

New insights into the evolutionary history of *Gnetum* (Gnetales)

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Abstract *Gnetum* (Gnetales: Gnetaceae) constitutes an evolutionarily isolated gymnosperm clade, comprising about 40 species that inhabit tropical areas of the world. While its closest living relative, the monotypic *Welwitschia*, has a well-documented fossil record from the Early Cretaceous, *Gnetum*-like fossils are rare and poorly understood. The phylogeny of *Gnetum* has been studied previously but the distant relationship to outgroups and the difficulty of obtaining plant material mean it is not yet fully resolved. Most species are tropical lianas with an angiospermous vegetative habit that are difficult to find and identify. Here a new phylogeny is presented based on nuclear and chloroplast data from 58 *Gnetum* accessions, representing 27 putative species, and outgroup information from other seed plants. The results provide support for South American species being sister to the remaining species. The two African species constitute a monophyletic group, sister to an Asian clade, within which the two arborescent species of the genus are the earliest diverging. Estimated divergence times indicate, in contrast with previous results, that the major lineages of *Gnetum* diverged in the Late Cretaceous. This result is obtained regardless of tree prior used in the BEAST analyses (Yule or birth-death). Together these findings suggest a correlation between early divergence events in extant *Gnetum* and the breakup of Gondwana in the Cretaceous. Compared to the old stem ages of major subclades of *Gnetum*, crown nodes date to the Cenozoic: the Asian crown group dates to the Cretaceous-Paleogene (K-Pg) boundary, the African crown group to the mid-Paleogene, and the South American crown group to the Paleogene-Neogene boundary. Although dispersal must have contributed to the current distribution of *Gnetum*, e.g., within South America and from Southeast Asian islands to the East Asian mainland, dispersal has apparently not occurred across major oceans, at least not during the Cenozoic.

Keywords Bayesian inference; birth-death prior; classification; node ages; phylogeny; Yule prior

Supplementary Material Electronic Supplement (Tables S1–S2) and alignment are available in the Supplementary Data section of the online version of this article at <http://www.ingentaconnect.com/iapt/tax>

■ INTRODUCTION

The Gnetales constitute three monotypic families and the genera *Ephedra* L., *Gnetum* L., and *Welwitschia* Hook.f. Based on morphological data, the three genera and the order have long been considered monophyletic (Hooker, 1863; Arber & Parkin, 1908; Crane, 1985) and this has been confirmed with molecular data (Chase & al., 1993; Price, 1996). Relationships among the three genera are well established based on morphological and molecular data; *Ephedra* is sister to a *Gnetum*-*Welwitschia* clade (Crane, 1985; Chase & al., 1993; Price, 1996). The three genera differ substantially from each other, morphologically as well as ecologically. While *Ephedra* and *Welwitschia* are open-habitat shrubs (in *Welwitschia* with arrested apical growth) that occur in arid, temperate to subtropical areas, *Gnetum* are ever-green lianas, or rarely trees, of moist tropical regions (Markgraf, 1929; Maheshwari & Vasil, 1961; Kubitzki, 1990; Won & Renner, 2006). The evolutionary history of the *Welwitschia* lineage is relatively well understood, due to the existence of well-documented *Welwitschia*-like fossils (e.g., described

by Crane & Upchurch, 1987; Duan, 1998; Rydin & al., 2003; Dilcher & al., 2005; Krassilov, 2009; Friis & al., 2014). These records indicate that the *Welwitschia* lineage was morphologically diverse and widespread in northern Gondwana during the Early Cretaceous, although it is today restricted to the Namib Desert in Africa. It can thus be assumed that also the *Gnetum* lineage has a long evolutionary history. In contrast, the crown group of extant species has been estimated to be comparatively young (late Oligocene to mid-Miocene, Won & Renner, 2006). Two Early Cretaceous fossils from the Yixian Formation of Liaoning (China) have been discussed as possible relatives of *Gnetum*, i.e., *Khitania columnispicata* (Guo & al., 2009) and *Siphonospermum simplex* (Rydin & Friis, 2010), but the respective phylogenetic positions of these fossils are uncertain.

In the most recent monograph of *Gnetum* (Markgraf, 1929), taxa are primarily classified based on differences in details of reproductive features. Plants of the genus (and the order) are functionally dioecious (Endress, 1996), and reproductive structures occur in whorled arrangements on spikes (Lotsy, 1899; Thoday, 1911; Pearson, 1912; Thompson, 1916; Pearson

& Thomson, 1917; Markgraf, 1929; Kubitzki, 1990; Endress, 1996). Each spike has a proximal bract pair and distally oriented collars (Strasburger, 1879), which may be funnel-shaped or flat and open (Markgraf, 1929, 1951, 1965; Fu & al., 1999a). Microsporangioophores typically have two microsporangia although deviations occur (Pearson, 1912, 1915; Markgraf, 1929). In male plants of most species, the uppermost whorl on each collar produces sterile and sometimes rudimentary ovules (Strasburger, 1872; Pearson, 1912, 1915).

According to Markgraf (1929), *Gnetum* consists of 30 species (Electr. Suppl.: Table S1). More recently, additional taxa have been described and in the World Checklist of Selected Plant Families (WCSP, 2014), 41 species are recognized (Electr. Suppl.: Table S1). Southeast Asia is considered the diversity “hotspot” of *Gnetum* (Kubitzki, 1990; Price, 1996; Won & Renner, 2006), but the genus is also present in South America and tropical Africa. A series of previous studies (Won & Renner, 2003, 2005a, b, 2006) investigated molecular evolution and phylogeny in *Gnetum*. Won & Renner (2006) addressed the phylogeny, divergence times and biogeography using two gene regions (*rbcL*, *matK*) and 9–13 *Gnetum* accessions (representing 9–12 putative species) plus outgroup taxa from across land plants. Based on initial results subsequent analyses, using six molecular markers (*rbcL*, *matK*, *trnT-trnF* region, rITS/5.8S, *LEAFY*, *nad1*) and 31 *Gnetum* accessions (representing 25 putative species), were conducted, rooted on South American *Gnetum*. The results indicated that *Gnetum africanum* Welw. is the next diverging species, sister to a clade comprising all Asian species, and that the two arborescent species are not “primitive” as had previously been suggested (Markgraf, 1929). However, some deep nodes are poorly supported and several questions remain, mainly regarding deep divergences, the phylogenetic placement of previously unsampled taxa, and species delimitation. Furthermore, although *Gnetum* is well studied in several respects, e.g., regarding embryogenesis (Coulter, 1908; Augustine & D’Souza, 1997; Friedman & Carmichael, 1998; Haycraft & Carmichael, 2001), life history (Lotsy, 1899; Pearson, 1912; Pearson & Thomson, 1917; Maheshwari & Vasil, 1961), anatomy and morphology (Berridge, 1911; Thoday, 1911, 1921; Duthie, 1912; Thompson, 1916; Rodin & Kapil, 1969; Fisher & Ewers, 1995; Endress, 1996) and palynology (Hesse, 1980; Gillespie & Nowicke, 1994; Yao & al., 2004; Tekleva & Krassilov, 2009), the data are often based on only one or a few species. Intraspecific variation and parallel evolution of similar traits (both of which are widespread in *Ephedra*; Ickert-Bond & Wojciechowski, 2004; Rydin & al., 2004, 2010; Huang & al., 2005; Rydin & Korall, 2009) have thus not been assessed in a modern phylogenetic framework. Also, species boundaries defined by Markgraf (1929, 1951, 1965) and other taxonomists (e.g., Fu & al., 1999a, b; Stevenson, 1999) have not been tested in a phylogenetic framework. Species boundaries are especially poorly defined in the core *Gnetum* clade in the tropical rainforests of Southeast Asia (e.g., Fu & al., 1999a, b), which has been suggested to have undergone recent radiation (Won, 2004; Won & Renner, 2006).

Here we build upon previous work, test hypotheses on deep divergences in *Gnetum* (Won & Renner, 2006), test the

monophyly of sections and subsections as defined in previous classifications (Endlicher, 1847; Griffith, 1859; Markgraf, 1929), address the phylogenetic positions of previously unsampled taxa, and assess the monophyly of species. To this end, we used expanded intraspecific sampling and molecular data from five markers, including two nuclear regions (18S, 26S) not previously utilized in phylogenetic studies of *Gnetum*. The study aims to provide new insights into the evolutionary history of *Gnetum*, as well as a framework for future work on the phylogeny, taxonomy, biogeography, and trait evolution in the group.

■ MATERIALS AND METHODS

Taxon sampling. — A 58-accession dataset was assembled. It comprised representatives from all major distribution areas, sections and subsections, and comprised 11 accessions of South American taxa, 6 accessions of African taxa, and 41 accessions of Asian taxa (Appendix 1). In total 27 currently recognised species of *Gnetum* were included, of which 6 have not been included in previous phylogenetic studies (*G. leyboldii* Tul., *G. camporum* (Markgr.) D.W.Stev. & Zanoni, *G. buchholzianum* Engl., *G. montanum* Markgr., *G. indicum* (Lour.) Merr. and *G. leptostachyum* Blume). Initial analyses were conducted using 39 outgroup taxa, representing lycopods, ferns, cycads, *Ginkgo*, conifers, *Ephedra* and *Welwitschia* (for GenBank accession numbers, see Appendix 2). For the final analyses, a more restricted outgroup sampling was employed, comprising *Welwitschia*, 16 *Ephedra* taxa, and 1 conifer, *Calocedrus* Kurz, on which the phylogeny was rooted. Divergence times were estimated based on a dataset of 20 *Gnetum* accessions, representing 19 putative species, spanning the known morphological variation and current distribution of the genus, and 39 outgroup taxa (see above and Appendix 2). The phylogeny was rooted on *Huperzia* Bernh. (Lycophyta).

Laboratory procedures and alignment. — Five molecular markers were selected with the aim to provide information on the deepest splits in the genus, as well as on relationships within subclades of the genus. To this end, the nuclear ribosomal DNA regions 18S, 26S, the internal transcribed spacers 1 and 2 (nrITS including 5.8S), and the chloroplast genes *rbcL* and *matK* were used. DNA was extracted, amplified and sequenced using standard procedures. Primers were newly developed for the present study or obtained from the literature (Table 1). In order to minimise problems with fungal contamination, the nrITS primers used (18SF and 26SR, Rydin & al., 2004) were designed to amplify on most land plants but not on fungi. Double bands were not observed following PCR amplification and double peaks were not observed in the original reading frames of the sequences. Sequence fragments were assembled using the Staden Package v.1.6 (Staden, 1996). The assembled sequences were aligned using MUSCLE v.3.8.31 (Edgar, 2004), with subsequent manual editing in Mesquite v.2.75 (Maddison & Maddison, 2011). All gene regions except nrITS could be confidently aligned against outgroup taxa. In *Gnetum* nuclear ITS1 is variable to the level at which homology assessment becomes uncertain (see also Won & Renner, 2005b)

Table 1. Primer sequences and references.

DNA region	Primer name	Primer sequence 5'–3'	Reference
18S forward	18S1	GCT TGT CTC AAA GAT TAA GCC	Rydin & al. (2004)
18S reversed	18Srev	CCT TCC TCT AAA CGA TAA GGT TC	Rydin & al. (2004)
26S forward	26S1	CGA CCC CAG GTC AGG CG	Kuzoff & al. (1998)
26S reversed	1229R	ACT TCC ATG ACC ACC GTC CT	Kuzoff & al. (1998)
ITS forward	18SF	GAA CCT TAT CGT TTA GAG GAA GG	Rydin & al. (2004)
ITS reversed	26SR	CCG CCA GAT TTT CAC GCT GGG C	Rydin & al. (2004)
<i>matK</i> forward	matK1-20F	CAG TTG TTA AAA TAA AAG GA	This study
<i>matK</i> reversed	matK f2	TCA ATG GAT GAG TAC TCG GC	Won & Renner (2006)
<i>matK</i> forward	matK1808R	TCC CCC CAA ACT GGG CTT GC	This study
<i>matK</i> reversed	Gn matK 1566R	AAA AAG CTC TGT AAA ATA AG	Won & Renner (2006)
<i>rbcL</i> forward	rbcL 5'	ATG TCA CCA CAA ACA GAG AC	This study
<i>rbcL</i> reversed	rbcL3rev	CCT TCC AGA CTT CAC AAG CAG C	Zurawski & Clegg (1987)

and was therefore only included for the Asian species. Nuclear 5.8S and nrITS2 were included for all species of *Gnetum*. Insertions and deletions were scored as missing data.

Parsimony analyses. — Each single-gene dataset was first analysed using equally weighted parsimony as implemented in PAUP* v.4.0b10 (Swofford, 2002). A heuristic search was conducted with the following settings: 100 random sequence additions and tree-bisection reconnection (TBR) branch swapping. A strict consensus of the most parsimonious trees was generated. Bootstrap support was calculated in PAUP*, with 1000 bootstrap replicates, each with 10 random sequence additions. No supported incongruence (measured as $\geq 70\%$ bootstrap support) was found among the gene trees and all the gene regions were therefore analysed in a combined supermatrix, comprising 7605 nucleotides.

Model selection and maximum likelihood analysis. — The fit of models of nucleotide sequence evolution to each single-gene dataset was tested using the Perl script MrAIC v.1.4.5 (Nylander, 2004) based on the software PhyML v.3.1 (Guindon & al., 2010). The optimal evolutionary model was selected from 24 candidate models using the corrected Akaike information criterion (AICc, Akaike, 1998). Similar tests were also carried out using jModeltest v.2.1.3, in which 88 candidate models are compared. The general time reversible model (Tavare, 1986)

with substitution rates drawn from a gamma distribution and an estimated proportion of invariable sites (GTR+I+ Γ) was preferred for all datasets (Table 2).

Maximum likelihood analysis was performed in PhyML on the combined, unpartitioned dataset using the subtree pruning and regrafting tree searching approach (Evans & Winter, 2006) and a parsimony tree as a starting tree. A fast likelihood-based method (aBayes) was chosen to obtain statistical support: a Bayesian-like transformation of an approximate likelihood ratio test (aLRT; Anisimova & Gascuel, 2006; Anisimova & al., 2011).

Bayesian analyses. — Bayesian inference of phylogeny was performed with the software MrBayes v.3.2.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist & al., 2012) on the nuclear and chloroplast datasets separately, and on both datasets together. The combined dataset was divided into two unlinked partitions (nuclear ribosomal regions, 4605 nucleotides; and chloroplast regions, 3000 nucleotides). Two parallel runs with four Markov Monte Carlo chains (MCMC) were run for 10^7 generations with a sampling frequency of 1000. Flat Dirichlet prior probabilities were selected for the substitution rates and the nucleotide frequencies. The prior probability of the shape parameter of the gamma distribution of rate variation was uniformly distributed in the interval (0.1, 50.0). A prior probability for the proportion of

Table 2. Sequence data description and model selection.

	18S	26S	ITS	<i>rbcL</i>	<i>matK</i>	Combined
Number of accessions in dataset	53	50	52	61	50	66
Number of newly generated accessions	37	39	39	34	37	186
Number of characters in dataset	1654	1234	1716	1335	1666	7605
Number of variable characters	245	353	792	311	987	2688
Number of phylogenetically informative characters	159	259	610	196	459	1683
Percent of informative characters (%)	10	21	36	15	28	22
Best-fitting evolutionary model (AICc weights) ^a	GTR+I+ Γ	GTR+I+ Γ	GTR+I+ Γ	GTR+I+ Γ	GTR+I+ Γ	GTR+I+ Γ

a AICc = corrected Akaike information criterion

invariable sites, uniformly distributed in the interval (0.0, 1.0), was also specified. Convergence of the two individual runs was evaluated in Tracer v.1.5 (Rambaut & Drummond, 2003) and AWTY (Nylander & al., 2008). The initial 2000 trees were removed as burn-in and the remaining trees summarized as a consensus tree. In addition, an analysis was performed on the combined dataset, using reversible jump MCMC (Huelsenbeck & al., 2004) as implemented in MrBayes v.3.2 (other settings as above).

Estimation of divergence times. — Divergence times were estimated in BEAST v.1.75 (Drummond & al., 2012) via the online cluster service Cyber-infrastructure for Phylogenetic Research (CIPRES, <http://www.phylo.org>; Miller & al., 2010). The dataset was partitioned and the substitution model specified as above. A strict clock was rejected using a posterior simulation-based analogue of the AIC (AICM; Baele & al., 2012). We therefore selected the UCLN model (Drummond & al., 2006), in which clock rates are independent among lineages and drawn from an underlying lognormal distribution, with a mean sampled from an exponentially distributed hyperprior with an initial value of 0.33 and mean of 0.33 and standard deviation sampled uniformly between 0 and 100 with an initial value of 1.0.

We explored the effect of different tree priors on node age estimates by comparing results of the birth-death (Kendall, 1948; Gernhard, 2008), Yule (Yule, 1925; Gernhard, 2008) and birth-death with incomplete sampling (Stadler, 2009) priors. The three models were compared and ranked based on the AICM in Tracer, by means of a comparison of the likelihood traces of posteriors obtained from the three independent MCMC analyses (one for each tree prior) with 1000 bootstrap replicates.

Next, three independent, full analyses were run using the birth-death tree prior. Each analysis was run for 100 million generations, sampling every 10,000. A random starting tree was selected, and topological constraints (Electr. Suppl.: Table S2) enforced, based on Rydin & Korall (2009), Leslie & al. (2012) and Bayesian results of the present study (see above). Convergence of runs was assessed in Tracer. The initial 10% of the sampled trees were removed as burn-in and post burn-in trees combined using log-Combiner v.1.75 (Drummond & al., 2012). Tree statistics were summarised on the maximum clade credibility tree with median node heights using Tree-Annotator v.1.75 (Rambaut & Drummond, 2003).

Calibrating the *Gnetum* tree. — Calibration to absolute time was accomplished using fossil data (nodes F1–F5; Electr. Suppl.: Table S2) and estimates on divergence times from the literature (nodes S1–S19; Electr. Suppl.: Table S2). For the former, we experimented with uniformly and lognormally distributed age priors, which yielded similar results. The latter was chosen for the final, full analyses. For secondary calibration (Ickert-Bond & al., 2009; Nagalingum & al., 2011; Leslie & al., 2012), normally distributed age priors were selected. We performed runs without the data to ensure that the priors do not dictate the posteriors or interact with each other (see, e.g., Heled & Drummond, 2012). Absolute age estimates of geological periods, epochs and ages follow interpretations in the GSA Geologic Time Scale (Gradstein & al., 2012).

The root node (crown group vascular plants, F1) was constrained based on the (unequivocally) oldest known members of either daughter lineage of the node, i.e., *Euphylllophyton bellum* S.G.Hao. from the Posongchong Formation (Yunnan, China) dated to the Early Devonian (Pragian; Hao, 1988) and phylogenetically placed along the stem leading to euphylllophytes (Kenrick & Crane, 1997), and *Asteroxylon mackiei* Kidst. & W.H.Lang (Kidston & Lang, 1920), an early lycopsid (Kenrick & Crane, 1997) from deposits that were radiometrically dated to the Early Devonian (Pragian, 408–411 Ma) by Rice & al. (1995). Based on these fossils, a lognormally distributed age prior with an offset of 408 Ma, a log mean of 0.5 (log stdev of 1.0) was assigned to the root node (F1), yielding a prior age distribution with a median of 410 Ma and a 97.5th percentile of 420 Ma.

Crown group euphylllophytes (node F2) was calibrated using the fossil *Pertica varia* Granoff & al. (Granoff & al., 1976), an early stem group member of seed plants (Kenrick & Crane, 1997). The fossil comes from the Battery Point Formation, which is considered to be of late Early Devonian age (mid- to late Emsian; Hoffman & Tomescu, 2013). Prior settings for node F2 were thus: offset of 393 Ma, a log mean of 1.5 (log stdev of 0.5), yielding a prior age distribution with a median of 398 Ma and a 97.5th percentile of 405 Ma.

The crown group monilophytes (node F3) was constrained using the fossil *Ibyka amphikoma* J.E.Skog & H.P.Banks from the late middle Devonian (Givetian; Skog & Banks, 1973), an early sphenophyte (Kenrick & Crane, 1997). Prior settings for node F3 were: offset of 383 Ma, a log mean of 1.0 (log stdev of 0.5), yielding a prior age distribution with a median of 386 Ma and a 97.5th percentile of 390 Ma.

The seed plants crown group (node F4) was constrained based on the oldest members of the clade, cordaites (Hilton & Bateman, 2006; Doyle, 2008), present from the Late Mississippian (Taylor & al., 2009). Prior settings for node F4 were: offset of 312 Ma, a log mean of 2.0 (log stdev of 1.0), yielding a prior age distribution with a median age of 319 Ma and a 97.5th percentile of 365 Ma.

The *Gnetum*-*Welwitschia* clade (node F5) was calibrated based on the extinct sister to *Welwitschia*, *Cratonia cotyledon* Rydin & al. (Rydin & al., 2003) from the lower part of the Crato Formation of the Ararape basin. The stratigraphy and age constraints of the basin have been debated and are poorly defined (Martill, 2007), but recent work assigns the Crato Formation to the late Aptian (e.g., Heimhofer & Hochuli, 2010). Prior settings for node F5 were thus: offset of 110 Ma, a log mean of 2.0 (log stdev of 0.5), i.e., a prior age distribution with a median age of 117 Ma and a 97.5th percentile of 130 Ma. Median values and 95% confidence intervals of all prior age distributions are summarized in Table S2 (Electr. Suppl.).

■ RESULTS

The phylogeny of *Gnetum*. — Results from the phylogenetic analyses did not reveal any supported topological incongruence among individual genes, nuclear versus chloroplast genes, or among the three different tree-building approaches.

There was also no conflict between results obtained with a priori model selection, and those obtained in the RJ-MCMC analyses. Adding a much larger set of outgroups, representing major clades of vascular plants, did not improve resolution within *Gnetum* compared to when Gnetales were analysed alone or when a single species of conifer was used as the outgroup. Relationships within *Gnetum* were generally well resolved in the likelihood and Bayesian analyses (see below), with only minor (i.e., unsupported) differences between these two analytical approaches. The results from the parsimony analysis were well supported within subclades of *Gnetum*, but relationships among these subclades were typically collapsed. Sequence data are described in Table 2.

Gnetum consisted of three major subclades, corresponding to their geographic distribution. A clade comprising South American taxa (clade A, Fig. 1) was sister to the remaining genus (clade D: LR [likelihood ratio] = 0.97, PP [posterior probability] = 0.99). African taxa (clade E: LR = 1.0, PP = 0.94) constituted the next diverging clade, sister to the Asian clade (clade F: LR = 1.0, PP = 0.93). The South American clade (clade A: LR = 1.0, PP = 1.0, BS [bootstrap support] = 99%) comprised a clade including *G. nodiflorum* Brongn. and *G. schwackeanum* Taub. ex Schenck (clade B: LR = 1.0, PP = 1.0, BS = 95%), sister to the remaining South American taxa (clade C: LR = 0.99, PP = 0.99). Within the latter, a clade comprising *G. leyboldii* and “*G. woodsonianum*” (accepted name: *G. leyboldii* var. *woodsonianum* Markgr.; LR = 1.0, PP = 1.0, BS = 67%) was sister to a clade comprising *G. camporum*, *G. urens* (Aubl.) Blume and *G. microstachyum* Spruce & Benth. ex Parl. (LR = 0.66, PP = 0.53). African taxa (clade E) were represented by *G. africanum* and *G. buchholzianum*. One accession of the latter taxon was nested among specimens of the former. A second accession of *G. buchholzianum* was sister to the remaining African taxa (LR = 1.0, PP = 0.99, BS = 87%). The Asian clade (clade F) comprised several well-resolved subclades. A clade comprising the arborescent species, *G. costatum* K.Schum. and *G. gnemon* L. (clade G: LR = 1.0, PP = 1.0, BS = 100%), was sister to the remaining Asian taxa (clade H: LR = 0.96, PP = 0.93). *Gnetum raya* Markgr. and *G. gnemonoides* Brongn. (clade I: LR = 1.0, PP = 1.0, BS = 100%) was the next diverging group. Its sister clade (clade J: LR = 1.0, PP = 1.0, BS = 87%) comprised two major clades. One of them (clade K: LR = 1.0, PP = 1.0, BS = 95%) consisted of *G. edule* (Willd.) Blume (LR = 1.0, PP = 1.0, BS = 100%), sister to a clade (clade L: LR = 1.0, PP = 1.0, BS = 95%) that comprised *G. latifolium* Blume, *G. neglectum* Blume and *G. leptostachyum* (clade M: LR = 1.0, PP = 1.0, BS = 91%) and a clade that comprised *G. tenuifolium* Ridl., *G. klossii* Merr. ex Markgr., *G. cuspidatum* Blume, *G. diminutum* Markgr. and *G. microcarpum* Blume (clade N: LR = 1.0, PP = 1.0, BS = 92%). *Gnetum latifolium* and *G. tenuifolium* were sister to the remaining taxa in their respective clades (LR = 1.0, PP = 1.0, BS = 80%; LR = 1.0, PP = 0.91, BS = 68%). A clade that comprised *G. parvifolium* (Warb.) W.C.Cheng, *G. indicum*, *G. luofuense* C.Y.Cheng, *G. montanum* and *G. hainanense* C.Y.Cheng was sister to clade K (clade O: LR = 1.0, PP = 1.0, BS = 91%). Resolution within this clade was poor. Furthermore, support for species monophyly was low in this clade, as in most subclades of the genus.

Node age estimates. — The birth-death and birth-death with incomplete sampling priors were the best-fitting to the data, with nonsignificant differences between them. In contrast, the Yule prior had a significantly worse fit to the data. Despite differences in model fit, node age estimates differed very little among the three approaches. The Yule prior yielded slightly older ages but age estimates overlapped almost entirely (Electr. Suppl.: Table S2). The results from the full analysis using the birth-death model are presented and discussed (Fig. 2). Effective age prior distributions, determined in the prior-only analysis, were consistent with those specified for all nodes except two: the *Ginkgo*-cycads clade (S1, effective calibration ca. 260 (230–320) Ma) and the Cupressophytes (S7, effective calibration ca. 250 (210–290) Ma). We deemed all effective prior distributions as acceptable based on the fossil record and age estimates in the literature (Magallón, 2010; Nagalingum & al., 2011; Leslie & al., 2012; Mao & al., 2012). The topological results were consistent with generally accepted views of vascular plant phylogeny, and with results of the undated phylogeny (Fig. 1), except for minor (i.e., unsupported) differences among Chinese taxa in clade O. The results were strongly supported across most of the nodes (Electr. Suppl.: Table S2), especially within *Gnetum*, with PP values of almost all nodes equal to 1.

Crown group *Gnetum* was dated to the Late Cretaceous (81 Ma; clade Q, 95% highest posterior density [HPD]: 64–98 Ma). In this epoch, the South American lineage diverged from the remaining taxa, which diverged into the African and Asian lineages 73 Myr ago (clade D, 95% HPD: 56–91 Ma). The age of the Asian crown group was estimated to 65 Ma (clade F, 95% HPD: 48–82 Ma), and the divergence of the arborescent lineage thus occurred around the Cretaceous-Paleogene (K-Pg) boundary. Node ages of major clades of *Gnetum* (clades A, E, G, H, I, J, K, O) dated to the Cenozoic. The lianoid Asian clade was dated to the Paleocene-Eocene boundary, 55 Ma (clade H, 95% HPD: 39–72 Ma). Clade J was dated to the late Eocene (35 Ma; 95% HPD: 22–51 Ma). Clade K, encompassing Malaysian and Indian species, was dated to the late Oligocene (26 Ma; 95% HPD: 14–39 Ma), whereas the Chinese species in clade O originated at the Paleogene-Neogene border (23 Ma; 95% HPD: 8–39 Ma). The African and the South American crown groups were found to be younger than the Asian crown group; the African clade was dated to the early Oligocene (33 Ma; clade E, 95% HPD: 10–58 Ma), and the South American clade to the early Miocene (22 Ma; clade A, 95% HPD: 8–39 Ma). For median posterior heights and 95% HPD of nodes outside of *Gnetum*, and results from analyses using the Yule and birth-death with incomplete sampling priors, see Electr. Suppl.: Table S2.

■ DISCUSSION

Deep evolutionary history of *Gnetum*. — Results of the present study provide strong support for a split between South American species and the remaining genus. There is no indication of conflicting information among gene regions regarding relationships, and this deep split in the genus is found regardless of approach. Confirming results of earlier work (e.g., Markgraf,

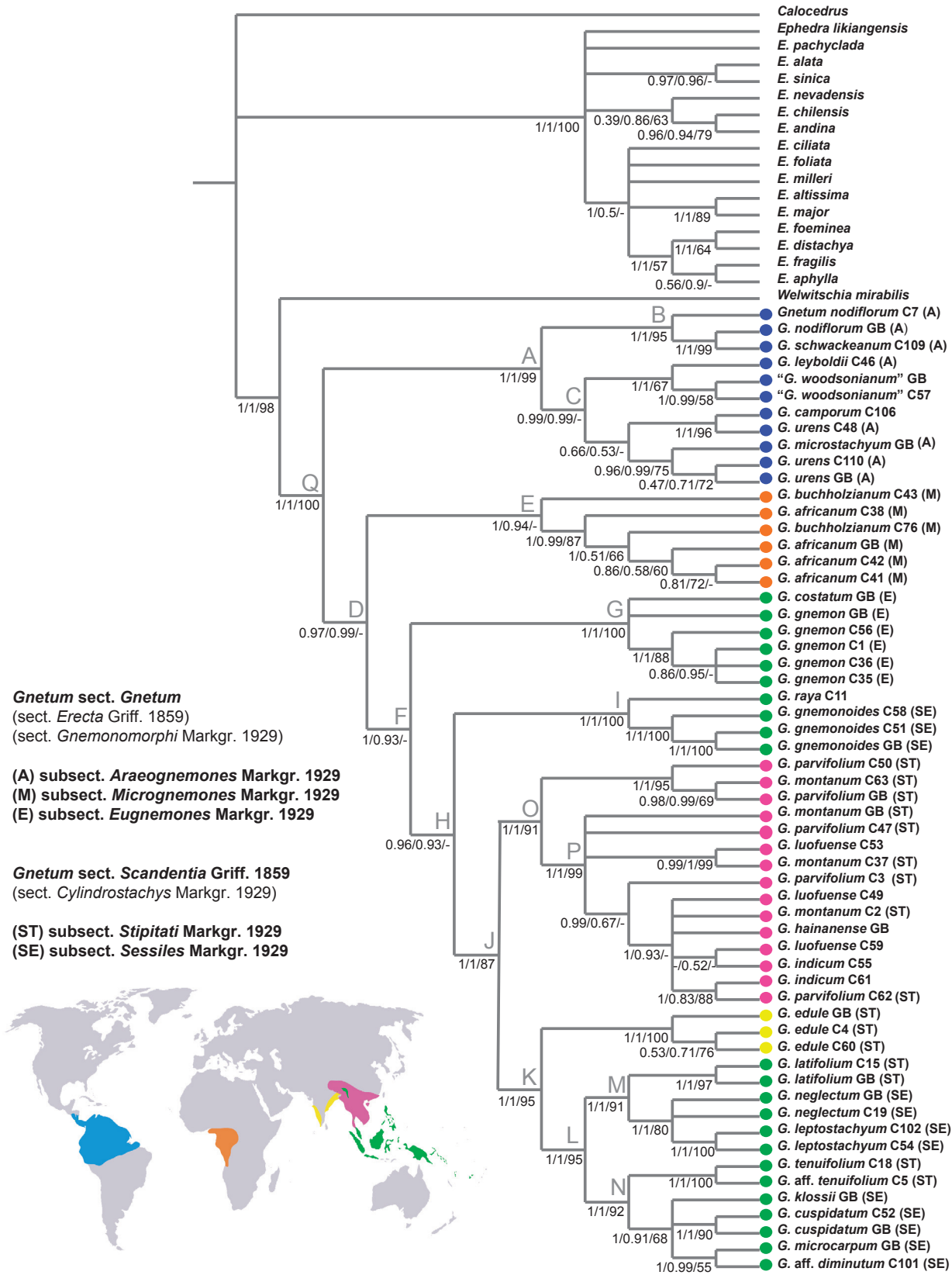


Fig. 1. Phylogeny of *Gnetum* based on combined analysis of three nuclear ribosomal gene regions (18S, 26S, nrITS) and two chloroplast genes (*rbcl*, *matK*) and Bayesian inference of phylogeny. Support values are given for each node (likelihood ratios/posterior probabilities/bootstraps values). The *Gnetum* specimens are labelled with coloured circles and abbreviations according to their respective distribution and classification (Griffith, 1859; Markgraf, 1929).

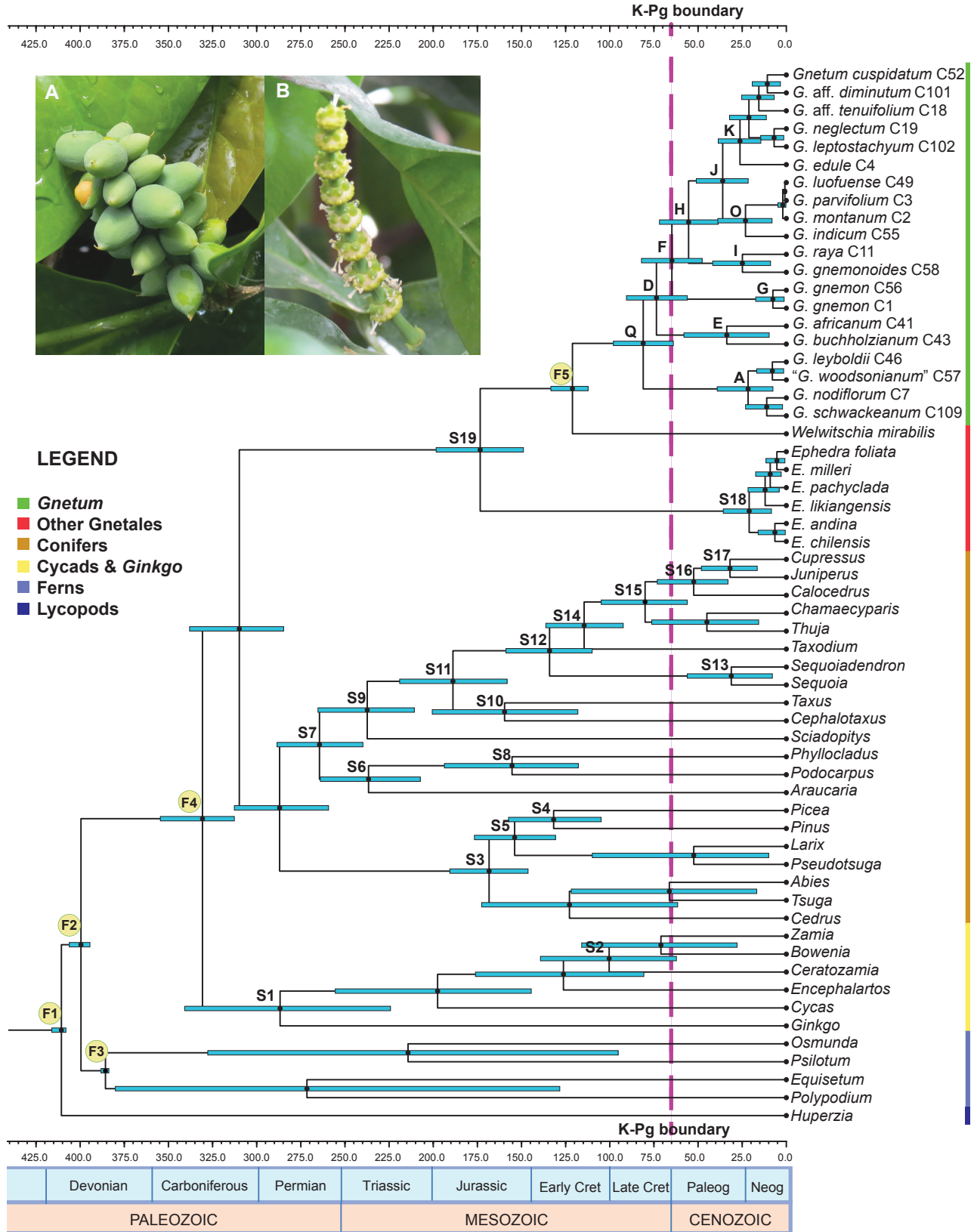


Fig. 2. Dated tree inferred from combined analysis of three nuclear regions (18S, 26S, nrITS) and two chloroplast genes (*rbcL*, *matK*) using a Bayesian framework and a relaxed molecular clock. Nodes marked F1 to F5 (in yellow circles) and S1 to S19 represent fossil calibration points and secondary calibration points, respectively. Light blue bars represent the node height 95% HPD intervals. The pink dashed line indicates the K-Pg boundary. Photos show (A) female and (B) male spikes of *Gnetum gnemon*.

1929; Won & Renner, 2006), subgroups of *Gnetum* reflect the geographical distribution of taxa. However, the resolution among and within clades differs from previous studies. The South American lineage, the African lineage, and the arborescent species (the Asian *G. gnemon* and *G. costatum*) all diverged early in the evolutionary history of crown group *Gnetum*. These clades, each strongly supported, are comparatively species poor; the majority of the diversity occurs in the lianoid, Asian clade (clade H).

Estimated divergence times of major clades are generally considerably older than those found previously. There is minor overlap in the confidence intervals of the age estimates presented here and those found by Won & Renner (2006) for their “Gnetales-sister” or “Anthophyte” topologies, but not when the Gnetales are sister to conifers or Pinaceae. Seed plant phylogeny is a difficult problem (e.g., Mathews, 2009). We accepted the results of our dating analysis (i.e., the “gnetifer” topology) and did not assess the effect of alternative placements of Gnetales because a close relationship between conifers and the Gnetales is generally accepted based on molecular data and is consistent with some morphological features (Doyle, 2008; Mathews, 2009). Furthermore, Won & Renner (2006) showed that age estimates for *Gnetum* are similar regardless of whether the Gnetales are nested within (“gnepine” topology) or sister to (“gnetifer” topology) conifers. There are several possible explanations for the older age estimates found in the present study, including methodological and sampling differences. For example, autocorrelated (Won & Renner, 2006) versus uncorrelated (this study) rates, where uncorrelated lognormal models have been demonstrated to lead to older ages than other methods (Magallón, 2010). Similarly, denser sampling can push back age estimates (see, e.g., Linder & al., 2005), although the sampling difference between the two *Gnetum* studies is not dramatic.

The temporal as well as topological results of the present study indicate that Gondwanan vicariance cannot be rejected as an explanation for the major biogeographical patterns. Dispersal is, however, also suggested based on our results, and has clearly been responsible for more recent geographical expansion within subclades of *Gnetum*; for example, dispersal between East Asia and Southeast Asia and across the South American continent. Several candidate dispersal vectors of *Gnetum* have been reported, including birds (Ridley, 1930), civet-cats (Markgraf, 1951), rodents (Forget & al., 2002), macaques (Corlett, 1996), and fish (Kubitzki, 1985). However, a mechanism by which long-distance dispersal over oceans might occur has to our knowledge not been clearly demonstrated. Some species of *Gnetum* have a corky layer in the seed (Maheshwari & Vasil, 1961; Rodin & Kapil, 1969), possibly allowing them to float. Nevertheless, with the potential exception of the origin of the Asian clade, we find no clear indication in our results that dispersal over oceans, such as the contemporary Atlantic, Pacific and Indian Oceans, has ever occurred.

Evidence for vicariance is rare (but see several recent studies, e.g., Mao & al., 2012; Pittermann & al., 2012; Beaulieu & al., 2013; Mennes & al., 2015). This is probably partly due to the age of the groups studied, frequently being too young to be

influenced by Gondwanan vicariance (Beaulieu & al., 2013), but also because long-distance dispersal cannot be ruled out as the mechanism underlying disjunct distributions, even for clades that are old enough to have been influenced by ancient vicariance events. Of particular interest, however, is that *Gnetum* comprises many old lineages, most of which contain only a few living species. This is akin to the pattern for some conifers, e.g., Podocarpaceae and some Cupressaceae (Leslie & al., 2012; Mao & al., 2012), but contrasts with patterns for other gymnosperm clades, where strikingly young crown ages have recently been inferred, e.g., for *Ephedra*, Pinaceae and cycads (Ickert Bond & al., 2009; Crisp & Cook, 2011; Nagalingum & al., 2011; Leslie & al., 2012). These contrasting patterns suggest that speciation and extinction processes have operated very differently among different gymnosperm clades, perhaps due to climatic or geographic differences (Leslie & al., 2012). Our ongoing research is addressing the generalities and some of the causes of these patterns in gymnosperms (A.M. Humphreys & C. Rydin, unpub.).

Previous and current classifications. — Endlicher (1847) divided *Gnetum* into two groups: *G.* sect. *Gnemon* (trees with erect stems) and *G.* sect. *Thoa* (woody shrubs; Electr. Suppl.: Table S1). He classified seven species. However, his conclusions conflict with subsequent work and current knowledge, and the classification is not useful. Another early classification scheme was postulated by Griffith (1859), who also divided *Gnetum* into two sections: *G.* sect. *Erecta* and *G.* sect. *Scandentia* (Electr. Suppl.: Table S1), based on a few species from the Tenasserim provinces in Southeast Asia (Griffith, 1859) (Electr. Suppl.: Table S1). Nearly half a century later, the taxonomy was revised by Markgraf (1929) and later further assessed (Markgraf, 1951, 1965), based on extensive sampling and field observations. *Gnetum* sect. *Erecta* (Griffith, 1859) corresponds to Markgraf’s (1929) *G.* sect. *Gnemonomorphi*. *Gnetum* sect. *Scandentia* (Griffith, 1859) corresponds to *G.* sect. *Cylindrostachys* of Markgraf (1929). Although Markgraf’s names have been widely accepted (Hutchinson & Dalziel, 1954; Phengklai & al., 1975; Lowe, 1984; Stevenson & Zanoni, 1991; Fu & al., 1999a; Rondón, 2000), Price (1996) pointed out that Griffith’s names have priority over those of Markgraf. Price (1996) nevertheless proposed the name *G.* sect. *Gnetum* instead of *G.* sect. *Erecta* Griff., based on the autonym rule of the *International Code of Nomenclature* (McNeill & al., 2012).

***Gnetum* sect. *Gnetum*.** — As in previous studies based on molecular data (Won & Renner, 2005a, b, 2006), our results resolve *G.* sect. *Gnetum* (as circumscribed by Markgraf, 1929 under the name *G.* sect. *Gnemonomorphi*) as paraphyletic; it comprises a basal grade of three clades (clades A, E and G, Fig. 1). The morphological similarities on which Markgraf’s (1929) circumscriptions are based (e.g., leaves often yellowish when dried, male spikes often with flat collars, and with clearly visible internodal areas between collars) vary among species in these clades, but are probably plesiomorphic features that reflect a shared ancestry of the entire genus rather than a close relationship of taxa referred to the section. However, the subsections of *G.* sect. *Gnetum* (Markgraf, 1929) are monophyletic (Fig. 1). *Gnetum* subsect. *Araeognemones* Markgr. (clade A) comprises the South American taxa, *G.* subsect.

Eugnemones Markgr. (clade G) corresponds to the arborescent taxa, and *G.* subsect. *Micrognemones* Markgr. (clade E) corresponds to the African taxa.

***Gnetum* sect. *Scandentia*.** — In contrast with previous studies based on molecular data (Won & Renner, 2003, 2005a, 2006), our results support monophyly of *G.* sect. *Scandentia* (as defined by Markgraf, 1929 under the name *G.* sect. *Cylindrostachys*). It corresponds to the lianoid Asian clade (clade H, Fig. 1), characterised by (male) spikes having densely positioned collars (Markgraf, 1929). As discussed above, however, the subsections of *G.* sect. *Scandentia* (*G.* subsect. *Sessiles* and *G.* subsect. *Stipitati*; Markgraf, 1929), defined by sessile versus stalked ovules and seeds, are not monophyletic according to our results (Fig. 1).

Relationships and divergence times within subclades of *Gnetum*. — The taxonomy of species and higher taxa needs to be rigorously studied for all subclades of *Gnetum*, using substantially increased sampling of taxa, and morphological as well as molecular data. Repeatedly, we find that clades with poorly supported species boundaries include both taxa that are morphologically variable and geographically widespread, which is sometimes reflected in the description of subspecific taxa, and taxa with a much more restricted distribution and low intraspecific variation. Some of these taxa are rare, or at least rarely collected, and are poorly described and known—there is a paucity of both literature and herbarium material for these species. Results in the present study highlight potential taxonomic problems and provide a framework for future taxonomic, phylogenetic and biogeographic work of individual subclades.

South American *Gnetum* (clade A). — South American taxa comprise a strongly supported clade, estimated to have originated in the Miocene. *Gnetum nodiflorum* and *G. schwackeanum* are sister to the remaining South American species (clade C). It is not clear from our results that they represent two separate species: *G. schwackeanum* is nested within *G. nodiflorum*. However, the species differ morphologically. *Gnetum nodiflorum* has smooth bark (Stevenson, 1999) and leaves that are dark green also when dry (Markgraf, 1929, 1965), whereas the bark of *G. schwackeanum* has prominent wings of cork (Stevenson, 1999) and leaves that may turn slightly black when dry (Markgraf, 1965). We have confirmed the identification of our specimen of *G. schwackeanum* (originally made by D. Stevenson), and a revision of these taxa may be needed.

Within clade C, *G. leyboldii* and “*G. woodsonianum*” (*G. leyboldii* var. *woodsonianum* Markgr.) are sisters. “*Gnetum woodsonianum*”, endemic to Colombia and Panama (Markgraf, 1965), is currently considered synonymous with *G. urens* (WCSP, 2014). Our results do not support such a treatment but are more in line with Markgraf’s (1965) opinion that “*G. woodsonianum*” is a variety of *G. leyboldii*. However, Markgraf (1965) reports morphological differences between the taxa: *G. leyboldii* has broadly elliptic leaves, which are smooth and shiny and has inflorescences with 3–4 whorls, whereas the leaves of “*G. woodsonianum*” are elliptic and not shiny, due to the occurrence of dense subepidermal fibres, and inflorescences have only 1–2 whorls (Markgraf, 1965). Considering these differences, “*G. woodsonianum*” and *G. leyboldii* could probably both be treated as species.

Remaining South American species included in the present study form a weakly supported clade, which comprises several specimens of *G. urens* and in addition *G. camporum* and *G. microstachyum*. The species boundaries appear unclear from our results, and nomenclatural treatments have differed in the past. *Gnetum microstachyum* has often been considered synonymous with *G. paniculatum* Spruce ex Benth. (Markgraf, 1929, 1965; Stevenson & Zanoni, 1991; Stevenson, 1999). *Gnetum urens* var. *camporum*, described by Markgraf (1971), was later ranked as a separate species, *G. camporum* (Stevenson & Zanoni, 1991; Stevenson, 1999). From our results it rather appears as if both *G. microstachyum* and *G. camporum* should be considered synonymous with *G. urens*. Additional study of a larger sample of specimens is needed to further elucidate species boundaries in the South American clade.

African *Gnetum* (clade E). — Only two species have traditionally been described from Africa: *G. africanum* and *G. buchholzianum*. The crown group is here estimated to be from the Oligocene, i.e., slightly older than the South American clade. The two African species differ morphologically from other species in the genus in that their male spikes lack the sterile ovules that are present in most species of *Gnetum* (Pearson, 1912, 1915; Markgraf, 1929). *Gnetum buchholzianum* is here included in a molecular study for the first time and while one accession of *G. buchholzianum* (C43) is sister to the remaining African accessions, the other specimen (*G. buchholzianum* C76) is nested among specimens of *G. africanum*. This result is somewhat surprising but should not be an artefact of misidentification; the material of accession C76 was originally identified by F. Markgraf and has been confirmed by us. Several authors have considered the two species significantly morphologically distinct to warrant species status (Pearson, 1912; Markgraf, 1929; Lowe, 1984; Clark & Sunderland, 2004; Ingram & al., 2012; Biye & al., 2014). For example, they differ in the shape of the collar on the male spikes and in the length and shape of the internodes in the spikes (Pearson, 1912; Markgraf, 1929). On the other hand, the two taxa have overlapping distributions in humid forests in Cameroon (Markgraf, 1929; Lowe, 1984; Biye & al., 2014), and hybridization may occur. Further, a recent revision of African *Gnetum* recognizes four species, thereby describing two new species (*G. latispicum* E.H.Biye, *G. interruptum* E.H.Biye, Biye & al., 2014), based primarily on male reproductive morphology. Further studies of the African lineage, including molecular phylogenetic analysis of all described species, are needed to clarify the diversity of *Gnetum* in Africa.

Asian *Gnetum* (clade F). — Asian taxa are monophyletic and the crown group is dated to the K-Pg boundary. It comprises two main sister clades: the arborescent (clade G) and the Asian, lianoid (clade H) taxa.

The arborescent Asian taxa (clade G). — The two arborescent species (*G. gnemon*, *G. costatum*) are strongly supported as a clade, sister to the remaining Asian taxa. These results differ from those of previous work. Markgraf (1929) suggested that the arborescent taxa are “ancestral” and among the earliest diverging taxa in the genus, whereas previous molecular phylogenetic studies have resolved the arborescent taxa as sister clade to the *G. gnemonoides*–*G. raya* clade (Won & Renner,

2005a, b, 2006). Although a sister relationship between the arborescent taxa and remaining *Gnetum* has been found in some molecular phylogenetic studies, e.g., using parsimony and/or in analyses with a much more restricted taxon sampling of *Gnetum* compared to the present study (e.g., Rydin & Korall, 2009; C. Rydin, pers. obs.), Markgraf's hypothesis on the arborescent taxa being "ancestral" must now be considered refuted.

Gnetum gnemon differs morphologically from *G. costatum* in having globose and short-tipped ovules, versus ovoid and long-tipped ovules in the latter (Markgraf, 1929, 1951). However, additional work on the status of these taxa using a substantially extended sampling is clearly needed. *Gnetum costatum* is endemic to New Guinea and the Solomon Islands with no described varieties, while *G. gnemon* is widely distributed in tropical regions of Southeast Asia, and several varieties are described (Markgraf, 1929; WCSP, 2014). Our results show the single sample of *G. costatum*, nested among several accessions of *G. gnemon*.

The lianoid Asian taxa (clade H). — The Asian lianoid clade is here resolved as monophyletic and it is also recognized based on morphology (Markgraf, 1929), characterised, e.g., by dense male spikes, in which internodes are not clearly visible between the collars (Markgraf, 1951). However, subdivision of the clade into species with sessile (*G.* subsect. *Sessiles*) versus stalked (*G.* subsect. *Stipitati*) ovules and seeds (Markgraf, 1929, see also Fig. 1) is not supported by our analyses. The sessile condition (of ovules and seeds) appears ancestral in the Asian lianoid clade, and stipitate ovules have probably evolved several times. The clade is estimated to have originated at the Paleocene-Eocene border, and *G. gnemonoides* and *G. raya* (clade I) are sister to remaining species in the Asian lianoid clade. As with *G. gnemon* (see above), *G. gnemonoides* has a broad distribution from the eastern Malaysian Peninsula to New Guinea (Markgraf, 1929, 1951), compared to its sister, *G. raya*, which is endemic to Borneo (Markgraf, 1967). Further, only one specimen of *G. raya* could be included in the present study and additional sampling of this poorly studied species would be necessary in future studies. Reduction to only one microsporangium on each microsporangiphore, instead of two, as is the prevailing condition in *Gnetum* (Griffith, 1859; Pearson, 1915), is reported for *G. gnemonoides* (Pearson, 1912; Markgraf, 1929). This may be a synapomorphy for the clade but we have not been able to obtain information on the number of microsporangia in each microsporangiphore for *G. raya*. Herbarium material that we have studied is restricted to female specimens and there is very little information in the literature on this species. Markgraf's (1967) description of *G. raya* is based entirely on female structures.

Clade K. — Our results resolved the Indian *G. edule* as sister to the remaining taxa in clade K. This differs from previous studies, in which the position of *G. edule* (syn. *G. ula*) is either unresolved with respect to other taxa in the equivalent of our clade K (Won & Renner, 2003), sister to the "latifolium clade" (Won & Renner, 2005a, b), or sister to the "cuspidatum clade" (Won & Renner, 2005a, b, 2006). *Gnetum edule* differs from most other species of clade K in that it has stipitate ovules and seeds (Markgraf, 1929, 1951).

Within clade L, *G. latifolium*, *G. neglectum*, and *G. leptostachyum* comprise a well-supported group (clade M). It corresponds to the "latifolium clade" of Won & Renner (2005a, b, 2006), which in their studies comprised *G. latifolium* and *G. neglectum*. Our results thus show that *G. leptostachyum* also belongs to the group, being most closely related to *G. neglectum*. *Gnetum latifolium* has a broad distribution in the Malay Archipelago and Peninsula, and New Guinea, and several varieties have been described (Markgraf, 1929, 1951). In contrast, *G. neglectum* and *G. leptostachyum* are endemic to Borneo with few (or no) varieties described (Markgraf, 1929, 1951). Further, also clade K exemplifies that the classical practice of dividing Asian species of *Gnetum* into groups based on stalked or sessile ovules and seeds (Markgraf, 1929) is not supported by our results; *G. latifolium* has stalked ovules and seeds, whereas *G. neglectum* and *G. leptostachyum* have sessile ovules and seeds (Markgraf, 1929, 1951).

A second subclade within clade K comprises *G. tenuifolium*, *G. klossii*, *G. cuspidatum*, *G. diminutum*, and *G. microcarpum* (clade N). It corresponds to the "cuspidatum clade" of Won & Renner (2005a, b, 2006), and their results indicate that clade N in addition comprises *G. macrostachyum* Hook.f. and *G. acutum* Markgr. (Won & Renner, 2006). In the present study, *G. tenuifolium* is well supported as sister to the remaining species, while Won and Renner (2006) found it to be unresolved with *G. macrostachyum* and *G. acutum*, sister to *G. cuspidatum*. As for clade M, some species in clade N have a relatively broad distribution in the Malay Peninsula and Archipelago (*G. tenuifolium*, *G. microcarpum*, *G. cuspidatum*), whereas other taxa are much more local, being endemic to parts of Borneo (*G. klossii* and *G. diminutum*; Markgraf, 1929). Resolution among these taxa is limited and additional information is needed to assess the taxonomy in the clade.

Clade O. — Clade O consists of taxa, which in contrast to those of clade K, are distributed mainly in continental Southeast Asia. The clade corresponds to the "hainanense clade", represented by *G. parvifolium* and *G. hainanense* in previous studies (Won & Renner, 2005a, b, 2006). Here, the clade is demonstrated to comprise five taxa, *G. parvifolium*, *G. luofuense*, *G. indicum*, *G. hainanense* and *G. montanum*. However, although resolution within the clade is generally poor, several of these taxa occur in more than one well-supported subclade—taxonomic revision is clearly needed. The status of several of these names has been questioned previously. For example, *G. indicum* was considered a doubtful species by Markgraf (1929, 1951), and he stated that specimens identified as such in fact belong to other species: *G. latifolium*, *G. formosum* Markgr. or *G. montanum* (Markgraf, 1951). The Chinese *G. luofuense* and *G. hainanense* constitute another example; these names have been questioned because of equivocal key characters (Fu & al., 1999a, 1999b). Our results tentatively support these arguments. Further, according to our results, the name *G. parvifolium* (Cheng, 1964) may represent several species. Thus, as with most clades in *Gnetum*, additional studies based on densely sampled morphological and molecular data are needed to further clarify species delimitation in clade O.

Concluding remarks. — While the *Welwitschia* lineage is well known from the Early Cretaceous, early diversity of *Gnetum* is poorly documented in terms of fossil evidence. However, stem relatives of *Gnetum* were clearly present in the Early Cretaceous, and our topological and temporal results indicate that early lineage diversification in the crown group occurred in the Late Cretaceous, possibly as a consequence of the breakup of Gondwana. Further studies are needed to assess species delimitation and morphological variation in all subclades of the genus.

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Appendix 1. Vouchers of studied species.

Species (synonymous name), geographic origin, collector and number of collection (herbarium) of material used in the present study, 18S, 26S, nrITS, *rbcl* and *matK*. An asterisk (*) denotes sequences newly produced for the present study; a dash (–) denotes missing information or sequences not available.

Calocedrus formosana Kurz, D85293, U90707, –, L12569, AF152179; *Ephedra alata* Decne., Egypt, *Abd Ealeem s.n.* (CAI), AY755698, AY755732, –, AY755805, KP997304*; *Ephedra altissima* Desf., Algeria, *Samuelsson 6227* (S S-C-628), AY755697, AY755731, –, AY755804, KP997305*; *Ephedra andina* Poepp. ex C.A.Mey, AY755670, AY755707, –, AY755782, –, *Ephedra aphylla* Forsk., Egypt, *W. Amer s.n.* (CAI), AY755695, AY755729, –, AY755802, KP997306*; *Ephedra chilensis* C.Presl., AY755691, AY755725, –, AY755799, AY492012; *Ephedra ciliata* Fisch. & C.A.Mey, Oman, *Ghazanfar 1914* (BR 13165406), AY755700, AY755776, –, AY755807, KP997307*; *E. distachya* L., AY755686, AY755719, –, AY755793, AY492013; *Ephedra foeninea* Forsk., Greece, *Rydin 332* (S), AY755677, AY755710, –, AY755784, KP997308*; *Ephedra foliata* Boiss. & C.A.Mey., AY755699, AY755734, –, AY755806, –, *Ephedra fragilis* Desf., Cyprus, *J. Walter 7840* (WU 2007-0002466), FJ957974, FJ957992, –, FJ958034, KP997309*; *Ephedra likiangensis* Florin, AY755690, AY755724, –, AY755798, –, *Ephedra major* Host, Spain, *Debreczy & al. 46206* (S S03-164), AY755702, AY755737, –, AY755809, KP997310*; *Ephedra milleri* Freitag & Maier-St., FJ957983, FJ958002, –, –, *Ephedra nevadensis* S.Watson, AY755688, AY755722, –, AY755796, AY492020; *Ephedra pachyclada* Boiss., AY755703, AY755738, –, AY755810, –, *Ephedra sinica* Stapf, AY755673, AY056488, –, AY056562, AY492024; *Gnetum* aff. *diminutum* Markgr., Sandakan (Malaysia), *Poster & Won SAN 151128* (MO 6064647), KP256588*, KP256625*, KP256664*, –, KP256702*; *Gnetum* aff. *tenuifolium* Ridl., Malaysia, *Lian V0080* (GB), KP256587*, KP256624*, KP256663*, KP256740*, KP256701*, *Gnetum africanum* Welw., Yaounde (Cameroun), *Tadjouteu 608* (BR 952296), KP256568*, KP256605*, KP256643*, KP256719*, KP256682*; *Gnetum africanum* Welw., Équateur (Democratic Republic of the Congo), *Luke 10375Z* (BR 952296), KP256567*, KP256604*, KP256642*, KP256718*, KP256681*; *Gnetum africanum* Welw., Haut-Ogooué (Gabon), *Niangadouma & Walters 194* (BR 503550), KP256566*, KP256603*, KP256641*, KP256717*, KP256680*; *Gnetum africanum* Welw., U43012, –, AY449543, AY296527, AY449631; *Gnetum buchholzianum* Engl., Yaounde (Cameroun), *Breitel 2002* (BR 13165097), KP256569*, –, –, KP256720*, –, *Gnetum buchholzianum* Engl., Cameroun, *Zenker 4134* (E), –, –, KP256721*, –, *Gnetum camporum* (Markgr.) D.W.Stev. & Zanoni (= *Gnetum urens* var. *camporum*), Potaro-siparuni (Guyana), *Henkel 1111* (NYBG 743502), –, –, KP256646*, KP256724*, –, *Gnetum costatum* K. Schum., AY755661, AY056497, –, AY296528, –, *Gnetum cuspidatum* Blume, Java (Indonesia), *Won 551* (KRB), KP256584*, KP256621*, KP256660*, KP256737*, KP256698*; *Gnetum cuspidatum* Blume, –, –, AY449547–AY449551, AY296530, AY449626; *Gnetum edule* (Wild.) Blume (= *Gnetum ula*, *Gnetum scandens*), India, *Maheshwari 63-0192* (UC), KP256582*, KP256619*, KP256658*, KP256735*, KP256696*; *Gnetum edule* (Wild.) Blume (= *Gnetum ula*, *Gnetum scandens*), (cult.), *Ickert-Bond s.n.* (ALA), KP256583*, KP256620*, KP256659*, KP256736*, KP256697*; *Gnetum gnemon* L. (= *Gnetum brunonianum*), (cult.), *Rydin s.n.* (S), KP256576*, KP256612*, KP256651*, KP256728*, KP256689*; *Gnetum gnemon* L. (= *Gnetum brunonianum*), (cult.), *Hou & Rydin 1002* (S), KP256578*, KP256614*, KP256653*, KP256730*, KP256691*; *Gnetum gnemon* L. (= *Gnetum brunonianum*), (cult.), *Hou & Rydin 1001* (S), KP256577*, KP256613*, KP256652*, KP256729*, KP256690*; *Gnetum gnemon* L. (= *Gnetum brunonianum*), (cult.), *Stevenson s.n.* (NYBG), KP256579*, KP256615*, KP256654*, KP256731*, KP256692*; *Gnetum gnemon* L. (= *Gnetum brunonianum*), AY755660, –, AY449558, AY296533, AY449621; *Gnetum gnetonoides* Brongn., Java (Indonesia), *Won 553* (KRB), –, KP256617*, KP256656*, KP256733*, KP256694*; *Gnetum gnetonoides* Brongn., Malaysia, *Poster & Won SAN 151121* (MO), KP256580*, KP256616*, KP256655*, KP256732*, KP256693*; *Gnetum gnetonoides* Brongn., –, –, AY296540, AY449625; *Gnetum hainanense* C.Y.Cheng ex L.K.Fu, Y.F.Yu & M.G.Gilbert, –, –, AY449565, AY296546, AY449623; *Gnetum indicum* (Lour.) Merr. (= *Gnetum parvifolium*), (cult.), *Stevenson s.n.* (NYBG), KP256598*, KP256635*, KP256674*, KP256749*, KP256711*; *Gnetum indicum* (Lour.) Merr. (= *Gnetum parvifolium*), (cult.), *Rydin s.n.* (S), KP256597*, KP256634*, KP256673*, KP256748*, KP256710*; *Gnetum klossii* Merr. ex Markgr., –, AY449582, AY449583, AY296550, –, *Gnetum latifolium* Blume, Malaysia, *Maxwell 78-227* (U), KP256585*, KP256622*, KP256661*, KP256738*, KP256699*; *Gnetum latifolium* Blume, –, –, AY449586, AB715153, –, *Gnetum leptostachyum* Blume, Sabah (Malaysia), *Poster & Won SAN 151114* (MO 6064655), KP256589*, KP256626*, KP256665*, KP256741*, KP256703*; *Gnetum leptostachyum* Blume, Sandakan (Malaysia), *Poster & Won SAN 151112* (MO), KP256590*, KP256627*, KP256666*, KP256742*, KP256704*; *Gnetum leyboldii* Tul., (cult.), *Al-Shehbaz & Lalumon 9501* (MO 82-0028), KP256572*, KP256608*, KP256647*, KP256725*, KP256685*; *Gnetum luofuense* C.Y.Cheng, Hong Kong (China), *Pang PCC48* (S), KP256592*, KP256629*, KP256668*, KP256743*, KP256706*; *Gnetum luofuense* C.Y.Cheng, Guangdong (China), *Won & Hang 600* (IBSC), KP256594*, KP256631*, KP256670*, KP256745*, KP256708*; *Gnetum luofuense* C.Y.Cheng, Hainan (China), *Ickert-Bond 1358* (ALA), KP256593*, KP256630*, KP256669*, KP256744*, KP256707*; *Gnetum microcarpum* Spruce & Benth. ex Parl. (= *Gnetum apiculatum*, *Gnetum neglectum* var. *microcarpum*), –, –, AY449589–AY449592, AY296558, –, *Gnetum microstachyum* Spruce & Benth. ex Parl. (= *Gnetum paniculatum*), –, –, AY296560, –, –, *Gnetum montanum* Markgr., AY755664, AY056496, –, AY056575, AF280994;

Appendix 1. Continued.

Gnetum montanum Markgr., Guangdong (China), *Cheng & Wang s.n.* (SZG), KP256600*, KP256637*, KP256676*, KP256750*, KP256713*; *Gnetum montanum* Markgr., Yunnan (China), *Yin s.n.* (HITBC), KP256595*, KP256632*, KP256671*, KP256746*, –; *Gnetum montanum* Markgr., (cult.), *Desitem 115-2* (E), KP256596*, KP256633*, KP256672*, KP256747*, KP256709*; *Gnetum neglectum* Blume, –, AY449600, AY296563, –; *Gnetum neglectum* Blume, Borneo (Malaysia), *van Niel s.n.* (U 0581657), KP256591*, KP256628*, KP256667*, –, KP256705*; *Gnetum nodiflorum* Brongn., Kalencia (Ecuador), *Balslev 84747* (GB), KP256570*, KP256606*, KP256644*, KP256722*, KP256683*; *Gnetum nodiflorum* Brongn., U42415, –, AY449601, AY296564, AY449622; *Gnetum parvifolium* (Warb.) W.C.Cheng (= *Gnetum scandens* var. *parvifolium*, *Gnetum montanum*), AY755662, AY755704, AY449604, AY296565, –; *Gnetum parvifolium* (Warb.) W.C.Cheng (= *Gnetum scandens* var. *parvifolium*, *Gnetum montanum*), Guangdong (China), *Cheng & Wang s.n.* (SZG), KP256601*, KP256638*, KP256677*, KP256751*, KP256714*; *Gnetum parvifolium* (Warb.) W.C.Cheng (= *Gnetum scandens* var. *parvifolium*, *Gnetum montanum*), Guangdong (China), *Al-Shehbaz & Lalumon s.n.* (MO, #93-0791-1), –, KP256640*, KP256679*, KP256753*, KP256716*; *Gnetum parvifolium* (Warb.) W.C.Cheng (= *Gnetum scandens* var. *parvifolium*, *Gnetum montanum*), Hong Kong (China), *Pang PCC49* (SUNIV), KP256599*, KP256636*, KP256675*, –, KP256712*; *Gnetum parvifolium* (Warb.) W.C.Cheng (= *Gnetum scandens* var. *parvifolium*, *Gnetum montanum*), (cult.), *Kato s.n.* (TI), KP256602*, KP256639*, KP256678*, KP256752*, KP256715*; *Gnetum raya* Markgr., Kalimantan (Malaysia), *Kessler 624* (U 0246872), KP256581*, KP256618*, KP256657*, KP256734*, KP256695*; *Gnetum schwackeanum* Taub. ex Schenck, Amazonas (Brazil), *Teixeira & al. 87* (NYBG 01843099), KP256571*, KP256570*, –, AY755782, –; KP256645*, KP256723*, KP256684*; *Gnetum tenuifolium* Ridl., Gombak (Malaysia), *Carlquist 8087* (U 0277388), KP256586*, KP256623*, KP256662*, KP256739*, KP256700*; *Gnetum urens* (Aubl.) Blume, Kabalebo (Surinam), *Bordenave & al. 8306* (NYBG 1843110), KP256573*, KP256609*, KP256648*, –, KP256686*; *Gnetum urens* (Aubl.) Blume, Amazonas (Columbia), *Kress & al. 91-3271* (MO #92-1080), KP256574*, KP256610*, KP256649*, KP256726*, KP256687*; *Gnetum urens* (Aubl.) Blume, U42417, –, AY449611, AY296569, AY449629; “*Gnetum woodsonianum*” (= *Gnetum leyboldii* var. *woodsonianum* Markgr.), (cult.), *Stevenson s.n.* (NYBG), KP256575*, KP256611*, KP256650*, KP256727*, KP256688*; “*Gnetum woodsonianum*” (= *Gnetum leyboldii* var. *woodsonianum* Markgr.), –, –, AY449617, AY296570, AY449628; *Welwitschia mirabilis* Hook.f., U43013, AY056484, –, AJ235814, AF280996.

Appendix 2. Vouchers of species sampled for the analysis of divergence times of clades.

Genus/species name, collector and number of collection (herbarium) of material used in the present study, 18S, 26S, ITS, *rbcl* and *matK*. An asterisk (*) denotes sequences newly produced for the present study; a dash (–) denotes sequences not available (for detailed voucher information and geographic origin of *Gnetum* specimens, see Appendix 1).

Abies Mill., DQ371809, AY056508, –, EU269028, JQ512383; *Araucaria* Juss., AF051792, FJ179544, –, U96467, AF456373; *Bowania* Hook., FJ179549, AY056480, –, AF531201, GQ203826; *Calocedrus* Kurz, D85298, EU161353, –, L12569, HM023982; *Cedrus* Duham., AB026936, AY056507, –, JQ512520, AF295025; *Cephalotaxus* Siebold & Zucc. ex Endl., D38241, EU161354, –, AB027312, HQ245920; *Ceratozamia* Brongn., EU161295, AY056482, –, –, –; *Chamaecyparis* Spach, EF053165, AY056506, –, L12570, JQ512405; *Cupressus* L., AF051797, EU161342, –, L12571, HM023992; *Cycas* L., D85297, DQ008667, –, AY056556, –; *Encephalartos* Lehmann, EU161302, EU161366, –, AF531206, –; *Ephedra andina* Poepp. ex C.A.Mey., AY755670, AY755707, –, AY755782, –; *Ephedra chilensis* C.Presl., AY755679, AY755725, –, AY755799, AY492012; *Ephedra foliata* Boiss. & C.A.Mey., AY755699, AY755734, –, FJ958030, –; *Ephedra likiangensis* Florin, AY755690, AY755724, –, AY755798, –; *Ephedra milleri* Freitag & Maier-St., FJ957983, FJ958002, –, –, –; *Ephedra pachyclada* Boiss., AY755703, AY755738, –, AY755810, –; *Equisetum* L., AF313576, EU161363, –, AB574691, –; *Ginkgo biloba* L., D16448, AY095475, –, AJ235804, HQ619815; *Gnetum* aff. *diminutum* Markgr., *Poster & Won SAN 151128* (MO 6064647), KP256588*, KP256625*, KP256664*, –, KP256702*; *Gnetum* aff. *tenuifolium* Ridl., *Carlquist 8087* (U 0277388), KP256586*, KP256623*, KP256662*, KP256739*, KP256700*; *Gnetum africanum* Welw., *Luke 10375Z* (BR 952296), KP256567*, KP256604*, KP256642*, KP256718*, KP256681*; *Gnetum buchholzianum* Engl., *Breteler 2002* (BR 13165097), KP256569*, –, –, KP256720* –; *Gnetum cuspidatum* Blume, *Won 551* (KRB), KP256584*, KP256621*, KP256660*, KP256737*, KP256698*; *Gnetum edule* Brongn., *Mareshwari 63-0192* (UC), KP256582*, KP256619*, KP256658*, KP256735*, KP256696*; *Gnetum gnemon* L., *Rydin s.n.* (S), KP256576*, KP256612*, KP256651*, KP256728*, KP256689*; *Gnetum gnemon* L., *Stevenson s.n.* (NYBG), KP256579*, KP256615*, KP256654*, KP256731*, KP256692*; *Gnetum gnemonoides* Brongn., *Poster & Won SAN 151121* (MO), KP256580*, KP256616*, KP256655*, KP256732*, KP256693*; *Gnetum indicum* (Lour.) Merr., *Stevenson s.n.* (NYBG), KP256598*, KP256635*, KP256674*, KP256749*, KP256711*; *Gnetum leptostachyum* Blume, *Poster & Won SAN 151114* (MO 6064655), KP256589*, KP256626*, KP256665*, KP256741*, KP256703*; *Gnetum leyboldii* Tul., *Al-Shehbaz & Lalumon 9501* (MO 82-0028), KP256572*, KP256608*, KP256647*, KP256725*, KP256685*; *Gnetum luofuense* C.Y.Cheng, *Pang PCC48* (SUNIV), KP256592*, KP256629*, KP256668*, KP256743*, KP256706*; *Gnetum montanum* Markgr., *Cheng & Wang s.n.* (SZG), KP256600*, KP256637*, KP256676*, KP256750*, KP256713*; *Gnetum neglectum* Blume, *van Niel s.n.* (U 0581657), KP256591*, KP256628*, KP256667*, –, KP256705*; *Gnetum nodiflorum* Brongn., *Balslev 84747* (GB), KP256570*, KP256606*, KP256644*, KP256722*, KP256683*; *Gnetum parvifolium* (Warb.) W.C.Cheng, *Cheng & Wang s.n.* (SZG), KP256601*, KP256638*, KP256677*, KP256751*, KP256714*; *Gnetum raya* Markgr., *Kessler 624* (U 0246872), KP256581*, KP256618*, KP256657*, KP256734*, KP256695*; *Gnetum schwackeanum* Taub. ex Schenck, *Teixeira & al. 87* (NYBG 01843099), KP256571*, KP256607*, KP256645*, KP256723*, KP256684*; “*Gnetum woodsonianum*” (= *Gnetum leyboldii* var. *woodsonianum* Markgr.), *Stevenson s.n.* (NYBG), KP256575*, KP256611*, KP256650*, KP256727*, KP256688*; *Huperzia* Bernh., X81964, DQ026522, –, DQ464224, –; *Juniperus* L., D38243, AY056504, –, L12573, KJ661402; *Larix* Mill., D85294, AY056502, –, AB501189, AB501189; *Osmunda* L., U18516, –, –, AB024948, –; *Phyllocladus* Rich. ex Mirb., D38244, EU161339, –, AB027315, AY442149; *Picea* A.Dietr., L01782, AY056510, –, AY056579, JX508309; *Pinus* L., X75080, AY056499, –, JQ512580, JQ512471; *Podocarpus* Labill., AF051796, DQ008664, –, JQ512600, JN627357; *Polypodium* L., DQ629431, U90712, –, KF909063, –; *Pseudotsuga* Carrière, AB026941, AY056498, –, JN854170, AF143439; *Psilotum* Sw., U18519, EU161326, –, AP004638, –; *Sciadopitys* Siebold & Zucc., D85292, EU161318, –, L25753, AB023994; *Sequoia* Endl., AY686598, U90701, –, L25755, AF152209; *Sequoiadendron* J.Buchholz., EU161296, AJ271027, –, AY056580, JQ512480; *Taxodium* Rich., EF053176, EU161346, –, JQ512606, HQ245912; *Taxus* L., D16445, AY056513, –, EF660721, JF725847; *Thuja* L., JF725733, AY056503, –, JQ512619, HQ245914; *Tsuga* Carrière, AB026942, AY056511, –, AY056581, JQ512502; *Welwitschia mirabilis* Hook.f., U43013, AY056484, –, AJ235814, AF280996; *Zamia* L., M20017, AF531256, –, AY056557, AB645727.