

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 *rbcL* and *atpB* 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 *rbcL* and *atpB* 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. *RbcL* 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 *atpB* dataset, 61 sequences were downloaded from GenBank. Besides the four species of Biebersteiniaceae, our *rbcL* 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). *RbcL* was amplified with the primers 1F, 636F, 724R, and 1326R (Olmstead et al. 1992, Fay et al. 1998) and *atpB* with the primers 2F, 611F, 766R, and 1494R (Hoot et al. 1995). A 50- μ l reaction mix contained 45 μ l ReddyMix polymerase chain reaction (PCR) mastermix at 2.5 mM MgCl₂ concentration (ABgene, Epsom, Surrey, UK), 1 μ l of two primers each (10 pmol), 1 μ l template DNA, and 2 μ l 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. <i>rbcL</i>	GenBank no. <i>atpB</i>
Sapindales			
Meliaceae	<i>Aglaia elaeagnoidea</i> Benth.	AY128209	
	<i>Aphanamixis polystachya</i> (Wall.) R.N. Parker	AY128213	
	<i>Azadirachta indica</i> A. Juss.	AY128215	
	<i>Capuronianthus mahafalensis</i> Leroy	AY128218	
	<i>Carapa guianensis</i> Aubl.	AY128219	
	<i>Cedrela odorata</i> L.	AY128220	
	<i>Chisocheton macrophyllus</i> King	AY128221	
	<i>Chukrasia tabularis</i> A. Juss.	AY128223	
	<i>Cipadessa baccifera</i> Miq.	AY128224	
	<i>Dysoxylum gaudichaudianum</i> (A. Juss.) Miq.	AY128227	
	<i>Ekebergia capensis</i> Sparrm.	AY128228	
	<i>Guarea glabra</i> Vahl	AY128229	
	<i>Khaya anthotheca</i> C.DC.	AY128231	
	<i>Lansium domesticum</i> Correa	AY128232	
	<i>Lovoa swynnertonii</i> E.G. Baker	AY128233	
	<i>Melia azedarach</i> L.	AY128234	
	<i>Munronia pinnata</i> (Wall.) Theob.	AY128236	
	<i>Naregamia alata</i> Wight & Arn.	DQ238059	
	<i>Nymania capensis</i> Lindb.	AY128238	AF066855
	<i>Quivisianthe papinae</i> Baill.	AY128239	
	<i>Ruagea pubescens</i> Karst.	DQ238057	
	<i>Sandoricum cf. koetjape</i> (Burm. f.) Merr.	DQ238068	
	<i>Swietenia macrophylla</i> King	AY128241	AJ235616
	<i>Toona</i> sp.	AY128243	
	<i>Trichilia emetica</i> Vahl	AY128244	AJ235629
	<i>Turraea sericea</i> Sm.	AY128245	
	<i>Vavaea amicorum</i> Benth.	DQ238066/67	
Simaroubaceae	<i>Ailanthus altissima</i> (Mill.) Swingle	AY128247	AF035895
	<i>Quassia amara</i> L.	AY128250	
	<i>Simarouba glauca</i> DC.	AY128252	AF235602
	<i>Soulamea soulameoides</i> (A. Gray) Noot.	U38923	
Rutaceae	<i>Adenandra uniflora</i> Willd.	AF066803	AF066832
	<i>Aegle marmelos</i> (L.) Correa ex Roxb.	AF066811	AF066839
	<i>Atalantia ceylanica</i> (Arn.) Oliver	AF066812	AF066840
	<i>Calodendrum capensis</i> Thunb.	AF066805	AF066834
	<i>Casimiroa edulis</i> La Llave	AF066808	AF066837
	<i>Chloroxylon swietenia</i> DC.	AF066802	AF066831
	<i>Choisya mollis</i> Standley	AF066800	AF066829
	<i>Chorilaena quercifolia</i> Endl.	AF066810	AF066838
	<i>Citrus glauca</i> (Lindl.) I.H. Burkill	AF066819	
	<i>Citrus japonica</i> Swingle	AF066799	
	<i>Citrus trifoliata</i> L.	AJ235806	

Table 1. (Continued)

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

Table 1. (Continued)

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

final extension for 10 min at 72°C. Alternatively, the *rbcL* 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 *rbcL* and *atpB* 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 *rbcL* and *atpB* 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 *rbcL*, 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 (*rbcL*) or one million cycles (*atpB*, *rbcL/atpB*), 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 *rbcL*, 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 *rbcL* 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 *rbcL* 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 (ν) 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 ν were set to 0.01, following Thorne's manual's recommendation that the time between root and tips multiplied by ν 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)		ii) NPRS (calibration 2)		iii) Bayesian		strict clock		Muellner et al. (2006)		Wikström et al. (2001)	
	direct	mean (s.d.)	direct	mean (s.d.)	mean (c.i.)	clock	direct	mean (s.d.)	direct	mean (s.d.)	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. <i>Cedrela/Toona</i>	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. Bursaraceae	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. Bursaraceae/ Anacardiaceae/ Anacardiaceae/ Kirkiaceae clade	95.3	83.6 (16.2)	98.3	93.6 (14.7)	74.1 (58.7–86.7)	108.3	-	-	-	-	-	-
10. Bursaraceae/ 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. <i>Biebersteinia</i> clade 1	25.2	30.8 (7.8)	26.0	36.1 (14.0)	32.6 (12.8–55.6)	15.3	-	-	-	-	-	-
14. <i>Biebersteinia</i> clade 2	5.0	8.2 (6.6)	5.2	9.6 (9.2)	12.6 (0.6–33.7)	1.9	-	-	-	-	-	-
15. <i>Biebersteinia</i> 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 (*rbcL*, *atpB*, *rbcL/atpB*) 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 *rbcL* 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 *rbcL* and *atpB* 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

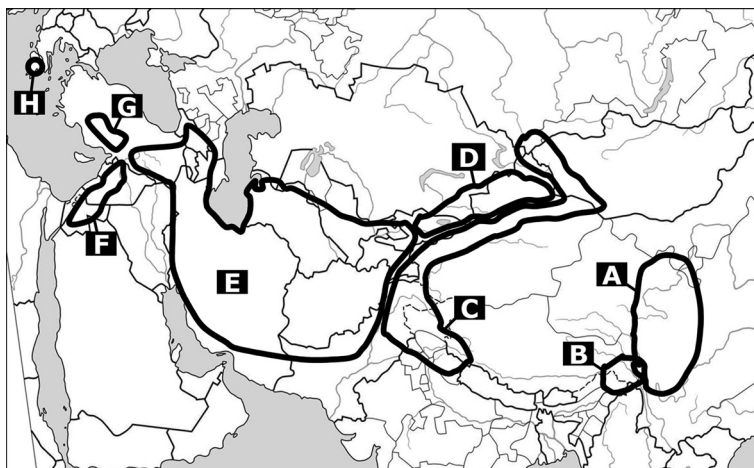


Fig. 1. Areas defined for the ancestral area analyses, based on the geological history of the area of occurrence and the extant distribution of the four species of *Biebersteinia*. The areas are: A Eastern Tibetan plateau including the Hengduan Mountains; B Eastern Himalaya; C Western Himalaya, Karakoram, 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	<i>rbcL</i>	<i>atpB</i>	<i>rbcL/atpB</i>
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 *rbcL* 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)

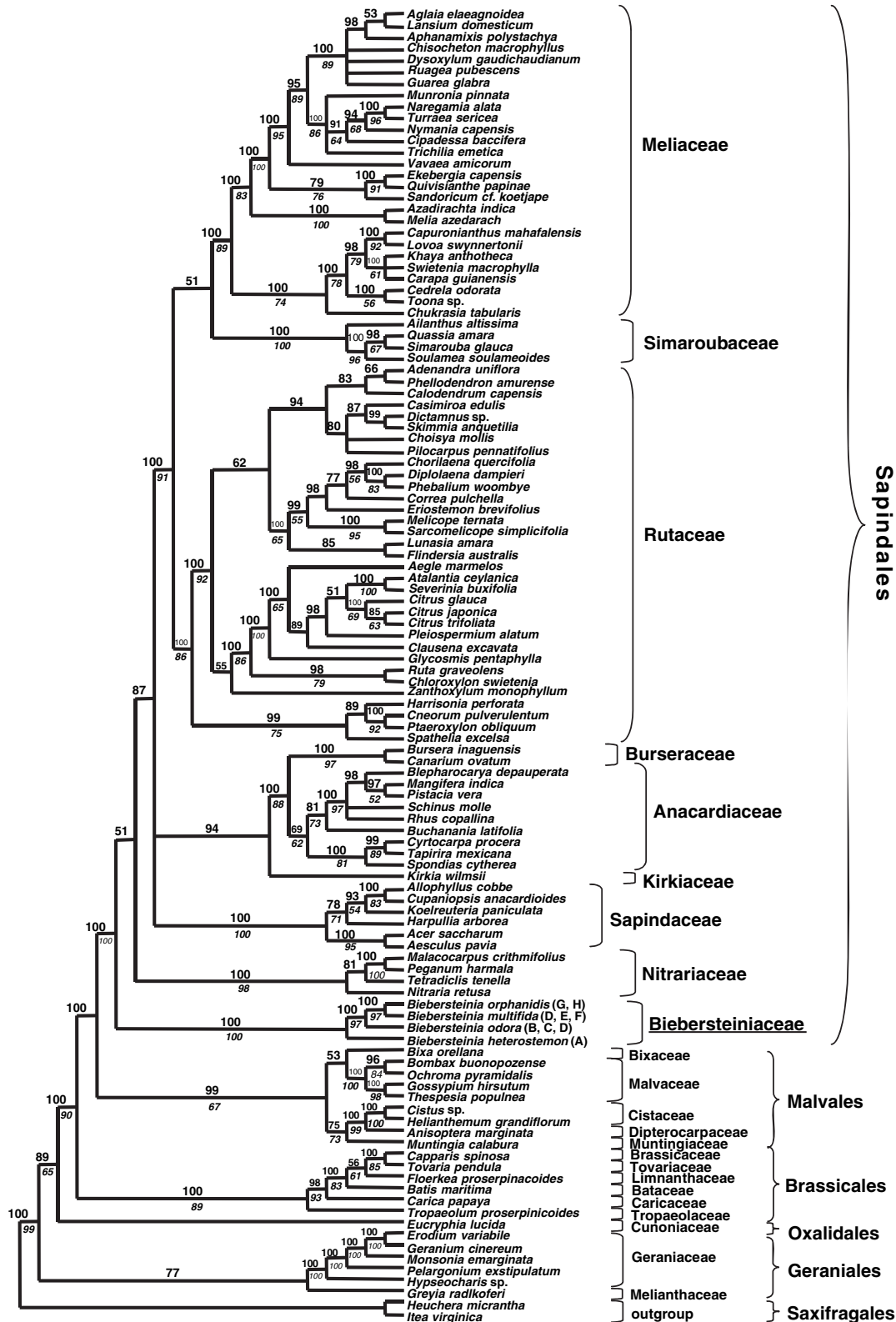


Fig. 3. NPRS chronogram based on a parsimony tree obtained from the *rbcL* data set used in Fig. 2 and calibrated with the *Biebersteinia* crown group set to 54.8 my. Nodes labelled 1–16 refer to locations in the tree for which age estimates are summarized in Table 2. **A, B** Fruits of *Ailanthus confucii*, 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

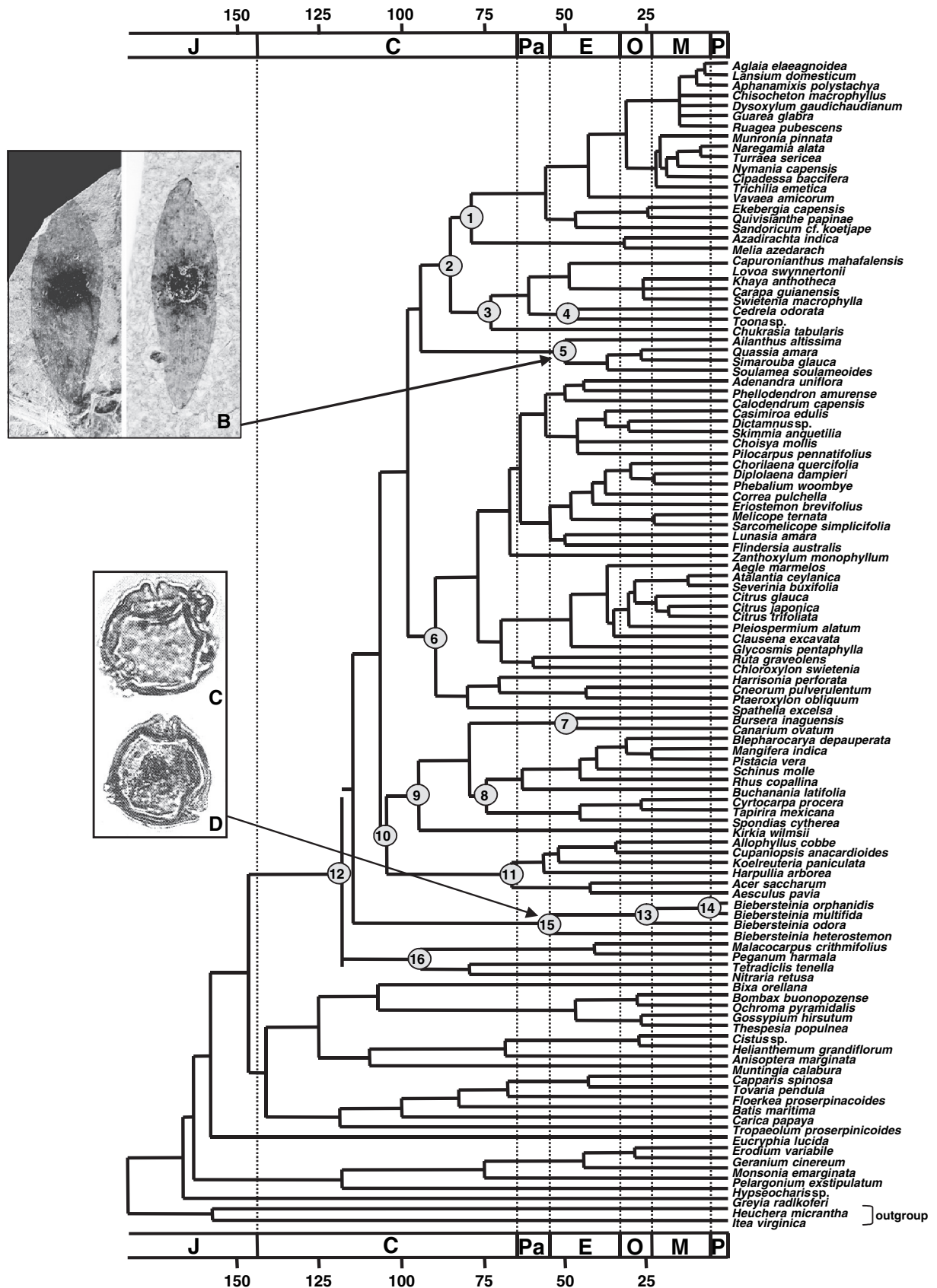
Areas	<i>G</i>	<i>L</i>	<i>G/L</i>
Area A: EAST TIB HENG	1	1	1.00
Area D: DZUN	2	2	1.00
Area B: EAST HIM	1	2	0.50
Area C: WEST HIM	1	2	0.50
Area E: SEM-AR MOUN	1	3	0.33
Area F: SE MED COAST	1	3	0.33
Area G: TAUR MOUN	1	3	0.33
Area H: SOUTH BALK PEN	1	3	0.33

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 Geraniaceae, 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



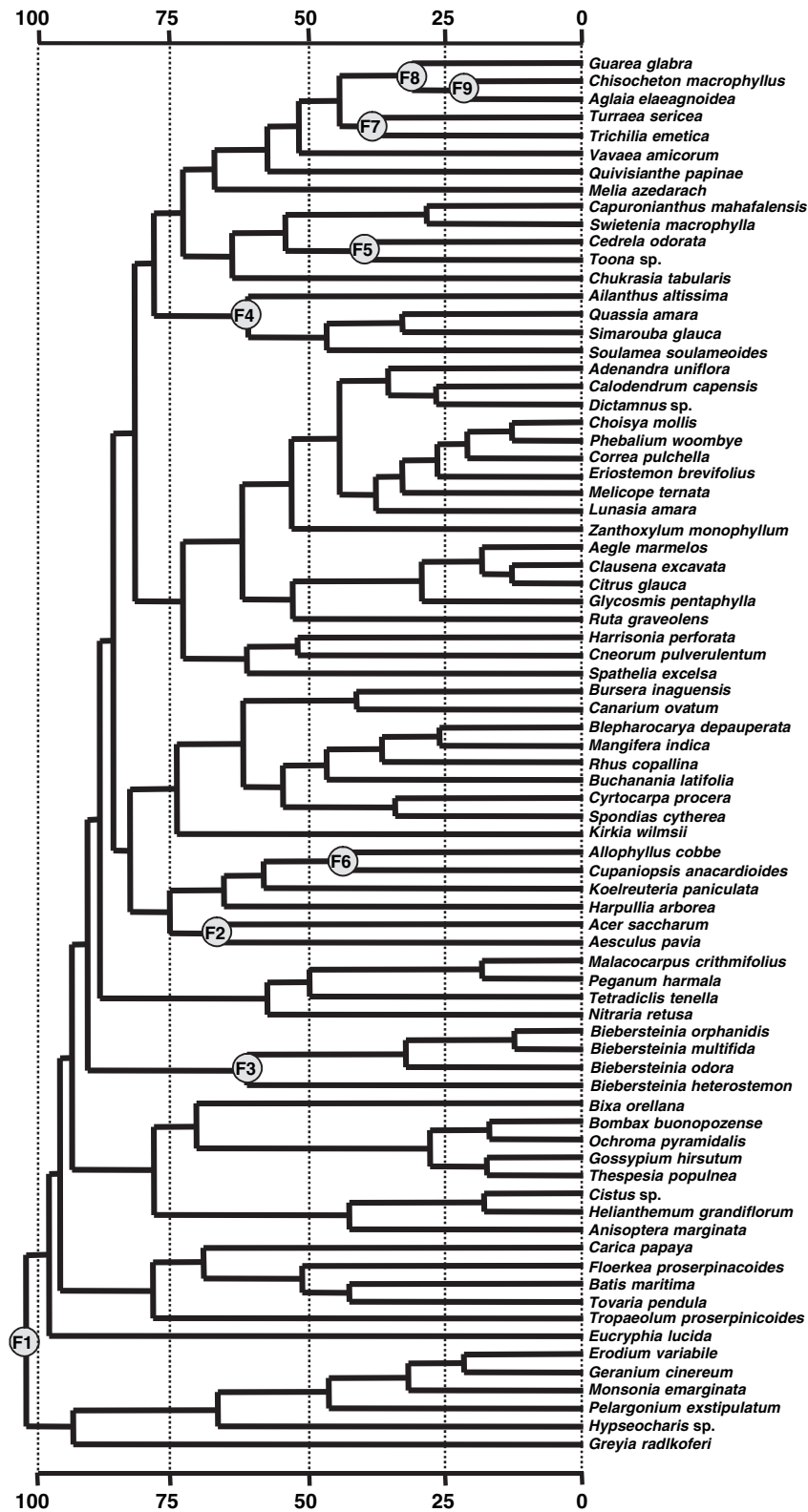




Fig. 4. Bayesian chronogram based on a maximum likelihood tree obtained from *rbcL* sequences of 78 ingroup and two outgroup taxa. Nodes labelled F1-F9 refer to the position of fossils used to constrain the relaxed clock (see text for details). Numbers along the bars indicate million years

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

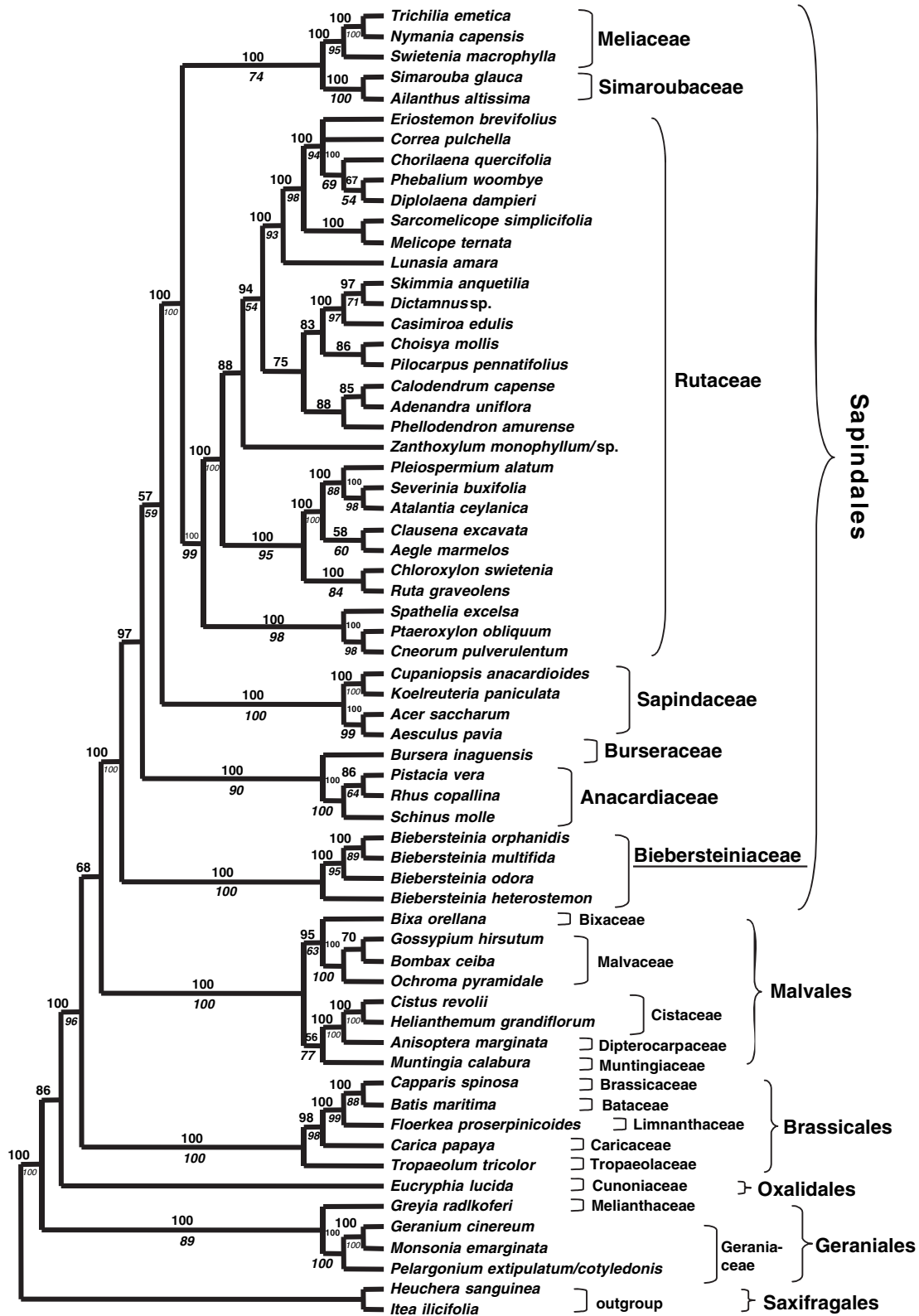


Fig. 5. Bayesian tree obtained from combined *rbcL* and *atpB* sequences of 62 ingroup and two outgroup taxa. Numbers above branches are Bayesian posterior probabilities, numbers below branches are bootstrap values (1000 replicates; italicized). Families and orders after APG II (2003)

younger ages than denser sampling (Sander-son 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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from the Neomugen Formation, and M. Watson (Edinburgh) and N. Turland (Saint Louis) for information on the distribution of *Biebersteinia*. Financial support for this study was provided by an EU Marie Curie Fellowship to ANM (MEIF-CT-2003–502194), and the Research Institute Senckenberg.

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