JSE Journal of Systematics and Evolution

Journal of Systematics and Evolution 49 (4): 315–329 (2011) doi: 10.1111/j.1759-6831.2011.00129.x

Research Article

Revisiting taxonomy, morphological evolution, and fossil calibration strategies in Chloranthaceae

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Abstract Chloranthaceae is one of the earliest diverging angiosperm families and is comprised of approximately 75 species in four genera (*Chloranthus*, *Sarcandra*, *Ascarina*, and *Hedyosmum*). This family has received considerable attention because of its seemingly primitive morphology, disjunct tropical distribution in Asia and America, and extensive fossil record from the Early Cretaceous. In the present study, we reconstructed the phylogeny of Chloranthaceae based on a combined dataset of three plastid DNA regions and 56 species. We then estimated divergence times in the family using two relaxed molecular clock methods (BEAST and penalized likelihood). We focused on testing the influence of fossil taxa in calibrating the molecular phylogeny, and on assessing the current taxonomy of the family in light of the phylogenetic results. Our results indicate that most intrageneric divisions within *Ascarina* and *Hedyosmum* are not monophyletic. The results from the dating analysis suggest that the *Hedyosmum*-like fossil *Asteropollis* represents a stem lineage of *Hedyosmum*, as has been suggested previously from morphological analyses. In contrast, our results indicate that the *Chloranthus*-like fossil *Chloranthistemon*, previously suggested on morphological grounds to be a stem relative of *Chloranthus*, may, instead, belong to the branch leading to the clade *Chloranthus* + *Sarcandra*. The median crown ages of *Chloranthus*, *Sarcandra*, *Ascarina*, and *Hedyosmum* estimated in the BEAST analysis were 26.3, 9.5, 31.0 and 45.8 million years ago (Ma), respectively, whereas the divergence between *Chloranthus* and *Sarcandra*, the splitting of *Ascarina* with the former two genera, and *Hedyosmum* separating from the three genera were estimated to 63.8, 95.7 and 111.1 Ma. The present study sheds further light on the temporal evolution of Chloranthaceae and exemplifies how molecular dating analyses may be used to explore alternative phylogenetic placements of fossil taxa.

Key words angiosperm evolution, *Asteropollis*, Chloranthaceae, *Chloranthistemon*, molecular dating, phylogeny.

Chloranthaceae is an early divergent angiosperm lineage, comprising four extant genera (*Chloranthus*, *Sarcandra*, *Ascarina*, and *Hedyosmum*) and approximately 75 species (Todzia, 1988; Eklund, 1999). Morphologically, *Chloranthus* and *Sarcandra* possess the simplest bisexual flower in angiosperms, which contains a single stamen (*Sarcandra*) or a tripartite androecium (*Chloranthus*) adnate to the abaxial side of the carpel. *Ascarina* and *Hedyosmum* bear the simplest unisexual flowers, with one to five stamens in the male flower and a single carpel in the female flower (the female flower of *Hedyosmum* also includes a tripartite tepal). Geographically, extant species of *Chloranthus* occur mainly in eastern and southeastern Asia, whereas *Sarcandra* inhabits tropical regions of southern, southeastern, and eastern Asia. Most extant *Ascarina* species are native to the southern Pacific Islands, except one disjunct species in Madagascar. *Hedyosmum* is disjunct in southeastern Asia and tropical America (summarized in Todzia, 1988). The family has long attracted the attention of evolutionary biologists owing to its simple reproductive structures as well as other seemingly "primitive" characters, ancient and numerous fossils, and pantropical disjunct distribution (summarized in Eklund et al., 2004). Therefore, understanding the evolution of Chloranthaceae may shed further light on the origin and diversification of the early angiosperms (e.g. Friis et al., 1986; Endress, 1987; Taylor & Hickey, 1992; Nixon et al., 1994; Eklund, 1999; Antonelli & Sanmartín, 2011).

The phylogenetic position of Chloranthaceae within angiosperms has remained contentious (Qiu et al., 1999; Zanis et al., 2002; Jansen et al., 2007; Moore

Received: 10 June 2010 Accepted: 28 February 2011
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et al., 2007). Based on plastid genomes, Chloranthaceae is placed as the sister of magnoliids with moderate support, just above the basal-most ANITA groups (Jansen et al., 2007; Moore et al., 2007). Within the family, molecular analyses consistently place *Hedyosmum* as the sister to all the three genera, followed by *Ascarina* being the sister to both *Chloranthus* and *Sarcandra* (Qiu et al., 1999; Zanis et al., 2002; Zhang & Renner, 2003; Antonelli & Sanmartín, 2011).

The intrageneric classification of *Chloranthus* has remained contentious prior to a series of thorough taxonomic revisions and morphological, cytological, and molecular studies (Kong, 2000a, 2000b, 2001; Kong & Chen, 2000; Kong et al., 2002a, 2002b). According to molecular studies based on full sampling (Kong & Chen, 2000; Kong et al., 2002a), the 10 species of *Chloranthus* were informally classified into two subgenera (*Chloranthus* and *Tricercandra*), with the former subgenus being further partitioned into two sections (*Chloranthus* and *Brachyuri*). These intrageneric taxa can be distinguished by some morphologic characters, such as the splitting extent of the "tripartite lobes" (Kong & Chen, 2000; Kong et al., 2002a).

Hedyosmum, the most species-rich genus in Chloranthaceae, has been investigated extensively using morphological and molecular evidence (Todzia, 1988; Doyle et al., 2003; Zhang & Renner, 2003; Eklund et al., 2004; Antonelli & Sanmartín, 2011). In a molecular study based on three plastid loci, Zhang & Renner (2003) included five out of approximately 45 *Hedyosmum* species and found the Asian endemic *Hedyosmum orientale* as sister to the remaining species. In a recent molecular study based on denser sampling (23 of approximately 45 species) and combination of two chloroplasts (*rbcL* and *rps16* intron regions) with the nuclear internal transcribed spacer (ITS; Antonelli & Sanmartín, 2011), the Asian endemic *H. orientale* appeared instead nested within the subgenus *Tafalla*, a relationship also obtained from a previous morphological analysis (Eklund et al., 2004). Further, in Antonelli & Sanmartín (2011), the section *Artocarpoides* was nested in section *Macrocarpa*, which, together, were nested within the species of section *Microcarpa.* The conflicts between the current intrageneric classification and morphological and molecular analyses from different loci indicate that intrageneric relationships in *Hedyosmum* need to be investigated further.

Ascarina (approximately 20 species) and *Sarcandra* (two species) have a large distribution range in Australasia and Asia, respectively. *Ascarina* has species that either possess one- or two-stamen male flowers; with one isolated species (endemic to Madagascar, up to 2000 km from other *Ascarina* in cloud mountain forest across southern Pacific Islands) having two to five stamens (usually three) and being traditionally treated as a separate subgenus or section (Swamy, 1953; Smith, 1976; Jérémie, 1980; Verdcourt, 1986; Eklund, 1999). The relationships among these taxa were only addressed in a morphological cladistic study and low resolution was obtained for the intrageneric relationships (Eklund et al., 2004). The relationships within *Ascarina* thus also need to be investigated further.

Chloranthaceous fossils show large morphological diversity during the Cretaceous and have been recovered from nearly all continents at numerous sites (summarized in Eklund, 1999; Eklund et al., 2004). Fossils have been used to calibrate the phylogeny of Chloranthaceae to unravel when the extant Chloranthaceae lineages diversified (Zhang & Renner, 2003; Antonelli & Sanmartín, 2011). Three fossil genera have particular potential to be used in such analyses. *Clavatipollenites* (Couper, 1958) can be traced back to the Barremian or even Hauterivian of the Early Cretaceous (Gübeli et al., 1984; summarized in Eklund, 1999). However, it is not entirely clear how one may use it as a calibration point because it is a morphologically complex group and not all fossil records assigned to *Clavatipollenites* can be definitely regarded as "chloranthaceous" (Doyle et al., 1975; Hughes et al., 1979; Eklund, 1999; Eklund et al., 2004). The other two sets of fossils, namely *Asteropollis* and *Chloranthistemon*, date to earliest Barremian/Aptian and Turonian, respectively. They consist of flowers, inflorescences, and pollens (Friis et al., 1994, 1999; Herendeen et al., 1993; summarized in Eklund, 1999) and appear to be of more certain taxonomic affinity with Chloranthaceae (e.g. Doyle et al., 2003; Eklund et al., 2004). The phylogenetic positions of these fossils among fossil and extant Chloranthaceae have been inferred based on detailed morphological cladistic analysis, which suggests that *Asteropollis* belongs to the stem or crown group of *Hedyosmum*, whereas *Chloranthistemon* was inferred to be on the stem to *Chloranthus* (Doyle et al., 2003; Eklund et al., 2004). The phylogenetic placements of these fossils are crucial for estimating absolute divergence ages within Chloranthaceae.

Based on the phylogeny of extant and fossil taxa inferred from morphological data (Doyle et al., 2003; Eklund et al., 2004), Zhang & Renner (2003) explored the influence of using either *Asteropollis* or *Chloranthistemon* as calibration points in the Chloranthaceae phylogeny. Their results showed large differences in the ages obtained. The diversification times of *Hedyosmum*, *Chloranthus*, and *Ascarina* were estimated to 29, 11 and 9 million years ago (Ma), respectively, when *Asteropollis* was used for constraining the node of *Hedyosmum* diverging from the remaining taxa. In contrast,

the same ages were estimated to 60, 22, and 18 Ma, respectively, when *Chloranthistemon* was chosen to constrain the node of most recent common ancestor (MRCA) of *Chloranthus* and *Sarcandra*. In the latter case, as pointed out by Antonelli & Sanmartín (2011), if one applied the substitution rates and node-to-tip distances provided in Zhang & Renner (2003) to calculate the crown age of the whole family, that age would be pushed back to 210–263 Ma. This age is considerably older than the angiosperm fossils found so far and predates the time frames suggested for large angiosperm groups from other dating analyses (e.g. Wikström et al., 2001; Moore et al., 2007, 2010; Smith et al., 2010). Whether the inconsistency of the ages obtained for the Chloranthaceae phylogeny was caused by an incorrect placement of the *Chloranthistemon* fossil or rather intrinsic problems with dating analyses, such as sensitivity to taxon sampling and different dating algorithms (Sanderson & Doyle, 2001; Near et al., 2005; Rutschmann, 2006; Rutschmann et al., 2007), requires further exploration.

To better understand the intrageneric relationships and temporal evolution of Chloranthaceae, in the present study we first reconstructed the phylogeny of the family based on single and combined datasets of three plastid loci, with improved species sampling compared with previous studies. Then, the diversification times within the family were estimated using two relaxed clock methods with several calibration strategies.

1 Material and methods

1.1 Plant materials

We sampled nine of the 10 species of *Chloranthus*, three samples including all the two species and one subspecies of *Sarcandra*, nine of 20 species of *Ascarina*, and 34 of approximately 45 species of *Hedyosmum*. The samples represent all the formally recognized intrageneric taxonomic units and span over most of the family's geographic range. For some species, we obtained samples from different localities. Sample information is listed in Table 1.

1.2 DNA extraction and PCR amplification

DNA was extracted from fresh or silica-dried leaves using the cetyltrimethylammonium bromide (CTAB) method and from specimen samples using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). Three plastid loci (*rbcL*, *rpl20–rps12*, and *trnL-F*) and the nuclear ribosomal ITS were amplified using the following PCR program: initial denaturation at 94 °C for 3 min, followed by 34 cycles of 30 s at 94 °C, 40 s at 52 °C, and 90 s (60 s for ITS) at 72 °C, with a final extension at 72 °C for 10 min. For extractions obtained from herbarium specimens, a second round of PCR amplification was performed using the combination of internal primers with the first-round PCR products as templates. The PCR products were purified using a TIANgel Midi Purification Kit (Tiangen Biotech) according to the manufacturer's instructions and then sequenced on an ABI 3730 DNA Sequencer (Applied Biosystems International, Foster City, CA, USA) using Big Dye Terminator (Applied Biosystems, Shanghai, China). The primers used for amplification or sequencing include were: 1F (ATGTCACCACAAACAGAAACT) and 1494R (GATTGGGCCGAGTTTAATTAC) from Olmstead et al. (1992); 359F (TACGAGCTCTACGTCTGGAG) and 970R (ATGATCTCCACCAGACATAC) designed in the present study for *rbcL*; *trnL* (CGAAATCGGTAGACGCTACG) and *trnF* (ATTTGAACTGGTGACACGAG) from Taberlet et al. (1991); external *trnXL* (GGATATGGCGAAATTGGTAG) and *trnXF* (AGGAACCAGATTTGAACTGG) designed in the present study for the *trnL-F* intergenic spacer and partial *trnL* region; *rpl20* (TTTGTTCTACGTCTCCGAGC) and rps12 (GTCGAGGAACATGTACTAGG) from Hamilton (1999); *rpl20b* (TAGTCCGTTCTTGCAAGAGT) and *rps12b* (GTCGAGGAACATGTACTAGG) designed in the present study for the *rpl20–rps12* intergenic spacer and partial *rpl20* region; and ITS1 (AGAAGTCGTAACAAGGTTTCCGTAGG) and ITS4 (TCCTCCGCTTATTGATATGC) from Baldwin (1992) for the ITS region.

1.3 Sequence alignment and phylogenetic analysis

In addition to the 114 new sequences generated in the present study, we also retrieved from GenBank 54 sequences from 24 species of Chloranthaceae and 15 sequences from *Calycanthus floridus*, *Drimys granadensis*, *Magnolia tripetela*, *Liriodendron tulipifera*, and *Illicium oligandrum*, which were chosen as outgroups according to recent phylogenomic studies (Jansen et al., 2007; Moore et al., 2007). All sequences were aligned with Clustal X 1.81 (Thompson et al., 1997) and adjusted manually with Bioedit 5.0.9 (Hall, 1999). The flank regions at both ends with many missing sites were excluded and the indels within sequences were filled by gaps. The incongruence length difference (ILD) test (Farris et al., 1994) was used to test for conflict among the three plastid loci and between the plastid loci and the nuclear ITS region. No conflict was found among the three plastid loci ($P > 0.3$), but a potential conflict was detected between the three combined plastid loci and the nuclear ITS region ($P < 0.01$). This conflict could have been caused by hybridization, incomplete

Table 1 Sources of plant material and GenBank accession numbers of the DNA sequences used in the present study

Taxon	*rbcL*	*trnL-F*	*rpl20–rps12*	Collector (voucher)/location
Ingroups				
Ascarina J. R. Forster & G. Forster				
coursii (Humbert & Capuron) J.F. Leroy & Jérémie	AY236844[a]	AY236747[a]	AY236721[a]	Ravelonarivo 1139 (MO)/Madagascar
lucida Hook. f.	AF238050[a]	AY236745[a]	AY236722[a]	Lange 3594 (AK)/New Zealand
marquesensis A.C. Smith	HQ336515		HQ336557	Feild/Hiva Oa, Marquesas, French Polynesia
marquesensis A.C. Smith	HQ336516	HQ336598	HQ336558	Feild/Nuku Hiva, Marquesas, French Polynesia
philippinensis C.B. Rob.	HQ336518	HQ336599	HQ336559	Feild/Mt Gumi, Morobe Province, Papua New Guinea
polystachya Foster	AY236842[a]	AY237816[a]	AY236732[a]	Feild/Tahiti
rubricaulis Solms-Laub.	HQ336519	AY236746[a]	HQ336560	Feild/Mt Dzumac, New Caledonia
solmsiana Schlechter. var. *grandifolia* Jérémie	HQ336520	HQ336600	HQ336561	Feild/Mt Dzumac, New Caledonia
sp.[b]	HQ336517	HQ336597	HQ336556	Feild/Mt Shungol, Morobe Province, Papua New Guinea
swamyana A.C. Smith	AY236843[a]	AY236748[a]	HQ336562	Feild/Des Vouex Peak, Taveuni, Fiji
Chloranthus Swartz				
angustifolius Oliver	AY236839[a]	AF364600[c]	AY236724[a]	Kong 97701 (PE)/China
erectus (Buch.-Ham.) Verdc.	AY236834[a]	AF329949[c]	AY236738[a]	Kong 97602 (PE)/China
fortunei (A. Gray) Solms-Laub.	AY236840[a]	AF364601[c]	HQ336563	Kong/MtWuzhishan, Ruyuan, Quangdong, China
henryi Hemsl.	AY236837[a]	AF364599[c]	AY236735[a]	Kong 97124 (PE)/China
japonicus Sieb.	L12640.2[d]	AF364603[c]	AY236723[a]	Chase 204 (NCU)/Japan
nervosus Coll. & Hemsl.	AY236841[a]	AF364602[c]	AY236733[a]	Kong 97603 (PE)/China
oldhamii Solms-Laub.	AY236838[a]	AF364598[c]	AY236734[a]	Wang 99001 (PE)/Taiwan, China
serratus (Thunb.) Roem. & Schult.	AY236836[a]	AF364596[c]	AY236736[a]	Kong 97402 (PE)/China
sessilifolius K.F. Wu	HQ336521	AF364597[c]	HQ336564	Kong/Mt Jinfoshan, Nanchuan, Chongqing, China
spicatus (Thunb.) Makino	AY236835[a]	AF329950[c]	AY236737[a]	Kong 97101 (PE)/China
Hedyosmum Swartz				
angustifolium (Ruiz & Pavón) Solms-Laub.	HQ336524	HQ336602	HQ336568	Persson 715 (GB)/Pasco, Peru
anisodorum Todzia	HQ336525	HQ336603	HQ336569	Feild/Trocha Union, Peru
arborescens Swartz	L12649.2[d]	HQ336604	AY236720[a]	Feild & Luke/St. Catherines, Jamaica
brasiliense Miquel	HQ336526	HQ336605	HQ336570	Antonelli & Andersson 297 (GB)/Rio de Janeiro, Brazil
brenesii Standl.	HQ336527	HQ336606	HQ336571	Feild/Costa Rica
bonplandianum Humboldt	HQ336528	AY236751[a]	AY236729[a]	Feild/Estacion Cacao, Guanacaste, Costa Rica
bonplandianum var. *callososerratum* Oerst.			HQ336572	Dwyer & Kirkbride 7839/Panama
columbianum Cuatrec.	HQ336529	HQ336607	HQ336573	Todzia 2431/Colombia
correanum D'Arcy & Liesner	HQ336530	HQ336608	HQ336574	Hammel 3064 (AAU)/Chiriquí, Panama
costaricense Burger	HQ336531	HQ336609	AY236718[a]	Feild 1023/Tapanti, Costa Rica
cuatrecazanum Occhioni	HQ336532	HQ336610	HQ336575	Gavilanes & Tivira 653-A (AAU)/Napo, Ecuador
cumbalense H. Karsten	HQ336533	HQ336611	HQ336576	Harling 25488 (GB)/Azuay, Ecuador
dombeyanum Solms-Laub.	HQ336534	HQ336612	HQ336577	Steinbach 543 (S)/Cochabamba, Bolivia
domingense Urban	HQ336535	HQ336613	HQ336578	Abbott 20902 (FLAS)/San Cristobal, Dominican Republic
domingense Urban	HQ336536		HQ336579	Feild/Pico Duarte, Dominican Republic
gentryi D'Arcy & Liesner		HQ336614	HQ336580	Dorr 8138/Venezuela
goudotianum Solms-Laub.	HQ336537	AY236754[a]	HQ336581	Pherson 15898 (MO)/Chiriqui, Panama
grisebachii Solms-Laub.	HQ336538	HQ336615	HQ336582	Abbott 19948 (FLAS)/Holguin, Cuba
lechleri Solms-Laub.	HQ336539	HQ336616	HQ336583	Solomon 13735/Bolivia
luteynii Todzia	HQ336540	HQ336617	HQ336584	Sodiro (P)/Pichincha, Ecuador
maximum (Kuntze) K. Schum	HQ336541	HQ336618	HQ336585	Feild/Wayquechas Reserve, Peru
mexicanum Cordemoy	HQ336542	HQ336619	HQ336586	Feild/Ojo de Agua, Talamancas, Costa Rica
neblinae Todzia			HQ336587	Maquire/Brazil
nutans Swartz	HQ336543		HQ336588	Veloz et al. 2903 (JBSD)/Pico Duarte, Dominican Republic
nutans Swartz	HQ336544	HQ336620		Feild & Luke/Vinegar Hill Trail, Jamaica
orientale Merr. & Chun	HQ336545	AY236749[a]	AY236730[a]	Kong/Mt Diaoluoshan, Lingshui, Hainan, China
parvifolium Cordemoy	HQ336546	HQ336621	HQ336589	Todzia et al. 2432 (AAU)/Cundinamarca, Colombia

Continued.

Table 1 Continued

Taxon	*rbcL*	*trnL-F*	*rpl20–rps12*	Collector (voucher)/location
peruvianum Todzia	HQ336547	HQ336622	HQ336590	Feild/Trocha Union, Peru
racemosum (Ruiz & Pavón) G. Don	HQ336548	HQ336623	HQ336591	Asplund 13151 (S)/Kosnipata Road, Peru
scaberrimum Standl.	HQ336549	HQ336624	HQ336592	Santamaría S-1029 (GB)/Alajuela, Costa Rica
scabrum (Ruiz & Pavón) Solms-Laub.	HQ336550	HQ336625	HQ336593	Andersson & Nilsson 2539 (GB)/Loja, Ecuador
spectabile Todzia	HQ336551	HQ336626	HQ336594	Qllgaard & Madsen 90562 (GB)/Zamora-Chinchipe, Ecuador
sp.[b]	HQ336555	HQ336628	HQ336596	Feild/Tono Alto, Peru
sprucei Solms-Laub.	HQ336552	AY236752[a]	AY236719[a]	Harling & Andersson 24138 (GB)/Zamora-Chinchipe, Ecuador
strigosum Todzia	HQ336553	HQ336627	HQ336595	Andersson & Nilsson 2412 (GB)/Sucumbíos, Ecuador
translucidum Cuatrec.	HQ336554	AY236753[a]	AY236728[a]	Harling & Andersson 21980 (GB)/Kosnipata Road, Peru
Sarcandra Gardner				
chloranthoides Gardner	AY236833[a]	AY236740[a]	HQ336565	Endress/Zurich (cultivated)
glabra (Thunb.) Nakai	HQ336522	AF329948[c]	HQ336566	Kong/China
glabra subsp. *brachystachys* (Blume) Verdc.	HQ336523	HQ336601	HQ336567	Kong/China
Outgroups				
Calycanthus floridus L.	AJ428413	AJ428413	AJ428413	
Drimys granadensis (L.f.) J.F. Gmel.	DQ887676	EU669556	DQ887676	
Liriodendron tulipifera L.	DQ899947	DQ899947	DQ899947	
Magnolia tripetala (L.) L.	AF206791	AY009073	AY727308	
Illicium oligandrum Merr. & Chun	EF380354	EF380354	EF380354	

[a]From Zhang & Renner (2003).
[b]New taxa that have not been formally described.
[c]From Kong et al. (2002a).
[d]From Qiu et al. (1993).

lineage sorting, or be simply an artifact of the test, whose usefulness and interpretation has been criticized by several studies (Darlu & Lecointret, 2002; Hipp et al., 2004; Ramírez, 2006; Quicke et al., 2007). Because our major aim here was to evaluate the effect of fossil placements on the dated phylogeny, we decided to exclude all ITS sequences from further analyses, using instead only the three plastid loci.

Phylogenetic analyses were performed using the maximum parsimony (MP) method in PAUP∗ version 4.0b10 (Swofford, 2002) and the Bayesian analysis in MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001). For the MP analysis, 1000 replicates of random stepwise addition with tree bisection–reconnection (TBR) branch swapping were performed using heuristic searches, with all most parsimonious trees saved at each replicate (MulTree on). Support for each branch was assessed using bootstrap analyses with 1000 bootstrap replicates, each with 10 stepwise additions and TBR branch swapping. Prior to the Bayesian analysis, the Akaike Information Criterion (AIC) implemented in ModelTest version 3.7 (Posada & Crandall, 1998; Posada & Buckley, 2004) was used to select the best fit model of molecular evolution, and different models were chosen for each plastid locus. In the Bayesian analysis, four chains of Markov chain Monte Carlo (MCMC) were run, sampling one tree every 100 generations for 10 million generations starting with a random tree. The first 50000 generations were excluded as burn-in.

1.4 Molecular dating analyses

In the molecular dating analysis, only 52 Chloranthaceae taxa were included, in which all the three plastid loci were available for each taxa. Furthermore, the indels were deleted from this dataset. Initial likelihood ratio tests for rate constancy were conducted for each locus in PAUP∗4.0b10. In the study of Zhang & Renner (2003), a strict molecular clock could not be rejected for the *rbcL* sequences, so that region was dated under a strict clock model. However, with the addition of more taxa, the null-hypothesis of a clock-like evolution was rejected in all cases ($P < 0.01$). Time estimations were therefore performed under a relaxed molecular clock of the penalized likelihood (PL) method in r8s 1.71 (Sanderson, 2002) and Bayesian relaxed clock (BRC) in BEAST version 1.5.3 (Drummond & Rambaut, 2007), respectively. For the PL analysis, the optimal smoothing parameter (λ) was determined by cross-validation. The input topology required by the method was the consensus phylogram from the MrBayes analysis, with magnoliids and *Illicium* chosen as outgroups according to recent phylogenetic studies (Qiu et al., 1999; Jansen et al., 2007; Moore et al., 2007, 2010). *Illicium* was subsequently

pruned, as required by r8s. Confidence intervals for node ages were calculated with the non-parametric bootstrap approach. In the BRC analysis (in Beast v.1.5.3), the relaxed clock method with uncorrelated lognormal distribution (UCLN) was used. The best fit substitution model for each of the loci was selected according to the result of ModelTest, under the AIC optimization criterion. Convergence of runs was evaluated in the software Tracer (in the same package as Beast v.1.5.3). After 30 000 000 generations of MCMC searches with one sample every 3000 generations, the effective sampling sizes (ESS) for all relevant parameters was larger than the minimum recommended 200. The post burn-in samples from two independent searches were combined to calculate the median times and 95% highest posterior densities (HPD) of ages, which could then be displayed in FigTree V.1.0 (Rambaut, 2006).

For calibration, the two fossil taxa *Asteropollis* and *Chloranthistemon* were used. *Asteropollis* has been dated originally to the Barremian or Aptian (i.e. around 120 Ma) based on a suite of flowers and inflorescences found in Portugal and some pollen fossils elsewhere (Friis et al., 1994, 1999; summarized in Eklund, 1999). However, the stratigraphic age of the Portuguese fossils where *Asteropollis* was found was complicated due to breaking down or turning over of the stratigraphic layers, meaning that they are more probably to date back to the Late Aptian–Early Albian (Hochuli et al., 2006). Moreover, the affinity of the pre-Albian *Asteropollis* pollens to *Hedyosmum* is questionable because the allegedly pre-Albian pollens have three chotomosulcate apertures that differ from the extant *Hedyosmum*, which have four to six (usually five) chotomosulcate apertures. Therefore, the earliest Albian fossils (ca. 112 Ma) were conservatively chosen as the earliest *Asteropollis* linked to *Hedyosmum* (J. A. Doyle & P. A. Hochuli, pers. comm., 2009; see also Antonelli & Sanmartín, 2011). The second fossil used for calibration, *Chloranthistemon*, is more certain to date to the Turonian and was used here to provide a minimum age of 90 Ma, as in the analyses of Zhang & Renner (2003) and Antonelli & Sanmartín (2011). The third and last calibration point used in this analysis was the splitting time between magnoliids and Chloranthaceae, as determined from previous phylogenetic analyses (140–160 Ma; Wikström et al., 2001; Moore et al., 2007, 2010).

2 Results

2.1 Phylogeny of Chloranthaceae

In our analysis, 55 *rbcL* sequences, 58 *rpl20–rps12* sequences, and 55 *trnL-F* sequences of Chloranthaceae were included. The *rbcL* sequences have an identical length of 1398 bp and contain 149 variable sites (10.7%) and 88 parsimony informative sites (6.3%) with a guanine and cytosine (GC) content of 44.9% (Table 2). The *rpl20–rps12* sequences have a length variation from 743 to 829 bp with a GC content of 35.4%, holding a total of 172 (19.2%) variable sites and 104 (11.6%) parsimony informative sites (Table 2). The *trnL-F* sequences have a variation of 827–982 bp in length with a GC content of 35.9%, including a total of 195 variable sites (17.7%) and 124 parsimony informative sites (11.3%) (Table 2). For the combined dataset of the three plastid loci, the matrix had a length of 3402 bp with a GC content of 39.8%, containing a total of 513 variable sites (15.1%) and 318 parsimony informative sites (9.4%; Table 2).

Table 2 Summary statistics of sequence characters for the three plastid loci of Chloranthaceae produced here

	rbcL	*rpl20–rps12*	*trnL-F*	Combined
Length before alignment (bp)	1398	743–829	827–982	
Length after alignment (bp)	1398	895	1100	3402
GC content (%)	44.9	35.4	35.9	39.8
No. variable sites (bp)	149 (10.7%)	172 (19.2%)	195 (17.7%)	513 (15.1%)
No. informative sites (bp)	88 (6.3%)	104 (11. 6%)	124 (11.3%)	318 (9.4%)

The phylogenetic reconstruction was first done based on each of the three loci using maximum parsimony. The monophyly of each of the genera and the intergeneric relationships of *Hedyosmum*, *Ascarina*, *Sarcandra*, and *Chloranthus* successively to be the sister of the rest were strongly supported by all three loci. In contrast, the intrageneric relationships were poorly resolved by any single locus, although several clades were retrieved in all three trees (see Figs. S1–S3 available as supplementary material to this paper). Because no obvious conflict was observed among the three trees, the combined dataset of the three loci was used for further phylogenetic reconstruction to improve resolution.

In the combined analysis, both MP and Bayesian phylogenetics were used. For the MP heuristic searches, only 500 000 best trees were saved. All the trees had 1130 steps with a consistency index (CI) of 0.74 and a retention index (RI) of 0.93. There were no obvious topological conflicts between the trees produced by the two methods, therefore only the Bayesian consensus tree is illustrated with both Bayesian posterior probabilities and bootstrap support values for nodes (Fig. 1). The combined tree strongly suggested the monophyly of each of the four genera (bootstrap support (BS) > 95%;

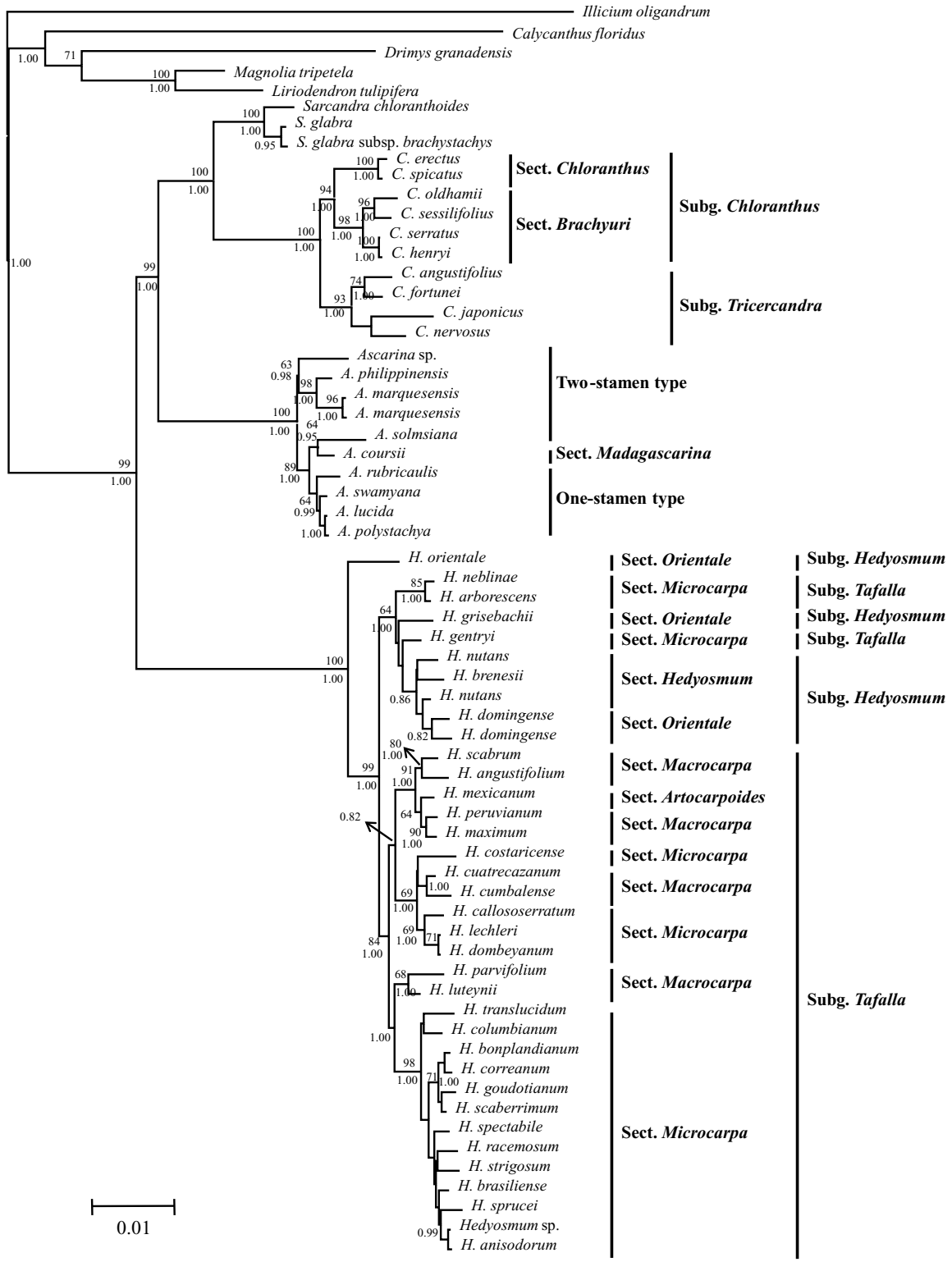

Fig. 1. The Bayesian majority consensus phylogram reconstructed from a combined dataset comprising *trnL-F*, *rpl20–rps12*, and *rbcL* sequences. Any BS (bootstrap support from 1000 bootstrap replications) values > 60% are shown above the branches, whereas PP (Bayesian posterior probability from 100000 post-burn-in trees) values > 0.80 are shown below the branches. Consistency index = 0.77; retention index = 0.95. The subgenus, section, and group delimitations refer to Todzia (1988, 1993) for *Hedyosmum*, Swamy (1953), Smith (1976) and Jérémie (1980) for *Ascarina*, and Kong (2000b) for *Chloranthus.*

posterior probability (PP) = 1.0) and an intergeneric relationship of *Hedyosmum*, *Ascarina*, *Sarcandra*, and *Chloranthus* successively to be the sister of the rest (BS > 95%; PP = 1.0), just as indicated by the previous single-locus analyses. Within *Chloranthus*, two main sister clades were retrieved: one (BS = 94%; PP = 1.0) consisted of the two evergreen subshrubs *C. spicatus* and *C. erectus* as sisters (BS = 100%; PP = 1.0) and a lineage (BS = 93%; PP = 1.0) constituted by two sister pairs, namely *C. oldhamii–C. sessilifolius* (BS = 96%; PP = 1.0) and *C. serratus–C. henryi* (BS = 100%; PP = 1.0); the other clade comprised the four herbs that possess long lobed stamen connectives (BS = 93%; PP = 1.0), among which *C. angustifolius* and *C. fortunei* form as sisters (BS = 74%; PP = 1.0), but their relationships to *C. japonicus* and *C. nervosus* were poorly resolved. As the sister group of *Chloranthus*, *Sarcandra* showed a relationship as *S. glabra* and *S. glabra* subsp. *brachystachys*, distributed in east and/or southeast Asia, grouped together and separating from *S. chloranthoides* only in India and Sri Lanka, although the relationship was supported by low support (BS < 60%; PP = 0.95).

Ascarina was divided into two clades: one (BS = 63%; PP = 0.98) consisted of a two-stamen new species not formally described and a two-stamen sister pair (BS = 98%; PP = 1.0) of *A. philippinensis* and *A. marquesensis*; the other clade (BS = 89%; PP = 1.0) was a mixed group that included two lineages. One lineage (BS = 64%; PP = 0.95) comprised *A. solmsiana* and *A. coursii* alone assigned to section *Madagascarina*, both of which possess two or more stamens; the other lineage was constituted by four one-stamen species, but their exact relationships were not well resolved, although *A. lucida* and *A. polystachya* as sisters obtained a high PPof 1.0.

Within *Hedyosmum*, the Asian endemic *H. orientale* was inferred to be the sister of a large monophyletic group made by all the other species from tropical America with BS 99% and PP 1.00. The tropical American clade was further divided into two subclades. In one subclade (BS = 64%; PP = 1.0), *H. gentryi*, *H. arborescens*, and *H. neblinae*, traditionally attributed to subgenus *Tafalla*, clustered instead with the taxa from subgenus *Hedyosmum* (i.e. *H. grisebachii*, *H. brenesii*, *H. nutans*, and *H. domingense*). The other subclade (BS = 84%; PP = 1.0) comprised most species from all three sections in subgenus *Tafalla* and several lineages could be further recognized. *Hedyosmum mexicanum* from section *Artocarpoides* was embedded in a lineage (BS = 91%; PP = 1.0) constituted by several species from section *Macrocarpa* (*H. scabrum*, *H. angustifolium*, *H. peruvianum*, *H. maximum*); however, the other species of Section *Macrocarpa* (*H. luteynii–H. parvilifolium*, *H.cuatrecazanum–H.cumbalense*) were grouped with other species from section *Microcarpa* (Fig. 1).

2.2 Calibration strategies and time estimates

The estimated times from PL for the deep node (Chloranthaceae/magnoliids divergence) were much older than those from BRC when single internal calibrations were used (see Table 3). Therefore, the estimated ages from PL only using internal fossil calibrations are not listed below.

According to previous cladistic analyses (Eklund et al., 2004), *Chloranthistemon* was placed on stem *Chloranthus*, thus giving a minimum age constraint of 90 Ma for the node of *Chloranthus*/*Sarcandra* divergence (Node D in Fig. 2). Using this point for calibration (Strategy I in Table 3), we estimated that the divergence time of Chloranthaceae and magnoliids (Node A in Fig. 2) was around 207.8 (148.5, 285.4) Ma (BRC), which largely exceeds the putative divergence time of Chloranthaceae and magnoliids (usually suggested between 140 and 160 Ma) inferred by large-scale phylogenetic analyses (Wikström et al., 2001; Moore et al., 2007, 2010). Apparently the placement of *Chloranthistemon* needs to be reconsidered.

Asteropollis has been regarded to have an affinity to *Hedyosmum*, but whether the fossil belongs to the stem *Hedyosmum* or its crown could not be inferred unambiguously from cladistic analyses, because both placements result in equally parsimonious trees (Doyle et al., 2003; Eklund et al., 2004). Therefore, *Asteropollis* could either calibrate the crown node (Node H in Fig. 2) or the stem node where *Hedyosmum* diverged from the rest (Node B). If this fossil is placed on the crown node of *Hedyosmum*, the divergence time between Chloranthaceae and magnoliids is estimated to 303.3 (213.0–461.4) Ma (Node A in Table 3), which far exceeds the accepted time frames of < 200 Ma for the crown node of extant angiosperms. Conversely, if it is used to calibrate stem *Hedyosmum* (Node B), the estimated time of 147.4 (125.6, 176.6) Ma (BRC) for the divergence of Chloranthaceae and magnoliids fits well with the time frames from larger analyses. Therefore, our results suggest that *Asteropollis* is more probably a stem relative of *Hedyosmum*.

The use of single fossil calibrations in the dating analysis reveal large incongruence in divergence ages, as pointed out by Zhang & Renner (2003) and replicated here despite denser sampling, longer sequences, and improved dating methods. It seems clear that the largest incongruence is retrieved when using *Chloranthistemon* to calibrate stem *Chloranthus* (rather than *Asteropollis* calibrating the stem *Hedyosmum*). This incongruence

Table 3 Estimated divergence times for eight major nodes (A–H, as indicated in Fig. 2) when different calibration strategies are used in the present study (Strategies I–VI) and compared with mean/median estimates from other studies (all ages are in million of years)

	Calibration strategy												Antonelli & Sanmartin (2011)		Zhang & Renner (2003)	
	I		II		III		IV		V		VI					
Node	PL	BRC	PL	BRC	PL	BRC	PL	BRC	PL	BRC	PL	BRC	PL	BRC	"Fossil A"	"Fossil B"
A	*1386.5 ± 637.2*	*207.8 (148.5–285.8)*	*1016.1 ± 120.0*	*303.3 (213.0–461.4)*	164.5 ± 11.1	147.4 (125.6–176.6)	150*	150*	271.5 ± 254.7	151.5 (121.3–192.4)	150*	145.6 (132.9–164.0)				
B	*914.0 ± 417.8*	*154.0 (113.8–208.0)*	*696.3 ± 31.7*	*265.8 (170.6–336.0)*	112*	112*	109.4 ± 6.2	111.5 (90.0–130.7)	179.9 ± 162.4	110.5 (94.5–135.2)	113.5 ± 2.9	111.1 (111.1–117.8)	110*	111.2	120*	210–263†
C	*477.9 ± 300.0*	*125.9 (100.0–169.3)*	*616.0 ± 7.1*	*222.4 (130.4–276.8)*	95.4 ± 5.3	92.3 (74.1–106.6)	93.3 ± 7.2	90.4 (68.1–111.8)	90*	90*	97.3 ± 5.2	95.7 (90.0–98.6)				
D	90*	90*	*461.9 ± 8.0*	*155.6 (89.1–195.6)*	65.1 ± 6.6	62.4 (44.6–81.9)	63.8 ± 7.3	61.8 (42.7–82.1)	61.1 ± 5.3	59.8 (44.4–74.4)	67.6 ± 6.8	63.6 (46.9–79.6)	90*			90*
E	*32.1 ± 7.2*	*24.9 (10.1–49.2)*	*222.9 ± 103.7*	*44.2 (13.9–75.6)*	23.4 ± 6.5	17.5 (6.9–33.5)	22.8 ± 6.5	17.6 (6.3–34.5)	21.9 ± 5.8	12.5 (5.0–24.3)	24.0 ± 6.8	9.5 (6.4–33.8)	9.91	6.88		
F	*40.5 ± 5.0*	*50.1 (35.0–67.9)*	*211.0 ± 18.4*	*74.5 (45.3–108.2)*	30.0 ± 4.5	34.1 (22.2–48.2)	28.9 ± 4.6	34.2 (21.6–50.0)	27.9 ± 4.0	31.8 (21.4–43.9)	30.2 ± 5.0	26.3 (23.5–48.9)	33.0	36.2	11 or 12	22
G	*148.5 ± 95.1*	*42.0 (23.2–66.7)*	*242.0 ± 17.0*	*60.3 (36.9–98.8)*	29.0 ± 5.3	29.0 (18.0–44.1)	28.2 ± 5.3	29.4 (16.9–46.2)	27.5 ± 4.8	26.1 (16.4–39.5)	29.5 ± 5.6	31.0 (18.6–45.1)	14.5	12.8	(9 or10) ± 6	(17 or 18) ± 13
H	*332.5 ± 163.3*	*71.1 (45.8–106.6)*	*112**	*112**	30.0 ± 5.3	49.6 (35.4–69.2)	28.8 ± 4.7	48.9 (33.5–71.2)	56.1 ± 63.6	48.0 (33.5–67.9)	29.8 ± 4.8	45.8 (35.0–69.1)	35.6	43.3	29 ± 11	(53 or 63) ± 20

The conflicting calibration strategies (I and II) and their estimated times are italicized.
The standard deviation of the age estimate from the penalized likelihood (PL; in r8s) method and the 95% highest posterior density in the parenthesis from Bayesian relaxed clock (BRC; in BEAST) are given. The first five strategies use a single calibration point, with fixed age marked with an asterisk. The last strategy uses three combined calibration points (A, B, C) with the age priors settled as: fixed 150 for A, > 112 for B, and > 90 for C in PL; normal distribution with mean = 150 and SD = 10 for Node A, lognormal distribution with offset = 111, mean = 0, and SD = 1.9 for B, and lognormal distribution with offset = 90, mean = 0, and SD = 1.9 for C.
One hundred random Bayesian phylograms after burn-in were used as input trees to estimate the mean times and standard deviations for the eight major nodes in the PL method.
†For node B in Zhang & Renner (2003) represents the age calculated by Antonelli & Sanmartin (2011) but using the same parameters and methods as in Zhang & Renner (2003).

could have been produced by an inappropriate placement of *Chloranthistemon* in the phylogeny. To further test this possibility, we estimated divergence times in Chloranthaceae using the previously estimated divergence time of magnoliids/Chloranthaceae as calibration and compared the estimated times with the fossil ages. The putative time (150 Ma as the mean value) of the divergence fo the Chloranthaceae and magnoliids was used for the calibration (Strategy IV in Table 3). Under this calibration, *Ascarina* splits from *Sarcandra* and *Chloranthus* (Node C in Fig. 2) around 93.3 ± 7.2 Ma (PL) and 90.4 (68.1, 111.8) Ma (BRC), slightly earlier than *Chloranthistemon*. *Hedyosmum* splits from the rest (Node H in Fig. 2) around 109.4 ± 6.2 Ma (PL) and 111.5 (90.0, 130.7) Ma (BRC), which are close to the earliest *Asteropollis*, whereas *Hedyosmum* diversified much more recently: 28.8 ± 4.7 Ma (PL) and 48.9 (33.5, 71.2) Ma (BRC). The *Sarcandra–Chloranthus* divergence (Node D in Fig. 2) is estimated at around 63.8 ± 7.3 Ma (PL) and 61.8 (42.7, 82.1) Ma (BRC), which are much younger than *Chloranthistemon.* These results suggest that *Asteropollis* is indeed likely to be an early member on the stem line to *Hedyosmum*. However, the results also suggest that *Chloranthistemon* probably occurred before the *Chloranthus–Sarcandra* divergence and is therefore unlikely to be a stem group of *Chloranthus* as suggested by previous cladistic studies (Doyle et al., 2003; Eklund et al., 2004).

Finally, by calculating squared differences, the set of calibration points constituted by the deep divergence of Chloranthaceae and magnoliids, *Asteropollis* for the stem *Hedyosmum*, and *Chloranthistemon* for the stem of both *Sarcandra* and *Chloranthus* (A, B and C, respectively, in Fig. 2) obtained a much lower squared error (17.4) than the other combinations (the second best score was 7241.48). The estimated times for the eight major nodes when single calibration of the three congruent points was used also agreed much better with each other (see Strategies III, IV, V in Table 3) than the former two proposed positions (Node D and H; Strategies I and II in Table 3). Based on these tests, the best congruent set of combined calibration points was used to estimate the internal times using PL and BRC methods, respectively (Strategy VI).

The final results under what we propose here to be the optimal calibration strategy infer that the times of *Hedyosmum* diverging from the other three genera, *Ascarina* separating from the other two genera and *Sarcandra* splitting from *Chloranthus* were around 113.5 ± 2.9 Ma and 111.1 (111.1–117.8) Ma; 97.3 ± 5.2 Ma and 95.7 (90.0–98.6) Ma; and 67.6 ± 6.8 Ma and 63.6 (46.9–79.6) Ma, respectively. The corresponding crown nodes ages are 29.8 ± 4.8 Ma and 45.8

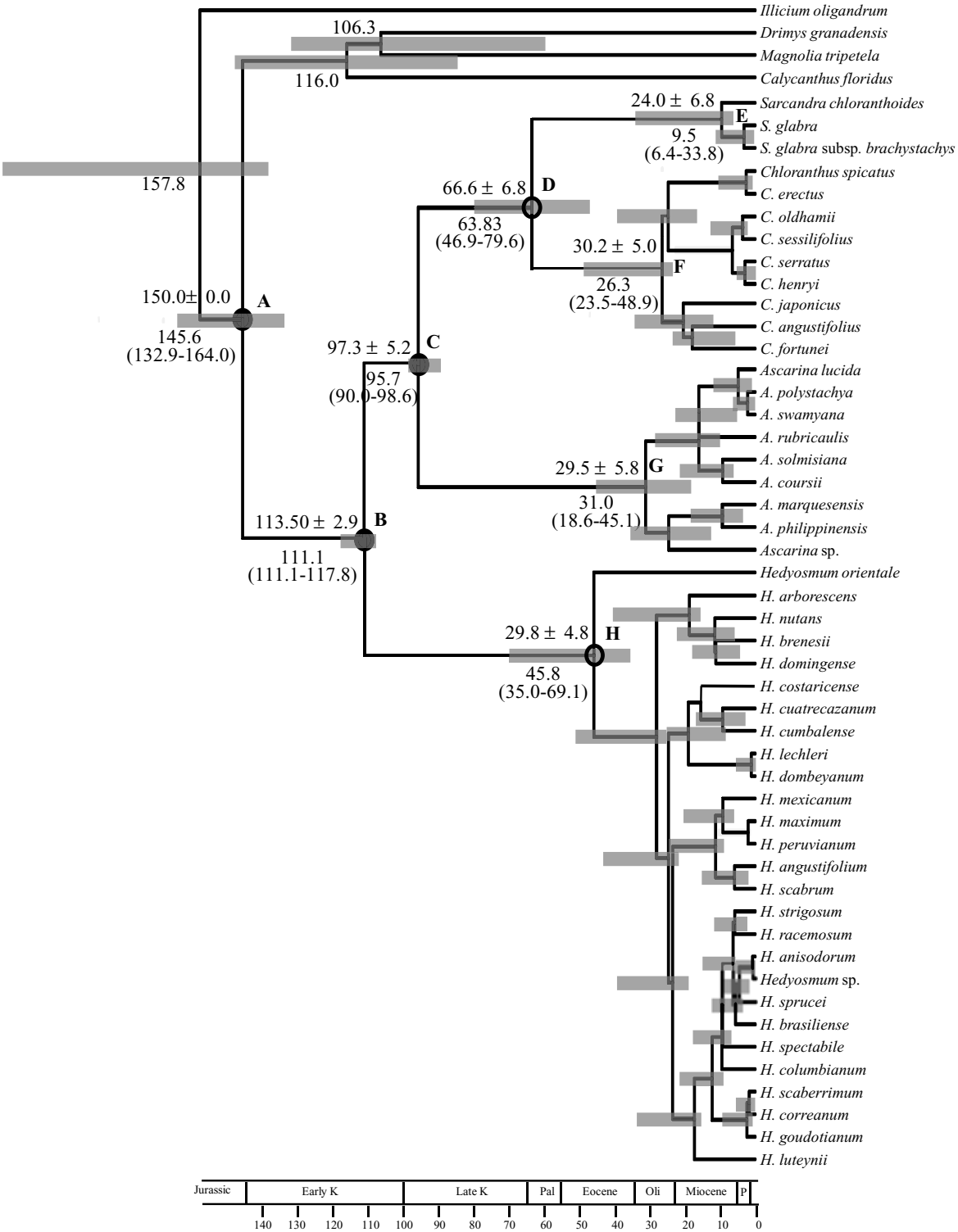

Fig. 2. Chronogram of Chloranthaceae reconstructed using the Bayesian uncorrelated lognormal method in BEAST, and the three combined calibration points (A, B, C). The prior ages of the three calibration points are set as follows: A is of normal distribution with mean = 150 and SD = 10; B is of lognormal distribution with offset = 111, mean = 0, and SD = 1.9; C is of lognormal distribution with offset = 90, mean = 0, and SD = 1.9. For the major nodes, the median ages with 95% highest posterior densities from BEAST and ages with standard errors from the penalized likelihood (PL) method (implemented in the software r8s) are given below and above the branches, respectively. Early K, Early Cretaceous; Late K, Late Cretaceous; Pal, Paleocene; Oli, Oligocene; P, Pleistocene.

(35.0–69.1) Ma for *Hedyosmum*; 29.5 ± 5.6 Ma and 31 (18.6–45.1) Ma for *Ascarina*; 30.2 ± 5.0 Ma and 26.3 (23.5–48.9) Ma for *Chloranthus*; and 24.0 ± 6.8 Ma and 9 (6.4–33.8) Ma for *Sarcandra*. These results show a pattern of early origin but recent diversification for all four extant genera. What has caused the long "temporal gap" between the stem and crown ages observed in Chloranthaceae, especially *Hedyosmum*, has been thoroughly explored by Antonelli & Sanmartín (2011) and seems to be attributable to gradual extinction coupled with rapid diversification during the uplift of the Andes (e.g. Antonelli et al., 2009; Hoorn et al., 2010).

3 Discussion

3.1 Relationships within Chloranthaceae

Based on the most comprehensive sampling of the Chloranthaceae to date, our phylogenetic evidence supports previous studies in the intergeneric and intrageneric relationships within *Chloranthus* (Qiu et al., 1999; Kong et al., 2002a; Zanis et al., 2002; Zhang & Renner, 2003; Antonelli & Sanmartín, 2011). However, the relationships we found within *Ascarina* and *Hedyosmum* do not support much of the traditional classifications within these genera and also differ from other recent molecular studies.

Ascarina comprises species with either one or two stamens per male flower and the far isolated *A. coursii*, which was usually treated solitarily as section *Madagascarina* (summarized in Eklund, 1999). The male flower of the two-stamen species is supported by one bract, whereas the male flower of the one-stamen species is supported by one bract and two bracteoles. *Ascarina coursii* has two to five stamens (usually 3) and one bract. In a later morphological cladistic analysis conducted by Eklund et al. (2004), *A. marquesensis*, a species with two stamens, clustered with a clade composed of one-stamen species, together forming a polytomy with other solitary two-stamen species and *A. coursii*. Our phylogenetic analyses based on nearly all the taxonomic groups and across much of the geographic range of *Ascarina* confirm the monophyly of the one-stamen taxa embedded within the polyphyletic two-stamen taxa as suggested by the previous cladistic analysis. However, in our phylogeny the non-monophyly of the two-stamen taxa was caused by the two-stamen *A. solmsiana* plus *A. coursii* as sisters, rather than *A. marquesensis* as suggested by the previous cladistic analysis, clustered with the monophyletic one-stamen taxa in one clade. The present phylogeny implies that one stamen is a derived state in *Ascarina*, reduced from ancestral two or more stamens as suggested by the previous cladistic analysis (Eklund et al., 2004).

Hedyosmum is the most diverse genus in Chloranthaceae, with more than 45 species in two subgenera (subgenus *Hedyosmum* and subgenus *Tafalla*; Todzia, 1988, 1993). In subgenus *Hedyosmum*, the pistillate inflorescence comprises solitary flowers with a characeous bract(s), whereas the pistillate inflorescence of subgenus *Tafalla* has cymules, each clustered by one or more flowers (Todzia, 1988). The classification into two subgenera was also recovered by recent morphological cladistic analysis based on over 100 characters (Doyle et al., 2003; Eklund et al., 2004). However, in a molecular study with dense sampling, *H. orientale*, traditionally attributed to subgenus *Hedyosmum*, was inferred as the sister to a large clade consisting of species from subgenus *Tafalla*, which, together, were sister to a clade comprising the remaining species of subgenus *Hedyosmum* (Antonelli & Sanmartín, 2011). Thus, the monophyly of subgenus *Hedyosmum* was rejected. The non-monophyly of subgenus *Hedyosmum* is again confirmed by the present study, but *H. orientale* was inferred to be sister to all other species in the genus. The topological conflict between different studies based on DNA sequences and morphology may reflect reticulate evolution of *H. orientale*, because the excessive phylogenetic information from ITS could have overshadowed the information from plastid markers, especially *rbc*L, which (as shown in Fig. S1) only contributed minimally to intrageneric resolution (only ITS and *rbc*L sequences could be obtained for *H. orientale* in the study of Antonelli & Sanmartín (2011)). This may also explain the possible conflict between ITS and the three plastid loci detected in our study. Because *H. orientale* has a large distribution range in Asia, from the boundary between China and Vietnam to Sumatra and to Sulawesi in Indonesia, samples from other regions should be added to the sample from Hainan (China) used in the present study to further test the monophyly and phylogenetic placement of *H. orientale.*

Apart from the topological conflict for *H. orientale*, both this and the study of Antonelli & Sanmartín (2011) revealed that subgenus *Hedyosmum* was polyphyletic to subgenus *Tafalla*, suggesting that cymules characteristic for subgenus *Tafalla* have derived from an ancestor with solitary flowers similar to subgenus *Hedyosmum*.

Our results also suggest that subgenus *Tafalla* is also non-monophyletic due to the three *Tafalla* species (*H. arborescens*, *H. gentryi*, *H. neblinae*) embedded within those from subgenus *Hedyosmum*. According to Todzia (1988), *H. arborescens*, *H. gentryi*, *H. neblinae*, and *H. pseudoandromeda* in subgenus *Tafalla* are closely related and assumed to be in one group

(*Pseudoandromeda*). By re-examining the morphologies described by Todzia (1988), it appears that *H. gentryi*, *H. neblinae*, and *H. pseudoandromeda* have cymules with only one flower. This structure could be modified from a solitary flower, similar to that in subgenus *Hedyosmum*, rather than reduced from a "real" cymule, homologous to those of subgenus *Tafalla*. In addition, the chartaceous floral bracts exclusively shared by these three species and those from subgenus *Hedyosmum* could support their affinities. Another species of the group, namely *H. arborescens*, has been described as having cymules with two or more flowers and flesh floral bracts that differ from subgenus *Hedyosmum*. However, owing to its general morphology, that species has been linked to *H. gentryi*, *H. neblinae*, and *H. pseudoandromeda*, which together formed a group in the cladistic analysis by Todzia (1988). Therefore, *H. arborescens* may be a hybrid species whose paternal line has cymules and flesh floral bracts but whose maternal line has a solitary flower and chartaceous bracts. The sister relationship of *H. arborescens* with other species from subgenus *Tafalla* in the study of Antonelli & Sanmartín (2011) is consistent with the hypothesis of its hybrid speciation. To investigate the incongruence between results derived from morphological and molecular data for *H. gentryi* and *H. neblinae*, evidence from nuclear or mitochondrial data is needed.

An additional area of conflict between traditional taxonomy and the present molecular phylogeny lies among the three sections within subgenus *Tafalla*. The present study suggests the eight *Macrocarpa* species characterized by one to three pistillate inflorescences and completely enclosing of the bract to the ovary are not monophyletic, but respectively grouped into three lineages with species from section *Microcarpa* (Fig. 1). The five sampled species of *Macrocarpa* formed a monophyletic group in the study of Antonelli & Sanmartín (2011), whereas in the present study they are separated into different lineages among other *Microcarpa* species. Therefore, the non-monophyly of *Macrocarpa* inferred here is also postulated to be caused by reticulate evolution of these taxa (e.g. due to hybridization or incomplete lineage sorting), as may have happened with *H. orientale* and *H. arborescens*.

The sole species in section *Artocarpoides*, *H. mexicanum*, characterized by its unique capitate pistillate inflorescence, is embedded in the lineage with several *Macrocarpa* species both in the previous and the present molecular studies. Such a result implies that the large capitate inflorescence evolved from a *Macrocarpa*-like ancestor, contradicting the hypothesis of non-homologous fusion of bract(s) to ovary in *Artocarpoides* and *Macrocarpa* proposed by Todzia (1988). Concerning *Microcarpa*, it appears to be clearly polyphyletic, with its taxa entangled with those from both subgenus *Hedyosmum* and other sections of *Tafalla.* Further phylogenetic analyses based on a larger dataset and species estimations using coalescence models, particularly after the addition of unlinked low-copy nuclear and mitochondrial sequences, may be necessary to shed further light on the relationships and evolutionary histories within *Hedyosmum*.

3.2 Complexity of calibrating Chloranthaceae with fossils

In molecular dating analyses, fossil calibration plays the most critical role for accurate time estimates. Among all the fossil calibration problems, how to place the fossil correctly in a given phylogeny is challenging and several empirical cases of problematic or questionable placements of fossils have been highlighted (e.g. Near et al., 2005; Rutschmann et al., 2007). This is an especially challenging task in Chloranthaceae owing to the isolated morphology for each of the four genera. The big morphological "gaps" among genera make it hard to reconstruct the ancestral morphologies on the stems, because the long branches are also associated with long times for morphological evolution and possible extinction of intermediate forms.

Independent time estimates based on different datasets, sampling strategies, and dating methods for large angiosperm lineages have converged in narrow ranges (e.g. 140–160 Ma for the Chloranthaceae–magnoliids divergence), which also coincide with most of the fossil evidence (e.g. Wikström et al., 2001; Moore et al., 2007, 2010; but see Smith et al., 2010). The arguably robust time frames for angiosperm evolution, in combination with well-resolved phylogenies of large angiosperm groups, thus provide an alternative (or at least complementary) approach for investigating the placement of uncertain fossil taxa, although care must be taken to avoid logical circularity.

We found that *Asteropollis* was more likely to belong to the stem *Hedyosmum*, one of the two probable positions suggested by previous morphological cladistic analyses (Doyle et al., 2003; Eklund et al., 2004), but that *Chloranthistemon* probably occurred before the divergence of *Chloranthus* and *Sarcandra*, despite its assignment to the stem of *Chloranthus* (Doyle et al., 2003; Eklund et al., 2004). The supported placement of *Asteropollis* on stem *Hedyosmum*, in addition to dense sampling, a relatively large dataset, and the use of recently developed dating methods, decreases the probability that the incongruence of *Chloranthistemon* was just a victim of dating methods, as has been suggested in other cases (e.g. Sanderson & Doyle, 2001).

Moreover, two lines of morphological evidence could also support or permit our assignment of *Chloranthistemon* to the stem of both *Sarcandra* and *Chloranthus*. First, two of the three taxa, namely *Chloranthistemon endressii* and *Chloranthistemon alatus*, which have three basally independent or completely independent stamens (Crane et al., 1989; Eklund et al., 1997), differed from the basally adherent stamen in most extant *Chloranthus*. The morphological reconstruction for the whole family also suggested that the common ancestor of *Chloranthus* and *Sarcandra* possessed more than one stamen (data not shown), which would be more similar to *Chloranthus* rather than *Sarcandra* with only one single stamen. Therefore, *Chloranthistemon* as the ancestor of both *Chloranthus* and *Sarcandra* appears fully possible.

Second, the spiral and perpendicular apertures of *C. endressii* and *C. alatus*, which have been regarded to be directly derived from *Ascarina*-like monosulcate aperture and were coded as monosulcate in previous morphological analyses, are also different from the polycolpate and polyporate apertures in extant *Chloranthus* and *Sarcandra*, whereas *Chloranthistemon crossmanensis* has a *Chloranthus*-like polycolpate aperture (Crane et al., 1989; Eklund et al., 1997; Doyle et al., 2003). If *Chloranthistemon* was on the stem of *Chloranthus*, the polycolpate pollen aperture of most *Chloranthus* and the polyporate aperture of *Sarcandra* would need to have been derived independently from a monosulcate form. However, if *Chloranthistemon* was on the stem of both *Chloranthus* and *Sarcandra*, the monosulcate condition is inferred to evolve into polyporate via polycolpate, just as observed in *C. erectus.* (According to the *Chloranthus* phylogeny, or with the addition of *C. crossmanensis* to the phylogeny, the polyporate aperture of *C. erectus* has obviously evolved from polycolpate, which all other *Chloranthus* species have.) This evidence, which could have been easily neglected owing to the same number of parsimony steps but representing different evolutionary paths for *Chloranthistemon* in the phylogeny, significantly supports the assignment of *Chloranthistemon* to the stem of *Chloranthus* and *Sarcandra*.

In the present case of *Chloranthistemon*, our assignment for its new phylogenetic position is supported not only by the dating results, but also by some of the morphological evidence. The previous placement of *Chloranthistemon* according to the morphological cladistic analysis could have been misled by plesiomorphy and/or homoplastic evolution between *Chloranthistemon* and *Chloranthus*. However, our placement of *Chloranthistemon* needs to be further confirmed by more unambiguous morphological or paleontological evidence.

The phylogenetic significance of different morphological characters, and their evolutionary paths, needs to be evaluated further. Further research should also focus on estimating species trees from several unlinked gene trees from the plastid and nuclear genomes to assess possible causes of conflicting results and to refine the estimations of divergence times, relationships, and morphological evolution in Chloranthaceae.

Acknowledgements The study was supported by National Natural Science Foundation of China (grants 30100011, 30530090, 30970210), as well as a National Science Foundation Grant (IOB-0714156) to TSF for fieldwork in Peru, French Polynesia, Papua New Guinea, and New Caledonia. The authors thank R. VALEGA, J. JANOVEC from the Botanical Research Institute of Texas, and M. N. RAURAU QUISIYUPANQUI from CATIE, Costa Rica for field work logistics in Peru and Jean-Yves MEYER (Delegation for Research, French Polynesia) for help coordinating the fieldwork in French Polynesia. Field sampling was performed with the permissions of the National Institute of Natural Resources (Peru), Province Nord (New Caledonia), National Environment and Planning Agency (Jamaica), Delegation for Research (French Polynesia), the National Research Institute (Papua New Guinea), and the Forest Research Institute, Lae (Papua New Guinea).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The majority consensus tree summarized from 8 660 000 trees produced by 100 bootstrap replications of heuristic searches based on the *rbcL* dataset using the maximum parsimony (MP) method. The numbers around the nodes are the bootstrap support values.

Fig. S2. The majority consensus tree summarized from 8 963 000 trees produced by 100 bootstrap replications of heuristic searches based on the *trnL-F* dataset using the maximum parsimony (MP) method. The numbers around the nodes are the bootstrap support values.

Fig. S3. The majority consensus tree summarized from 9 017 300 trees produced by 100 bootstrap replications of heuristic searches based on the *rpl20–rps12* dataset using the maximum parsimony (MP) method. The numbers around the nodes are the bootstrap support values.

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