe papinae
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Tropaeolum proserpinicoides
Eucryphia lucida
Erodium variabile
Geranium cinereum
Monsonia emarginata
Pelargonium exstipulatum
Hypseocharis sp.
Greyia radlkoferi
100
75
50
25
0

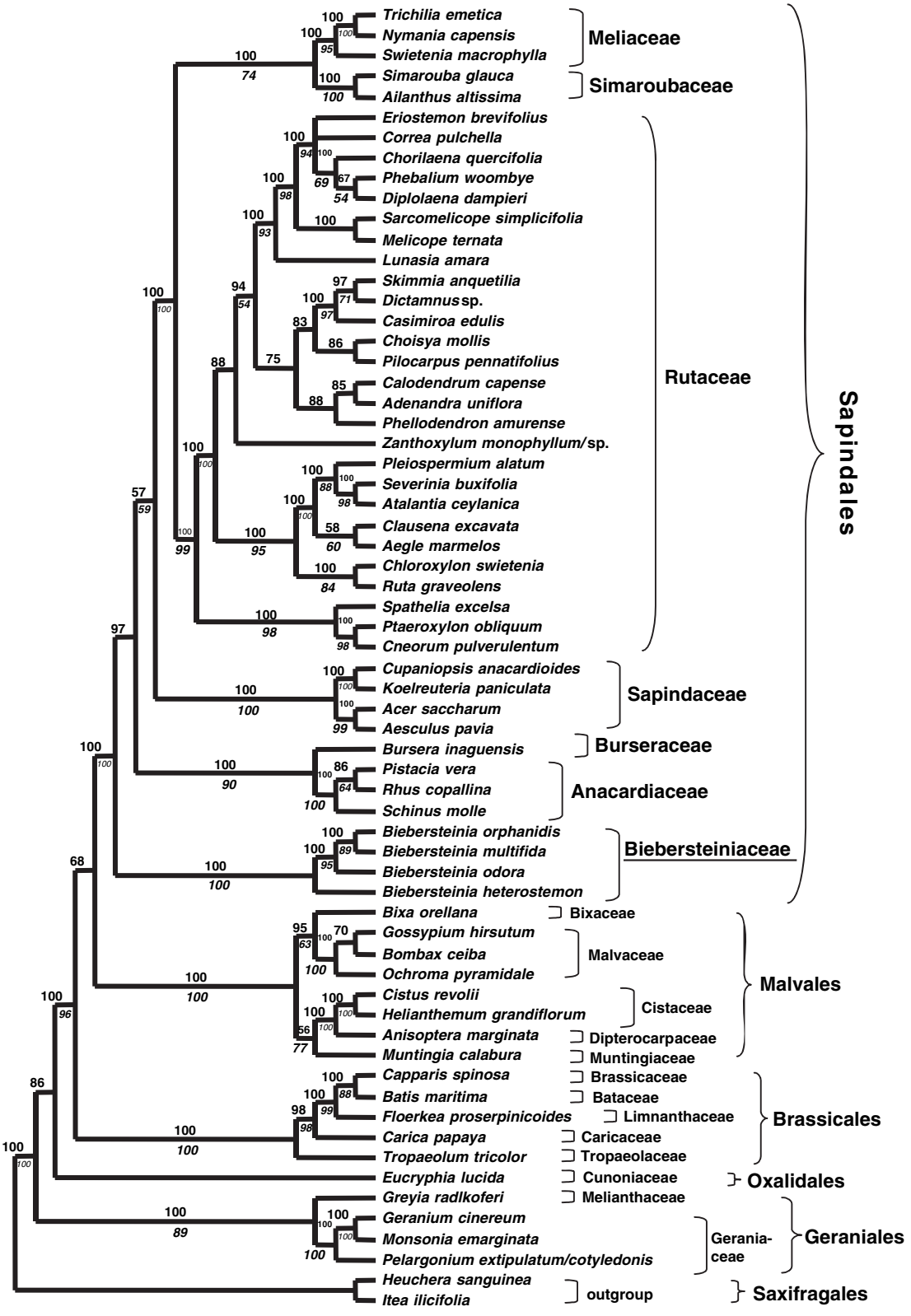
Fig. 4. Bayesian chronogram based on a maximum likelihood tree obtained from *rbc*L sequences of 78 ingroup and two outgroup taxa. Nodes labelled F1-F9 refer to the position of fossils used to constrain the relaxed clock (see text for details). Numbers along the bars indicate million years

the Irano-Turanian floristic province changes to the Mediterranean province (Takhtajan 1986). While all *Biebersteinia* species are perennials, only these two have tuberous rhizomes, while the ancestral condition appears to be scarcely thickened roots. *Biebersteinia odora* differs from the other three species in being adapted to alpine conditions, frequently growing above 4500 m near glaciers. The fruits of the four species of *Biebersteinia* differ in the way they are attached to the receptacle, but without knowing the fruit morphology of the closest relatives the evolutionary direction of character change cannot yet be inferred.

The divergence time estimates for the crown group of Biebersteiniaceae obtained with NPRS (Table 2) are in good agreement with the *Biebersteinia* pollen fossil (when this is not used as a constraint). The Biebersteiniaceae stem lineage appears to date back at least to the Late Paleocene, if not the Early Paleocene (Table 2), thus predating the collision of India with southern Asia and the uplift of large mountain chains and plateaus (Barron and Harrison 1980, Summerfield 1991, Lee and Lawver 1995, McLoughlin 2001). Further diversification then took place in the Oligocene and Miocene. Given the long stem lineage and the small number of extant species, it is extremely likely that *Biebersteinia* has suffered much extinction. However, it is precisely the small size of the clade that prevents a lineage-through-time analysis, which might otherwise permit estimating levels of extinction.

The ranges of the four species of *Biebersteinia* exhibit several disjunctions. The Mediterranean species *B. orphanidis* is disjunct across the Aegean Sea, in mountains of southern Greece and Turkey. The alpine species *B. odora* (above) is known from the Karakorum Mts., Pamir Mts., Ala Tau, the Tien Shan and the Altai Mts. to the north, but has not been collected in Nepal (Mark Watson, Royal Botanic Garden Edinburgh, personal communication, 2006) nor is it known from Bhutan (Grierson and Long 1987). The species re-appears in the eastern Himalaya, in the region of Shugden Gompa, where Kingdon-Ward collected it in 1933 (deposited in the herbarium BM, seen by DV). The range of *B. heterostemon* extends from the eastern and north-eastern part of the Tibetan plateau south to the Hengduan Mountains, to the east and to the north of the Brahmaputra river, which separates it from *B. odora* (except for the apparently isolated population of the latter near Shugden Gompa). The Brahmaputra, known as the Yarlong Tsangpo in its upper reaches, also is a biogeographic barrier in other plant groups, e.g. in *Roscoea* (Ngamriabsakul et al. 2000), Morinaceae (Bell et al. 2003), and *Pleione* (Gravendeel et al. 2005), as well as for elephants (Vidya et al. 2005).

Sapindalean clades ranked as families – much older than previously assumed. To provide an "initial hypothesis of angiosperm diversification times," Wikström et al. (2001) used a tree from a three-gene phylogenetic analysis by Soltis et al. (1999, 2000) that included 560 angiosperms and seven outgroup taxa, representing ca. 75% of the angiosperm families (APG 1998), and applied NPRS with a single fossil-based constraint, namely the divergence of Fagales from Cucurbitales based on the occurrence of *Protofagacea* and *Antiquacupula* in the Campanian and Late Santonian of Georgia. Their dataset included five genera of Sapindaceae, one genus (*Ailanthus*) of Simaroubaceae, two of Meliaceae, three of Rutaceae, and one genus each of Burseraceae (*Bursera*) and Anacardiaceae (*Schinus*). Taxon sampling affects age estimates, with sparse sampling leading to


Trichilia emetica
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Chloroxylon swietenia
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Spathelia excelsa
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Rhus copallina
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Biebersteinia heterostemon
Biebersteiniaceae
Sapindales
Bixa orellana
Bixaceae
Gossypium hirsutum
Bombax ceiba
Ochroma pyramidale
Malvaceae
Cistus revolii
Helianthemum grandiflorum
Cistaceae
Anisoptera marginata
Dipterocarpaceae
Muntingia calabura
Muntingiaceae
Malvales
Capparis spinosa
Brassicaceae
Batis maritima
Bataceae
Floerkea proserpinicoides
Limnanthaceae
Carica papaya
Caricaceae
Tropaeolum tricolor
Tropaeolaceae
Brassicales
Eucryphia lucida
Cunoniaceae
Oxalidales
Greyia radlkoferi
Melianthaceae
Geranium cinereum
Monsonia emarginata
Pelargonium extipulatum/cotyledonis
Geraniaceae
Geraniales
Heuchera sanguinea
Itea ilicifolia
outgroup
Saxifragales

Fig. 5. Bayesian tree obtained from combined *rbc*L and *atp*B sequences of 62 ingroup and two outgroup taxa. Numbers above branches are Bayesian posterior probabilities, numbers below branches are bootstrap values (1000 replicates; italicized). Families and orders after APG II (2003)

younger ages than denser sampling (Sanderson et al. 2004, Linder et al. 2005). This is borne out by a comparison of the ages obtained here with a sample of 85 genera with those in Wikström et al. (2001) with a sample of 13 genera. Estimates differ by 50% and more, for example, the minimum age of the Meliaceae crown group in Wikström et al. was 36 my, while we obtained an age of ca. 85 my (likewise for Rutaceae 38 my versus ca. 90 my; Sapindales 62 my versus ca. 120 my; Table 2). For Burseraceae, by contrast, all studies so far yield similar ages. Wikström et al. (2001) estimated the Burseraceae crown group age as 52 ± 5 my, based on three genes and one species of Burseraceae (including representatives of six families of Sapindales); Weeks et al. (2005) estimated it as 60 ± 1.9 my, based on two genes from 50 species representing 13 genera of Burseraceae (including two families of Sapindales); and we obtained ages of 56.3 my (strict clock), 48.1 ± 9.3 my (NPRS), or 41.4 my (c.i. = 20.2–65.2; Bayesian clock), based on one gene and two species from as many genera (including representatives of all nine families of Sapindales). These and other molecular dating results highlight the need for dense taxon sampling as well as the importance of checking results against other types of evidence if time estimates are to be trusted. Sapindales, with their rich fossil record, provide an exceptional system for comparing different clock approaches because they permit such critical cross-validation.

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