A fossil-calibrated relaxed clock for *Ephedra* indicates an Oligocene age for the divergence of Asian and New World clades and Miocene dispersal into South America

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Abstract   *Ephedra* comprises approximately 50 species, which are roughly equally distributed between the Old and New World deserts, but not in the intervening regions (amphitropical range). Great heterogeneity in the substitution rates of Gnetales (*Ephedra*, *Gnetum*, and *Welwitschia*) has made it difficult to infer the ages of the major divergence events in *Ephedra*, such as the timing of the Beringian disjunction in the genus and the entry into South America. Here, we use data from as many Gnetales species and genes as available from GenBank and from a recent study to investigate the timing of the major divergence events. Because of the tradeoff between the amount of missing data and taxon/gene sampling, we reduced the initial matrix of 265 accessions and 12 loci to 95 accessions and 10 loci, and further to 42 species (and 7736 aligned nucleotides) to achieve stationary distributions in the Bayesian molecular clock runs. Results from a relaxed clock with an uncorrelated rates model and fossil-based calibration reveal that New World species are monophyletic and diverged from their mostly Asian sister clade some 30 mya, fitting with many other Beringian disjunctions. The split between the single North American and the single South American clade occurred approximately 25 mya, well before the closure of the Panamanian Isthmus. Overall, the biogeographic history of *Ephedra* appears dominated by long-distance dispersal, but finer-scale studies are needed to test this hypothesis.

Key words   biogeography, *Ephedra*, relaxed molecular clock dating, uncorrelated rates model.

Efforts to date the evolutionary divergences of the five extant seed plant lineages (*Gingko* L., cycads, gymnosperms, Gnetales, and angiosperms) with molecular clocks have been hampered by the still unresolved relationships between them (for a summary, see Mathews et al., 2009, in press). Another problem is the marked difference in the rate of molecular evolution among seed plant lineages. The Gnetales in particular have unusually high or low substitution rates (depending on genus) in all datasets examined so far (Sanderson et al., 2000; Magallón & Sanderson, 2002, 2005; Mathews, 2009). For example, the *rbcL* substitution rate in *Ephedra* L. is approximately 10-fold slower than that in its sister clade *Gnetum* (Renner & Grimm, 2008). Such heterogeneity among lineages, which is not accommodated by molecular substitution models, presents a challenge for molecular clock dating, whether strict or relaxed. In response to this challenge, local and relaxed clock methods have been proposed that permit different parts of a tree to have different rates (Rambaut & Bromham, 1998; Thorne et al., 1998; Yoder & Yang, 2000; Kishino et al., 2001; Rannala & Yang, 2007). Of these relaxed clock approaches, several rely on a Bayesian framework and assume that substitution rates are autocorrelated between branches, meaning that rate changes occur gradually between ancestors and descendants as a clade diversifies. Other Bayesian clock models assume that branch-specific rates are drawn from a single underlying distribution, such as a log normal, gamma, or exponential distribution, the parameters of which are estimated from the data (Drummond et al., 2006; Rannala & Yang, 2007). Studies that have tested the performance of the different approaches have found that relaxed clock models with uncorrelated rates can outperform other approaches (Ho et al., 2005; Drummond et al., 2006; Lepage et al., 2007; but see Ho, 2009).

The Gnetales comprise *Ephedra* L., *Gnetum* L., and *Welwitschia* Hook.f. and are one of the five major groups of extant seed plants. Studies over the past 17 years have been unable to securely resolve the phylogenetic relationships of Gnetales with the four other seed plant lineages (Mathews et al., 2009, in press). Most recently, plastid sequence data have placed the Gnetales

as sister to all non-Pinaceae conifers or cupressophytes ("gnecup" clade; Nickrent et al., 2000; Doyle, 2006; Chumley et al., 2008; Braukmann et al., 2009; Rydin & Korall, 2009).

Resolving species-level relationships within *Ephedra* has been equally problematic. However, in contrast with the many well-supported, yet often mutually incompatible hypotheses on seed plant phylogeny, relationships in *Ephedra* have been largely unresolved owing to few informative characters in investigated gene regions and substantial plasticity in gross morphological traits (Ickert-Bond & Wojciechowski, 2004; Rydin et al., 2004; Huang et al., 2005). A recent study, with denser species sampling, provides support for several subgeneric clades (Rydin & Korall, 2009), but the deepest divergences in the genus are still ambiguous.

Divergence times from molecular clock analyses for the most recent common ancestors of living Gnetales genera range from 8–32 mya under a strict clock for Ephedra (Huang & Price, 2003) to 10–11 or 14 mya under a strict clock (Won & Renner, 2003, 2006) or 26–38 mya under a relaxed clock for Gnetum (Won & Renner, 2006). Estimates for the Gnetales crown group range from 120–131 mya (relaxed clock; Ickert-Bond & Wojciechowski, 2002) to 189 mya (relaxed clock; Schneider et al., 2004). Recent paleobotanical discoveries have further stirred up discussions about the age of *Ephedra* (Yang et al., 2005; Rydin et al., 2006; Friis et al., 2009), with some authors suggesting that Cretaceous fossil seeds resemble living species of *Ephedra* and may date the divergence of crown group *Ephedra* to ca. 125 mya (Yang et al., 2005; Rydin et al., 2006; Y. Yang, Institute of Botany, Beijing, pers. comm., 2008). However, to date, no ephedroid seed fossil has been unambiguously placed within crown group *Ephedra*, and these fossils are therefore of limited use as calibration points in molecular dating analyses. Conversely, coalified *Ephedra* seeds from the Drewry's Bluff locality of the Patuxent Formation in Virginia, USA, and from Buarcos and Torres Vedras localities in Portugal, which date to the late Barremian to early–middle Aptian age, have been assigned to stem group *Ephedra* based on two preserved features: (i) in situ *Ephedra*-type pollen, including discarded upcurled exines, which show that the pollen had germinated inside the ovules; and (ii) preserved papillae formed by the inner epidermis of the seed envelope. A combination of these features is unique to *Ephedra* (Rydin et al., 2006).

Here we apply relaxed molecular clock dating, using an uncorrelated rates model, to an *Ephedra* dataset that represents all the major clades found by Rydin and Korall (2009) with the goal of inferring the most probable age of the *Ephedra* crown group as well as the timing of key divergence events in the genus. The radiation of extant *Ephedra* is interesting because of its disjunct distribution in deserts north and south of the tropics, but not in the intervening regions (a classic amphitropical range; see Wen & Ickert-Bond, 2009). The genus comprises approximately 50 species, which are more-or-less equally distributed between the Old and New World deserts. In light of recent palaeobotanical evidence (above), availability of large molecular datasets for both *Ephedra* and *Gnetum*, as well as new approaches to dating that take into account topological uncertainty and rate heterogeneity among lineages, in the present paper we provide new age estimates for *Ephedra* and discuss their implications for the evolution of the genus.

1   Material and methods

1.1   Taxon sampling

To assemble the most useful phylogenetic dataset of *Ephedra* we included as many taxa and genes as possible from GenBank. We used PHYLOTA (Sanderson et al., 2008; http://loco.biosci.arizona.edu/pb/) to extract GenBank data. The browser returned 10 phylogenetic informative clusters. The genes comprising these clusters have been variously used for phylogenetic inference of the Gnetales and include the *atpB* gene (Rydin et al., 2002), the *rbcL* gene (Rydin et al., 2002, 2004; Rydin & Källersjö, 2002; Huang & Price, 2003; Won & Renner, 2003, 2006; Huang et al., 2005; Wang et al., 2005; Rydin & Korall, 2009), the *matK* gene (Won & Renner, 2003, 2006; Huang et al., 2005), the *rps4* gene (Ickert-Bond & Wojciechowski, 2004; Rydin et al., 2004; Rydin & Korall, 2009), the *psbA-trnH* intergenic spacer (IGS) (Techen et al., 2006), the *trnL* gene, and the *trnL–trnF* IGS (Long et al., 2004), as well as nuclear ribosomal 18S (Rydin et al., 2002, 2004; Wang et al., 2005; Rydin & Korall, 2009), 26S (Rydin et al., 2002, 2004; Rydin & Korall, 2009), and internal transcribed spacer (ITS) 1 and ITS2 (Ickert-Bond & Wojciechowski, 2004; Rydin et al., 2004; Huang et al., 2005; Wang et al., 2005; Won & Renner 2005, 2006; Rydin & Korall, 2009). We excluded the cluster of the *chlB* gene (Boivin et al., 1996) from further consideration because it only contained four taxa. In addition to the clusters returned by PHYLOTA, we added the plastid *rpl16* intron and the *trnS*[UGA]–*trnfM*[CAU] intron data from Rydin and Korall (2009). Because of the tradeoff between increasing gene and taxon sampling and limiting the amount of missing data, we reduced the initial matrix from 265 to 95 accessions. The reduced matrix included the most complete coverage

for the genes used while maintaining sampling of the geographic and taxonomic diversity of *Ephedra*. This matrix included 53 accessions of *Ephedra*, 13 accessions of *Gnetum*, *Welwitschia mirabilis*, and 28 accessions of other gymnosperm taxa comprising the outgroup. Information regarding vouchers and GenBank accession numbers is given in Table 1.

1.2 Sequence and phylogenetic analyses

Maximum likelihood (ML) searches were performed in RAxML 7.2.1 (Stamatakis, 2006), using the GTR+Γ model. Model parameters were estimated over the duration of runs and searches started from random parsimony trees. Statistical support was measured by ML bootstrapping in RAxML, with 100 replicates.

1.3 Estimation of divergence time

We used a Bayesian relaxed clock as implemented in BEAST 1.4.8 (Drummond et al., 2006; Drummond & Rambaut, 2007). To reduce topological uncertainty in parts of the tree, which prevented the Markov chain Monte Carlo (MCMC) chains from reaching a stationary state, we gradually reduced the 95-taxon matrix to 67, 54, 46, and 42 taxa (with varying taxon combinations), and we also took out partitions with more than 30% empty cells, which left a matrix of 7736 aligned nucleotides. After tuning the operators using the auto-optimization option in BEAST, analyses used a speciation model that followed a Yule tree prior, with rate variation across branches uncorrelated and lognormally distributed. The MCMC chains were run for between 40 and 60 million generations (burn-in 10%), with parameters sampled every 1000th step. Results from individual runs were combined as recommended, and effective sample sizes for all relevant estimated parameters and node ages were above 100. Because the oldest described ephedroid fossils place somewhere along the stem lineage of *Ephedra* (see above), we used a single constraint, namely a lognormal prior probability that the split between *Gnetum* and *Welwitschia* is at least 110 mya old (with a 95% confidence interval of 10 mya), based on the welwitschioid fossil seedling *Cratonia cotyledon* (Rydin et al., 2003) from the Early Cretaceous of Brazil. This fossil is slightly younger than the oldest *Ephedra* seeds (125 mya) and clearly belongs to crown group Gnetales, based on the presence of an embryo feeder and a unique venation pattern, shared by the fossil and *Welwitschia*.

2 Results

The ML tree obtained from the 10 locus–95 taxon dataset shows relationships within *Ephedra* (Fig. 1) that are similar to those recovered by Rydin and Korall (2009), although species sampling in the present study is smaller. *Ephedra foeminea* is sister to the rest of *Ephedra*. The next diverging clade is one of strictly Mediterranean taxa (*E. altissima*, *E. aphylla*, *E. milleri*, *E. alata* and *E. fragilis*), sister to the rest of *Ephedra* ("core *Ephedra*" sensu Rydin & Korall, 2009). Core *Ephedra* comprises several subclades of Mediterranean and Asian distribution (e.g. *E. laristanica* and *E. foliata*), but there is no statistical support for their precise composition and relationships. However, the New World clade of *Ephedra* is strongly supported (bootstrap (BS) 94%) and consists of North American and South American clades. The large substitution rate heterogeneity among Gnetales is evident from the phylogram (Fig. 1), particularly the long branches leading to the three genera compared with the significantly reduced branch lengths within *Ephedra*.

Relationships among the fewer species included in the molecular clock runs (Fig. 2) differ in part from those obtained from the ML analysis (Fig. 1) and have slightly better statistical support because the matrix includes many fewer missing nucleotides. Table 2 lists divergence times obtained for key nodes within *Ephedra* (labeled 3–9 in Fig. 2). Although deep divergences have originated in the Oligocene, most of the tip clades have diverged more recently in the Late Miocene or Pliocene (Fig. 2).

3 Discussion

The results of the present study provide strong evidence for a recent radiation of extant *Ephedra*. Given the few clear morphological differences among species, it has been suggested that the lack of molecular divergence in *Ephedra* plastid genomes may be the result of hybridization and polyploidization, which appears to be rampant in the genus (Cutler, 1939; Ehrendorfer, 1976; Choudry, 1984; Wendt, 1993). Plants with montane distribution also frequently exhibit rapid diversification, likely because of small-scale habitat heterogeneity (Bell & Donoghue, 2005; Hughes & Eastwood, 2006).

Studies with a comprehensive species sampling of *Ephedra* (the Bayesian analysis in Rydin & Korall, 2009; the ML analysis in the present study) indicate a basal grade of Mediterranean species and thus a possible origin of the crown group of *Ephedra* in the Mediterranean region (northern Africa, southern Europe, the Near East). However, these basal divergences still have little statistical support owing to the limited signal in the loci so far included (Ickert-Bond & Wojciechowski, 2004; Ickert-Bond et al., 2009; Rydin & Korall, 2009; present study). Parsimony

Table 1 Voucher information and GenBank accession numbers

	Voucher	Distribution	18S	26S	atpB	rbcL	rps4	matK	ITS	rpL16	trnS to trnfM	psbA, psbA-trnH, trnH
E. alata Decne.	*C303 Anderberg 480* (S)	Mediterranean	AY755698	AY755732		AY755805	AY755851		AY755774	FJ958074	FJ958162	
E. altissima Desf.	*Bot. Dep. SU C7688* (S)	North Africa	AY755697	AY755731		AY755804	AY755850		AY755773	FJ958073	FJ958161	
E. americana Humb. & Bonpl. ex Willd.	*Ickert-Bond 1105* (ASU)	South America					AY591464		AY599143			
E. andina Poepp. ex C.A. Mey.	*Chase 10140* (K)	South America	AY755670	AY755707	AY056538	AY056573, AY755782*	AY755821		AY755744	FJ958045	FJ958128	
E. antisyphilitica Berl. ex C.A. Mey.	*Huang20_1* (GA)	US, Mexico				AY492031		AY492008	AF429442			
E. aphylla Forssk.	*Anderberg 853* (S)	Mediterranean	AY755695	AY755729		AY755802	AY755848			FJ958071	FJ958159	
E. aspera Engelm. ex S. Wats.	*Huang s.n.* (GA)	North America				AF489532		AY492010	AF429443			
E. boelkei F.A. Roig	*Ickert-Bond 1252* (ASU)	South America					AY591473		AY599175			
E. breana Phil.	*Ickert-Bond 1234*	South America					AY591472		AY599177			
E. californica S. Watson	*Stedge O68_154* (O)	North America	AY755676*	AY755708	AY056533	AY056569	AY755827		AY755750	FJ958050	FJ958135	AY849358
E. chilensis C. Presl.	*Forbes 49_0542* (UC)	North America	AY755691	AY755725		AY755799	AY755844		AY755767	FJ958067	FJ958155	
E. compacta Rose	*Puente 1901* (ASU)	Mexico					AY591474		AY599157			
E. coryi Reed	*Ickert-Bond 953* (ASU)	North America					AY591461		AY599153			
E. cutleri Peebles	*Ickert-Bond 1006* (ASU)	North America					AY591456		AY599156			
E. distachya L.	*Rydin 69* (S)	Asia–Europe	AY755686	AY755719		AY755793	AY755838		AY755761	FJ958061	FJ958149	
E. fasciculata A. Nelson	*Ickert-Bond 513* (ASU)	North America					AY591457		AY599180			AY849360
E. fedschenkoae Pauls.	*Ickert-Bond s.n.* (ASU)	Central Asia					AY591442		AY599158			AY849350
E. foliata Boiss. & C.A. Mey.	*Thulin 9975* (UPS)	Mediterranean	FJ957969	FJ957988		FJ958030	FJ958109		FJ958008	FJ958085	FJ958173	
E. foliata Boiss. & C.A. Mey.	*Thulin 10745* (UPS)	Mediterranean	FJ957971	FJ957990		FJ958032	FJ958111		FJ958010	FJ958087	FJ958175	
E. frustillata Miers	*Chase 10218* (K)	South America	AY755674	AY056490	AY056528	AY056564	AY755825		AY755748	FJ958048	FJ958131	
E. funerea Coville & Morton	*Ickert-Bond 473* (ASU)	North America					AY591454		AY599168			
E. gerardiana Wall. & Florin	*Chase 10141* (K)	Central Asia	AY755671	AY056486	AY056524	AY056560	AY755822		AY755745	FJ958046	FJ958129	
E. gracilis Phil.	*Ickert-Bond 1201* (ASU)	S. America					AY591465		AY599150			
E. intermedia Schrenk & C.A. Mey.	*Rydin 66* (S)	Central–east Asia	AY755683	AY755716		AY755790	AY755835		AY755758	FJ958058	FJ958146	

Table 1 Continued

Taxon	Voucher	Distribution	18S	26S	atpB	rbcL	rps4	matK	ITS	rpL16	trnS to trnfM	psbA, psbA-trnH, trnH
E. laristanica Assadi	Assadi & Sardabi 41781 (KAS)	Iran					AY591437		AY599126			
E. laristanica Assadi	Davis & Bokhari D56211B (E)	Iran	FJ957980						FJ958020	FJ958096	FJ958182	
E. likiangensis Florin	Forbes 94_0389 (UC)	China	AY755690	AY755724		AY755798	AY755843		AY755766	FJ958066	FJ958154	AY849357
E. lomatolepis Schrenk & C.A. Mey.	Baitulin (UPS)	Central–east Asia	FJ957967	FJ957986		FJ958028	FJ958108		FJ958006	FJ958083	FJ958171	
E. major Host	Uggla (S)	Mediterranean–central Asia	FJ957976	FJ957994		FJ958035	FJ958117		FJ958016	FJ958092	FJ958178	
E. milleri Freitag & Maier-Stolte	E 7667	Oman	FJ957983	FJ958002			FJ958121		FJ958024	FJ958100	FJ958186	
E. minuta Florin	Rydin 63 (S)	China	AY755681	AY755714		AY755788	AY755833		AY755756			
E. monosperma JG. Gmel. ex C.A. Mey.	Hurka & Neuffer12182 (KAS)	Central–east Asia					AY591443		AY599139			
E. monosperma JG. Gmel. ex C.A. Mey.	Chase 10142 (K)	Central–east Asia	AY755672	AY056525, AY056487	AY056525	AY056561	AY755823		AY755746			
E. multiflora Phil. ex Stapf	Icker-Bond 1211 (ASU)	South America					AY591471		AY599173			
E. nevadensis S. Watson	Forbes 66_1033 (UC)	North America	AY755688	AY755722		AY755796	AY755841		AY755764	FJ958064	FJ958152	
E. ochreata Miers	B380819 (B)	South America										
E. pachyclada Boiss.	Danin S-2455 (S)	West Asia	AY755703	AY755738		AY755810	AY591463 AY755857		AY599176 AY755779	FJ958080	FJ958168	AY849362
E. pedunculata Engelm. ex S. Watson	Icker-Bond 920 (ASU)	Mexico, Texas					AY591460		AY599144			
E. regeliana Florin	Wundisch 956 (KAS)	Central–east Asia					AY591449		AY599160			
E. rhytidosperma Pachom.	Wang 518	China	DQ028781			DQ028779		DQ028780	DQ028782			
E. rupestris Benth.	Forbes 87.1368 (UC)	South America	AY755689	AY755723		AY755797	AY755842		AY755765	FJ958065	FJ958153	
E. sarcocarpa Aitch. & Hemsl.	Allen & Esfandri 2703 (S)	Central Asia	FJ957977	FJ957995			FJ958118	FJ958093	FJ958017	FJ958093	FJ958179	
E. saxatilis Florin	S. Hedin C-218	Central Asia	FJ957981	AY056491	AY056529	AY056565	AY755826		FJ958022	FJ958098	FJ958184	AY849364
E. sinica Stapf	J. Schonenberger s.n. (S)	East Asia	AY755675						AY755749	FJ958049	FJ958134	
E. somalensis Freitag & Maier-Stolte	Thulin10925A (UPS)	Horn of Africa	FJ957966						FJ958004	FJ958081	FJ958169	
E. strobilacea Bunge	Aellen & Esfandri 2703 (S)	Central Asia	FJ957978						FJ958018	FJ958094	FJ958180	

Table 1 Continued

	Voucher	Distribution	18S	26S	atpB	rbcL	rps4	matK	ITS	rpL16	trnS to trnfM	psbA, psbA-trnH, trnH
E. strobilacea Bunge	*Rechinger 27161* (US)	Central Asia	AY599162				AY591448		AY599162			
E. torreyana S. Watson	*04_487* (S)	North America	AY755684	AY755717		AY755791	AY755836		AY755759	FJ958059	FJ958147	
E. transitoria Riedl	*Collenette 9095B* (?)	West Asia		FJ957999					FJ957999	FJ958097	FJ958183	
E. triandra Tul.	*Ickert-Bond 1227* (ASU)	South America					AY591468		AY599165			
E. trifurca Torr.	*MO04630447* (MO)	North America	AY755687	AY755720		AY755794	AY755839		AY755762	FJ958062	FJ958150	
E. tweediana Fisch. ex C.A. Mey.	*Forbes 66.0742* (UC)	South America	AY755692	AY755726		AY755800	AY755845		AY755768	FJ958068	FJ958156	
E. viridis Coville	*Huang37_1* (GA)	North America				AY492050		AY492028	AF429436			
Gnetum africanum Welw.	—	Tropical Africa	U43012			AY296527						
G. costatum K. Sch.	*Chase 10219* (K)	Asia	AY755661	AY056497		AY056576	AY755812			FJ958102	FJ958132	
G. cuspidatum Blume	—	Asia				AY296530	AY591430					
G. gnemon L.	*Swenson et al. s.n.* (S)	Asia	AY755660	AF036488		L12680	AY755811			FJ958101	FJ958122	
G. gnemonoides Brongn.	—	Asia				AY296539	AY591429					
G. indicum Merr.	*E00130257* (E)	Asia	AY755663	AY056495		AY056574	AY755814			FJ958104	FJ958139	
G. leyboldii Tul.	—	South America	L24045			U72820	AY591432					
G. montanum Markgr.	*E00130261* (E)	Asia–Australia	AY755664	AY056496		AY056575	AY755815			FJ958105	FJ958140	
G. nodiflorum Brongn.	—	Asia	U42415			AY296564						
G. parvifolium (Warb.) W.C. Cheng	*Rydin s.n.* (S)	Asia	AY755662	AY755704		AY056577	AY755813			FJ958103	FJ958133	
G. schwackeanum Taub. ex Schenck	—	Asia				AY296567						
G. ula Brongn.	—	Asia	U42417			AY296568	AF313610					
G. urens Blume	—	South America				AY296569						
Welwitschia mirabilis Hook. f	*Stedje 67-1177* (O)	Namibia	AF207059	AY056484		AJ235814	AY188246			FJ958106	FJ958137	
Araucaria Juss.	—		AF051792	U90690		U96467	AY188260					
Calocedrus Kurz	—		D85293	U90707		L12569	AY188281					
Cephalotaxus Siebold & Zucc. ex Endl.	—		D38241	U90697		AF227461	AY188264					
Chamaecyparis Spach	—			AY056506		L12570	AY188283					

Table 1 Continued

|	Voucher	Distribution	18S	26S	atpB	rbcL	rps4	matK	ITS	rpL16	trnS to trnfM	psbA, psbA-trnH, trnH
Cupressus L.	—		AF051797			L12571	AY188282					
Juniperus L.	—		D38243	AY056504		L12573	AY188279					
Metasequoia Miki	—		L00970	AY056512		AJ235805	AY188268					
Phyllocladus Rich. ex Mirb.	—		D38244			AB027315	AY188258					
Podocarpus Labill.	—		AF051796	U90685		AF307931	AY188252					
Sciadopitys Siebold & Zucc.	—		D85292	U90698		L25753	AY188262					
Sequoia Endl.	—		AY686598	U90701		L25755	AY188266					
Sequoiadendron J. Buchholz	—					AY056580	AY188267					
Taxodium Rich.	—		EF053176	U90702		AF127427	AY188270					
Taxus L.	—		D16445	AY056513		AJ235811	X84145					
Thuja L.	—			AY056503		L12578	AY188276					
Thujopsis Siebold & Zucc.	—			AY056505		L12577	AY188277					
Bowenia Hook.	—			AY056480		L12671						
Ceratozamia Brongn.	—			AY056482		AY056558						
Cycas L.	—		D85297	U90674		L12674	EU016841					
Cycas revoluta Thunb.	—		AB029356	U90673		AY056556	AF313609					
Dioon sp. Lindl.	—			AY056483		AF531203						
Encephalartos Lehm.	—			AY056479		L12676						
Zamia furfuracea L. f.	—		AB029357	U90677		AF202959						
Zamia pumila L.	—		M20017	AY056481		AY056557	AY188209					
Ginkgo biloba L.	—		D16448	U90672		AJ235804	AF313611					
Abies Mill.	—			AY056508		AB029646	AY188224					
Cedrus Duham.	—		AB026936	AY056507		X63662	AY188222					
Larix Mill.	—		D85294	AY056502		X63663						
Picea asperata Mast.	—		L07059	AY056509		AY056578	AY188226					
Picea breweriana S. Watson	—			AY056510		AY056579						
Pinus cembra L.	—			U90681		AB019795						
Pinus L.	—		AF051798	U90680		AB019819	AF313612					
Pinus mugo Turra	—			AY056500		AB063372						
Pinus peuce Griseb.	—			AY056499		AB019803						
Pinus strobus L.	—			AY056501		AB019798	AY188212					
Pinus wallichiana A.B.Jacks.	—		X75080			X58131						
Pseudotsuga Carrière	—		AB026941	AY056498		X52937	AY188223					
Tsuga Carrière	—		AB026942	AY056511		AY056581	AY188220					
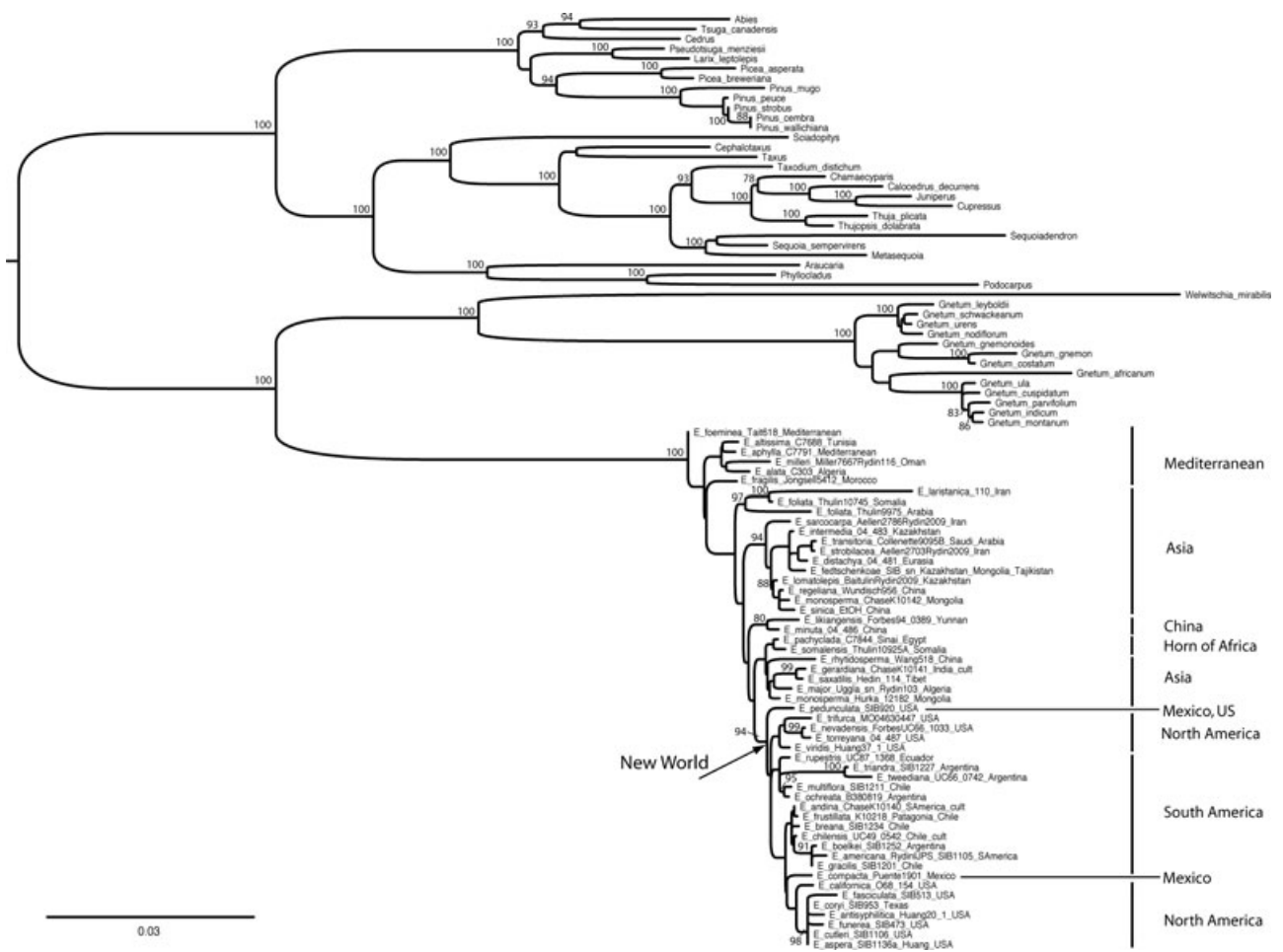
Fig. 1. Maximum likelihood (ML) phylogeny, inferred from 10 combined chloroplast and nuclear ribosomal DNA (nrDNA) loci for 95 taxa of gymnosperms; branch lengths computed using RAxML. The ML bootstrap support values above 80% are indicated above the branches. The biogeographic distribution of *Ephedra* taxa is shown on the right.

analysis (Rydin & Korall, 2009), as well as Bayesian analysis of the reduced dataset in the present study (Fig. 2), results in a Mediterranean clade sister to the remaining *Ephedra*. The respective divergence may have taken place some 30 mya (Fig. 2, node 3; Table 2). Within the Mediterranean clade, the Near Eastern *E. foliata* (Arabia and Somalia) and *E. laristanica* (Iran) split from the western Mediterranean species ca. 26 mya. Turning to the (mostly) Asian clade of *Ephedra*, the prevailing pattern appears to be westward dispersal (Fig. 3), with an estimated divergence of a strictly Chinese clade from the rest of the Asian/African clade at 28 mya (Fig. 2, node 5; Table 2). Dispersal into the Horn of Africa from the Asia 1 clade (Fig. 2, node 9; Table 2) may date back to 16 mya.

New World species are monophyletic and estimated to have diverged from their mostly Asian sister clade some 30 mya. This timeframe is corroborated by many

other Beringian plant disjunctions (for a review, see Wen & Ickert-Bond, 2009). In turn, the New World species split into a North American and a South American clade (Fig. 2), which appear to have diverged approximately 25 mya; that is, well before the closure of the Panamanian Isthmus (Fig. 2, node 7; Table 2). These results mirror other studies indicating that significant dispersal took place between Mesoamerica and South America before the closure of the Isthmus of Panama during the Oligocene or Miocene (e.g. mammals: Marshall & Sempere, 1993; Melastomeae: Renner & Meyer, 2001; *Ruprechtia* (Polygonaceae) and *Nissolia* (Leguminosae): Pennington et al., 2004; and *Platymiscium* (Leguminosae): Saslis-Lagoudakis et al., 2008).

Dispersal in *Ephedra* may have been facilitated by the ovulate bracts, which, in some species of *Ephedra*, are bright red and fleshy and indicative of endozoochory (Stapf, 1889; Freitag & Maier-Stolte, 1994; Danin,
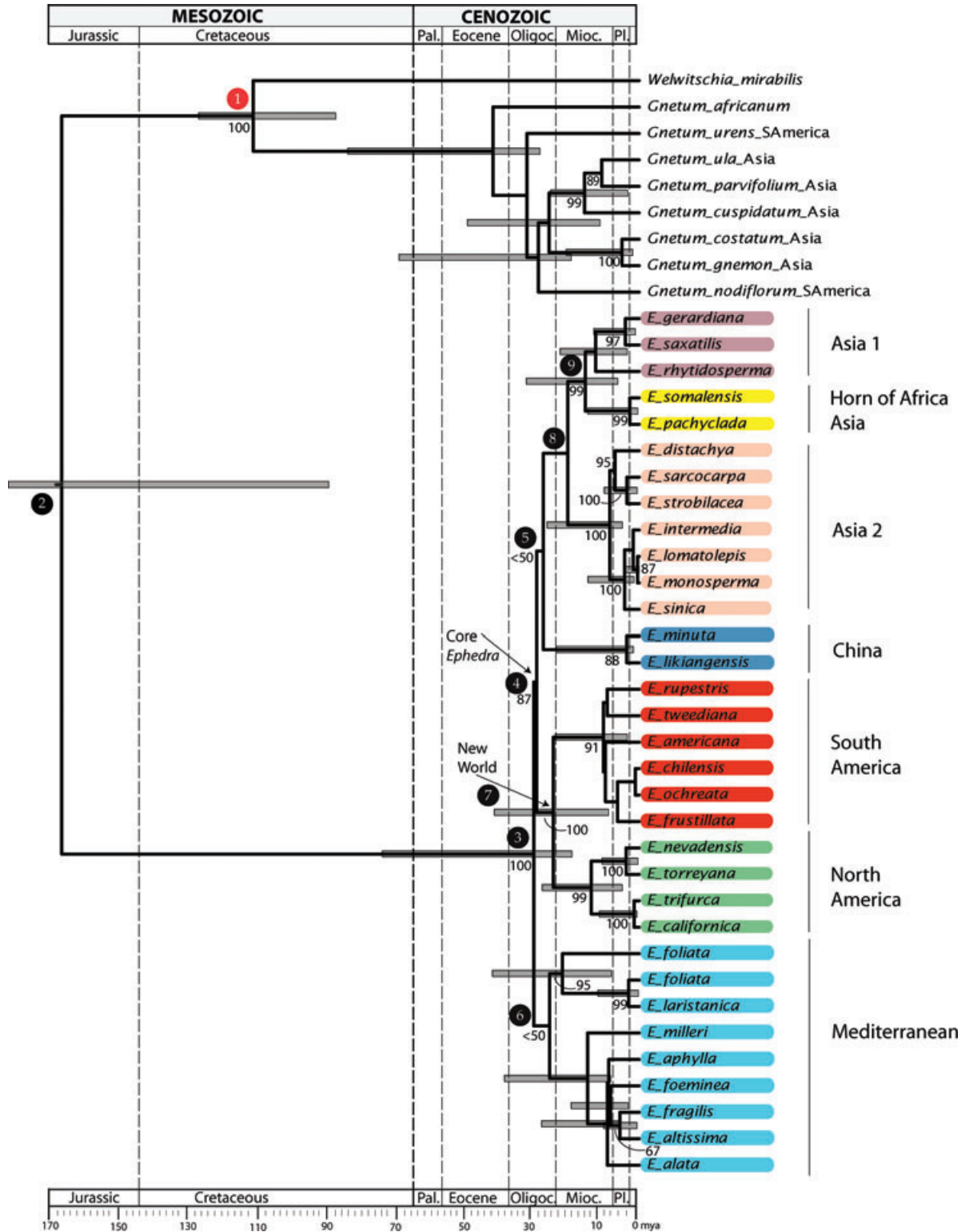
Fig. 2. Chronogram based on 42 accessions of *Ephedra*, *Gnetum*, and *Welwitschia* from combined chloroplast and nuclear ribosomal DNA (nrDNA) loci obtained under a model of uncorrelated rate change using one fossil-based constraint (see text for details). Node heights are median ages, with gray bars indicating the 95% highest posterior density intervals (see Table 2 for details). The maximum likelihood bootstrap support values are indicated below the branches. The distribution of *Ephedra* taxa is indicated on the right with node labels (black circles) pointing to particular nodes of biogeographic interest (see Table 2 and text for details). Pal., Paleocene; Oligoc., Oligocene; Mioc., Miocene; Pl., Pleistocene.

Table 2 Time estimates (in million years) and confidence intervals for significant nodes for the crown group *Ephedra* obtained from combined analysis of the 7736-nucleotide matrix under an uncorrelated rates molecular clock (see Material and Methods)

Node no.	Clade name	Fossil evidence	Age (million years)		95% Highest posterior density intervals
			Fossil	Estimated	
1	Split: *Welwitschia* vs. *Gnetum*	*Cratonia cotyledon*	110*	111.35	87.21, 127.01
2	Divergence of *Ephedra* from *Gnetum* and *Welwitschia*	*Ephedra archaerhytidosperma*	125	166.61	90.62, 192.34
3	Divergence Mediterranean clade from core *Ephedra*			30.39	20.55, 73.5
4	Divergence of NW clade from the rest of core *Ephedra*			29.56	8.84, 41.53
5	Divergence of China clade from rest of mixed Asia clade			27.63	14.45, 49.36
6	Divergence of Middle Eastern/Horn of Africa from African Mediterranean members			25.8	15.37, 55.53
7	Divergence of North American clade from South American clade			24.78	8.84, 41.53
8	Divergence of Asia 2 clade from combined Asia 1/Horn of Africa and Asia clade			20.61	14.35, 49.36
9	Divergence of Asia 1 clade from Horn of Africa/Asia clade			15.51	6.18, 32.5

*Used as a constraint.
n.a., not available.
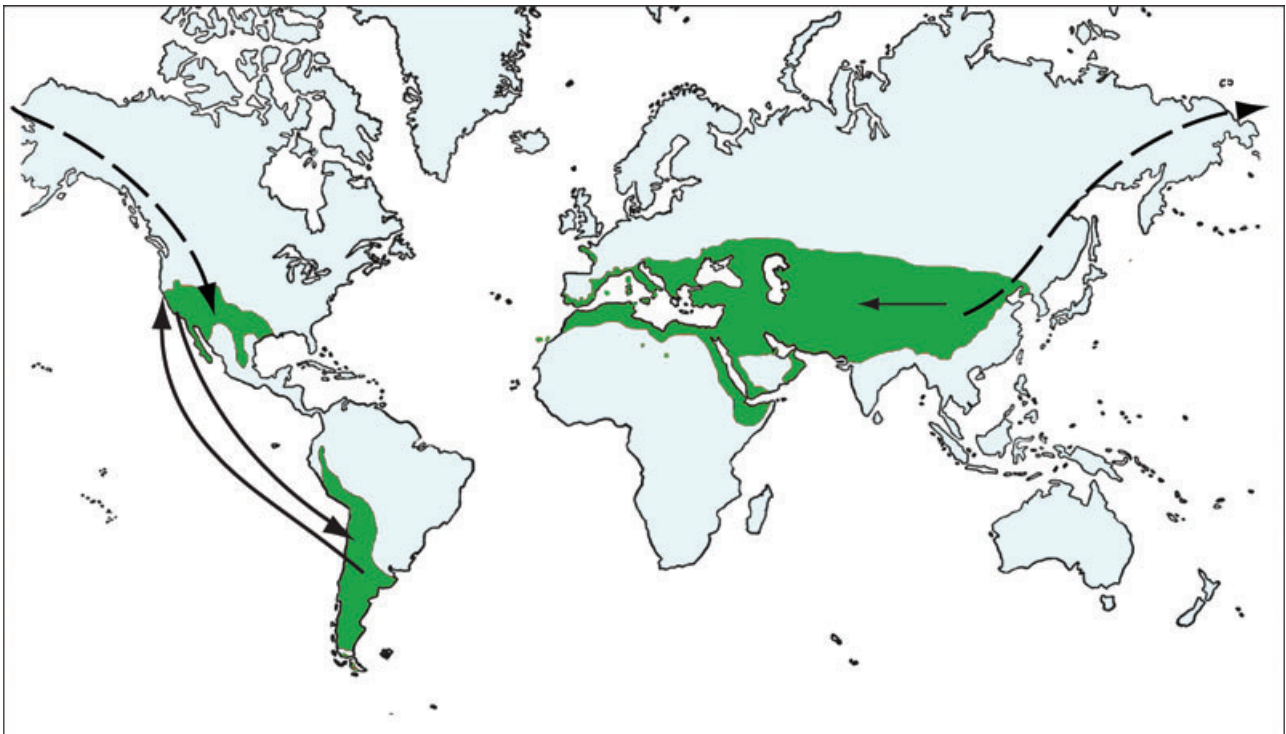
Fig. 3. Distribution of Ephedra (green shading) and hypothesized intercontinental (solid arrows) and intracontinental dispersal routes (dashed arrows).

1996; Hódar et al., 1996). Bird dispersal has also been observed directly (Ridley, 1930; Hollander et al., 2009). In contrast, dry, wing-bracted strobili are adapted for anemochory (Stapf, 1889; Danin, 1996). The seeds of North American *E. aspera*, *E. californica*, *E. funerea*, *E. nevadensis*, and *E. viridis* are not fleshy and their ovulate bracts are not winged. Seeds of these "intermediate bracted" taxa often accumulate at the stem base, and seed-caching rodents have been observed as dispersers (Ickert-Bond, 2003; Ickert-Bond & Wojciechowski, 2004; Hollander & Vander Wall, 2009;

Hollander et al., 2009). Wind-dispersed *Ephedra* typically inhabit marginal habitats, such as hyperarid deserts or dry salt lakes devoid of animal life (Danin, 1996), and, in general, dispersal biology in *Ephedra* appears to relate to habitat, rather than being phylogenetically conservative (Hollander et al., 2009).

Recent studies have found that reliable topologies may be obtained even in the face of large amounts of missing data (e.g. Wiens, 2003, 2006; McMahon & Sanderson, 2006; Smith et al., 2009). However, for molecular clock dating, missing data present a so-far

insurmountable challenge. This is because estimation of divergence time depends on accurate estimates of branch lengths, which can only be obtained with large numbers of nucleotides (Sanderson, 1998). When the BEAST dating runs failed to reach stable distributions, we first reduced the number of empty cells by deleting data partitions that lacked sequences for more than 30% of the included species; next, we deleted species that lacked sequences for more than five loci. Even so, a combined run length of 108 million generations was needed for each parameter to converge on a stationary distribution.

A caveat with all molecular clock dating is that the absolute ages obtained depend on the calibration used. An earlier study that concentrated on *Gnetum* and only included three species of *Ephedra*, using a Bayesian relaxed clock and an auto-correlated model, explored the effects of three different constraints (Won & Renner, 2006). In one experiment, these authors used 125-mya-old *Ephedra* seeds to constrain the crown group age of *Ephedra*. This had the effect of roughly doubling within-*Gnetum* estimates compared with the ages obtained when these seeds were assigned to the *Ephedra* stem (Won & Renner, 2006, table 1). In the present study, we initially included representatives of all major lineages of gymnosperms so that we could constrain the *Ephedra* stem to a minimum of 125 mya. However, this introduced the problem of the uncertain placement of Gnetales within seed plants, a problem that Won & Renner (2006) circumvented by conducting dating runs under four different seed plant topologies.

With just the Gnetales included, as in Fig. 2, one cannot infer a Bayesian probability distribution around the split between *Ephedra* and *Gnetum*/*Welwitschia*. Instead, we decided to rely exclusively on the *Cratonia cotyledon* fossil from the Early Cretaceous of Brazil, the assignment of which is unambiguous because it clearly represents the *Welwitschia* stem group (Rydin et al., 2003). This calibration yielded an age of 167 mya (91–192 mya confidence interval) for the divergence between *Ephedra* and the other two genera. This age range is too large to be very useful, but fits the placement of Gnetales within conifers, perhaps as sister to the non-Pinaceae conifers ("gnecup" clade; Nickrent et al., 2000; Doyle, 2006; Chumley et al., 2008; Braukmann et al., 2009; Rydin & Korall, 2009), and also with the *Ephedra* pollen and seed fossil record.

The present biogeographic analysis (Figs. 2, 3) corroborates other molecular studies that have found New World clades of Oligocene age evolving out of Asian paraphyletic residuals, which is the classic pattern of Beringian disjunctions (for summaries, see Wen & Ickert-Bond, 2009). Our work also adds to a growing body of studies reporting long-distance dispersal between arid floras in North and South America (Moore et al., 2006). Finer-scale studies are now needed to test the broad-brush biogeographic scenario for *Ephedra* developed here.

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