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Int. J. Plant Sci. 164(5 Suppl.):S383–S392. 2003.
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1058-5893/2003/16405S-0013$15.00

THE DEEPEST SPLITS IN CHLORANTHACEAE AS RESOLVED BY CHLOROPLAST SEQUENCES

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Evidence from the fossil record, comparative morphology, and molecular phylogenetic analyses indicates that Chloranthaceae are among the oldest lineages of flowering plants alive today. Their four genera (ca. 65 species) today are disjunctly distributed in the Neotropics, China, tropical Asia, and Australasia, with a single species in Madagascar but none in mainland Africa. In the Cretaceous, Chloranthaceae occurred in much of Laurasia as well as Africa, Australia, and southern South America. We used DNA sequence data from the plastid *rbc*L gene, the *rpl20-rps12* spacer, the *trn*L intron, and the *trn*L-F spacer to evaluate intra-Chloranthaceae relationships and geographic disjunctions. In agreement with earlier analyses, *Hedyosmum* was found to be sister to the remaining genera, followed by *Ascarina* and *Chloranthus* + *Sarcandra*. Bayesian and parsimony analyses of the combined data yielded resolved and well-supported trees except for polytomies among Andean *Hedyosmum* and Madagascan-Australasian-Polynesian *Ascarina*. The sole Asiatic species of *Hedyosmum*, *Hedyosmum orientale* from Hainan, China, was sister to Caribbean and Neotropical species. Likelihood ratio tests on the *rbc*L data set did not reject the assumption of a clock as long as the long-branched outgroup *Canella* was excluded. Two alternative fossil calibrations were used to convert genetic distances into absolute ages. Calibrations with *Hedyosmum*-like flowers from the Barremian-Aptian or *Chloranthus*-like androecia from the Turonian yielded substitution rates that differed by a factor of two, illustrating a perhaps unsolvable problem in molecular clock–based studies that use several calibration fossils. The alternative rates place the onset of divergence among crown group (extant) species of *Hedyosmum* at 60 or 29 Ma, between the Paleocene and the Oligocene; that among extant *Chloranthus* at 22 or 11 Ma; and that among extant *Ascarina* at 18 or 9 Ma, implying long-distance dispersal between Madagascar and Australasia-Polynesia.

Keywords: *Ascarina*, biogeography, Chloranthaceae, *Hedyosmum*, long-distance dispersal, molecular clock, *Sarcandra*.

Introduction

Morphologists and paleobotanist are fond of Chloranthaceae because of their unusual flowers, occasionally vesselless xylem, and deep fossil record (Endress 1986, 1987; Friis et al. 1986; Carlquist 1987, 1990, 1992*a*, 1992*b*; Crane et al. 1989; Herendeen et al. 1993; Eklund et al. 1997; Eklund 1999; Doyle et al. 2003). During the past 2 decades, fieldwork has begun on the family's pollination and mating systems (Endress 1987; Ma et al. 1997; Luo and Li 1999; von Balthazar and Endress 1999; Wang et al. 1999; Tosaki et al. 2001; see Endress 2001 for a summary). From a molecular phylogenetic viewpoint, however, Chloranthaceae have received little attention, with the notable exception of studies of *Chloranthus* by Kong and Chen (2000) and Kong et al. (2002*a*). Kong et al. (2002*a*) obtained a resolved and well-supported phylogeny for the 10 species of *Chloranthus* (recognized in their forthcoming monograph) on the basis of combined sequences from chloroplast and nuclear regions and rooted with a species of *Sarcandra*. However, Chloranthaceae comprise four genera, *Ascarina*, *Chloranthus*, *Hedyosmum*, and *Sarcandra*, with ca. 65 species altogether, 40–50 of them in *Hedyosmum* (Todzia 1988; C. A. Todzia, personal communication, 2002). The five-gene data set of Qiu et al. (1999, 2000), the first to include all four genera, yielded the relationships *Hedyosmum* (*Ascarina* [*Chloranthus* + *Sarcandra*]). A subsequent 11-gene analysis that included the same four species of Chloranthaceae and six additional genes yielded the same topology (Zanis et al. 2002). The molecular topologies had high bootstrap support but contradicted nonmolecular interpretations of the deepest splits in Chloranthaceae (Eklund 1999; Stevens 2001; but see Doyle and Endress 2000). Ecological differences between these pairs undoubtedly have influenced their morphological evolution and have obscured ancestral relationships. For example, *Chloranthus* and *Sarcandra* have bisexual flowers and are insect pollinated, while *Ascarina* and *Hedyosmum* have unisexual flowers and are wind pollinated (Endress 1987; also the pollination studies cited above). Unisexual (diclinous) flowers are strongly correlated with wind pollination probably to optimize dispersal and capture of wind-borne pollen (Renner and Ricklefs 1995). In environments favoring wind pollination, dicliny and a monoecious or dioecious sexual system may therefore be strongly favored, leading to repeated transitions as lineages enter such environments. One can thus readily imagine that *Ascarina* and *Hedyosmum* acquired their unisexual, wind-pollinated flowers independently of each other.

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Manuscript received November 2002; revised manuscript received February 2003.

In terms of vegetative ecology, most Chloranthaceae are shrubs or treelets in low to high montane wet forests, usually in naturally disturbed habitats (Feild et al., in press). One species of *Hedyosmum* (*Hedyosmum orientale*) and eight species of *Chloranthus* are perennial herbs (Todzia 1988; Kong et al. 2002*a*). Noteworthy is the loss of vessels from the aboveground xylem but not the roots of *Sarcandra* (Carlquist 1987).

The contradictory generic relationships inferred from some morphological and molecular analyses have prompted two new investigations. One is a morphological analysis of 131 characters for living and fossil Chloranthaceae by Doyle et al. (2003). The other is this analysis of chloroplast spacer, intron, and gene sequences for a representative sample of species. Both studies support the generic relationships found by Qiu et al. (1999) and Doyle and Endress (2000). Although taxon sampling in the morphological analysis is twice that of the molecular analysis, both recover nearly the same relationships where they overlap. Doyle et al. (2003) discuss past and novel views on inter- and intrafamily relationships of Chloranthaceae, with particular emphasis on inflorescence and androecium evolution. Here, we focus instead on the implications of molecular data for the ages of the deepest splits and geographic disjunctions in Chloranthaceae.

In principle, Chloranthaceae are well suited for molecular-clock dating because of their extensive fossil record, which permits multiple calibrations of genetic distances in a tree. The caveat refers to the well-understood weaknesses of molecular clock–based estimates. These are threefold. First, noise is introduced by using fossils to provide minimum ages for particular nodes even though morphology could have changed much earlier than the point of divergence of the gene sequences that are the basis of those nodes. Second, rate variation among taxa and/or positions along the sequenced strands can invalidate the assumptions of the method. Third, the substitution process is stochastic in nature (Hillis et al. 1996; Sanderson 1998; Sanderson and Doyle 2001; Wikström et al. 2002). All estimates therefore have large error margins and, like all evidence, need to be evaluated critically in the light of additional data.

As reviewed by Doyle et al. (2003) and also Eklund (1999), the fossil record of Chloranthaceae probably starts with Early Cretaceous *Ascarina*-like *Clavatipollenites* pollen from an almost worldwide range of localities. *Clavatipollenites* grains have recently been found associated with *Couperites* fruits from the Early Cenomanian of North America (Pedersen et al. 1991), which may represent a lineage basal in, or possibly just outside, extant Chloranthaceae (Doyle et al. 2003). It is doubtful, therefore, that *Clavatipollenites* provides a minimum age of extant *Ascarina*; rather, it seems to have evolved along the stem lineage of Chloranthaceae, and once the sister clade of Chloranthaceae is identified, pollen can help constrain the age of the family (see "Material and Methods" for Chloranthaceae outgroups).

At least two other sets of fossils, however, constrain intra-Chloranthaceae nodes. *Hedyosmum*-like female flowers with attached pollen (*Asteropollis*) from the Barremian-Aptian of Portugal (Friis et al. 1994, 1997) provide a minimum age for crown group *Hedyosmum*, and *Chloranthus*-like androecia from the Turonian of New Jersey and the Santonian-Campanian of Sweden (Friis et al. 1986; Crane et al. 1989; Herendeen et al. 1993; Eklund et al. 1997; Eklund 1999) provide a minimum age for crown group *Chloranthus*. Three kinds of androecia have so far been described, *Chloranthistemon alatus*, *Chloranthistemon crossmanensis*, and *Chloranthistemon endressii*, and they possess a strongly zygomorphic tripartite bauplan that among living angiosperms is known only from *Chloranthus* (Herendeen et al. 1993; Kong et al. 2002*b*). *Chloranthistemon alatus* and *C. endressii* are in addition known from inflorescences and flowers that also fit with *Chloranthus*.

Age estimates for major splits in the Chloranthaceae could clarify how long the single species of *Hedyosmum* in China (*H. orientale*) has been isolated from congeners in the Caribbean and Central and South America and since when *Ascarina* has occupied its Madagascan-Australasian-Polynesian range.

Material and Methods

Taxon Sampling

Our sampling includes five of the 10 species of *Ascarina*, nine of the 10 species of *Chloranthus* recognized by Kong et al. (2002*a*), five of the 40–50 species of *Hedyosmum* (Todzia 1988; C. A. Todzia, personal communication, 2002), and one of the two species of *Sarcandra* (for vouchers and GenBank accession numbers of all species with their geographical provenance, see table 1). We could not obtain *trn*L-F spacer sequences (ca. 350 bp) for *Hedyosmum bonplandianum* and *Hedyosmum translucidum* and therefore used *Hedyosmum goudotianum trn*L-F to complement their *rbc*L and *rpl20* data. All three species represent subgenus *Tafalla* (Todzia 1988), and we are not addressing intra-*Tafalla* relationships. *Hedyosmum* is the focus of an ongoing molecular phylogenetic study by L. Andersson (personal communication, 2002), who contributed two *rbc*L sequences (table 1).

Trees were rooted with Canellaceae, Winteraceae, and Magnoliaceae (table 1). These taxa were chosen on the basis of two considerations. First, an ongoing analysis of nine genes provides weak support for a position of Chloranthaceae as sister to the eumagnoliids, namely Canellales (=Winterales), Piperales, Laurales, and Magnoliales (Y.-L. Qiu, personal communication, 2002; eumagnoliids is here used *sensu* Qiu et al. 2000). Other analyses of three, five, and 11 genes show Chloranthaceae in a polytomy with Canellales, Piperales, Laurales, Magnoliales, *Ceratophyllum*, and monocots (Qiu et al. 1999, 2000; Soltis et al. 2000; Zanis et al. 2002). Chloroplast spacer and intron sequences of some of these potential outgroups (where available) could not be aligned unambiguously, resulting in the necessary exclusion of sections that are informative in the ingroup. Because of this problem and because our concern is with intra-Chloranthaceae relationships, we selected a representative group of eumagnoliids whose sequences were reliably alignable with those of Chloranthaceae. The taxa returned by GenBank BLAST searches in October 2002 as most similar to submitted sequences of *Hedyosmum* and *Chloranthus* were Canellaceae, Winteraceae, and Magnoliaceae.

DNA Isolation, Amplification, and Alignment

Total genomic DNA was isolated from silica-dried leaves using DNeasy kits (QIAGEN, Valencia, Calif.), NucleoSpin-Plant kits (Macherey-Nagel, Düren), or the CTAB method (Doyle and Doyle 1987), with the modifications that 4% CTAB was used instead of 2% and 8 μL of RNase were added to each sample before incubation. Ten DNA aliquots were received from colleagues (table 1), each with voucher details. In all, we sequenced four chloroplast regions, but one of them (the *rpl16* intron) lacks some taxa and is therefore not included here. All data sets have been submitted to GenBank and are accessible to others (table 1; GenBank numbers for the *rpl16* sequences are AY237819–AY237825). The *rbc*L gene was amplified by PCR using the primers developed by Olmstead et al. (1992). To amplify the *trn*L intron and adjacent spacer before the *trn*F gene, we used the universal primers c, d, e, and f of Taberlet et al. (1991). The *rpl20-rps12* spacer was amplified with primers *rpl20* and *rps12* of Hamilton (1999).

Amplifications were usually performed in 25 μL volumes, with 15.3 μL deionized sterile water, 1.5 μL of 25 μmol/L $MgCl_2$ solution, 2.5 μL 10 × buffer (Promega, Madison, Wis.), 2 μL of a 2.5 μmol/L dNTP solution in equimolar ratio, 1 μL of each primer at 10 pmol/μL, 1 unit (0.2 μL) polymerase (Promega), and 1 μL of the template DNA. PCR conditions were a pretreatment of 3 min at 94°C, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min, with a posttreatment of 7 min at 72°C. PCR products were purified either by running the entire product on a low-melting-point agarose gel and then recovering the DNA with QIAquick Gel Extraction Kits (QIAGEN) or by using QIAquick PCR Purification kits directly without a prior gel purification step. Cycle sequencing of the purified PCR products used the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems [ABI], Norwalk, Conn.). The sequencing profile consisted of an initial treatment of 2 min at 96°C, 60 cycles of 15 s at 96°C, 12 s at 48°C, and 4 min at 60°C, and a final incubation of 4 min at 60°C. The dye was removed by 2 μL 3 mol/L NaOAc (pH 4.6) and 50 μL ethanol precipitation, and samples were then run on the ABI 377 automated sequencer of the Department of Biology at the University of Missouri–St. Louis.

Sequences were aligned manually using Sequencher version 4.1.2 (GeneCodes Corporation, Ann Arbor, Mich.) and exported as NEXUS files. The *trn*L intron and *trn*L-F spacer, the *rpl20-rps12* spacer, and the *rbc*L gene sequences were analyzed separately and then combined. Three short stretches in the *trn*L region and one 50-bp strech in the *rpl20-rps12* spacer that were ambiguous in alignment were excluded from all analyses.

Phylogenetic Analyses

All analyses were conducted with test version 4.0.b10 of PAUP* (Swofford 2002). Bayesian analyses relied on MrBayes version 2.01 (Huelsenbeck and Ronquist 2001) and ran for 100,000 generations, sampling trees every tenth generation and discarding the first 4000 trees as burn-in. Parsimony analyses used branch-and-bound searches. All multiple trees were kept, and zero-length branches were collapsed. For parsimony analyses, DNA insertions or deletions were treated as missing data; for maximum likelihood (ML) analyses, all gapped and ambiguous characters were excluded; and for the Bayesian analysis, most gapped characters were removed by hand before analysis because MrBayes does not provide a character exclusion option. Bootstrap values were estimated from 10,000 replicates of full heuristic searches using tree bisection-reconnection (TBR) branch swapping. ML and Bayesian analyses were performed using the general time reversible (GTR) model (Yang 1994), which fit the data significantly better than did less parameter-rich models (data not shown). The rate matrix, proportion of invariable sites (P_{inv}), and shape parameter were estimated simultaneously using four rate categories and empirically observed base frequencies. All ML searches were heuristic and used TBR swapping, with the "multiple trees" and "steepest descent" options in effect. The initial model estimation search used a neighbor-joining tree as the starting tree. Parameter estimation reached completion after 1708 rearrangements, and the estimated parameters were then used in searches (each running to completion) that used neighbor-joining trees as a starting tree, with or without clock enforcement.

Molecular Clock Analyses and Calibration

Substitution accumulation in spacer and intron regions is poorly understood and therefore difficult to model. The *trn*L intron, the sole group I intron in the chloroplast genome (Kelchner 2002), is especially problematic in its secondary and tertiary structure (Besendahl et al. 2000). By contrast, the *rbc*L gene is well understood (Kellogg and Juliano 1997), and, where it does not violate tests for clocklike substitution accumulation, it has been widely used for time estimation within angiosperms (for critical discussion, see Sanderson and Doyle 2001; Wikström et al. 2002). We therefore restricted molecular clock analyses to the *rbc*L data. Excluded from all ML analyses were 23 missing or ambiguous characters. Likelihood ratio testing (LRT) was used to assess whether substitution rates in sister groups can be modeled as clocklike. The LRT statistic is calculated as $-2\ (\ln L_0 - \ln L_1)$, where L_0 and L_1 are the likelihoods under the null (clock) and alternative (nonclock) hypotheses, respectively. The significance of this value is judged by comparing it to a χ^2 distribution with n degrees of freedom, where n is the number of included taxa minus 2.

Fossils used to convert genetic branch lengths (as output by PAUP* after clock-enforced ML searches) into absolute times were those mentioned in "Introduction" (alternative fossil calibrations are described in "Results"). Absolute dates for geological periods are from Berggren et al. (1995). Standard deviations (SD) on the molecular clock estimates were calculated by estimating the SD of the distance from a calibration node to the tips of the tree and then using this value to obtain the SDs of the estimated ages. According to the binomial distribution, the number of nucleotide substitutions (S) is equal to the product of the total number of nucleotides in a sequence (N) times the proportion of nucleotides substituted (p). Thus, $S = Np$. The SD of this value is the square root of $Np(1-p)$, or $\mathrm{SD}(S) = \sqrt{Np(1-p)}$. The SD of the number of nucleotides substituted divided by the total number of nucleotides is the SD of the proportion of nucleotide substitutions. Thus, $\mathrm{SD}(p) = \sqrt{p(1-p)/N}$.

Table 1

Sources of Plant Material and GenBank Accession Numbers for the Phylogenetic Analysis of Chloranthaceae

Taxon	*rpl20-rps12* spacer	*trn*L-*trn*F spacer	*trn*L intron	*rbc*L gene	Voucher/source/country of provenance
Ingroup:					
Ascarina J. R. Forster & G. Forster:					
A. coursii (Humbert & Capuron) J.-F. Leroy & Jérémie (*Ascarinopsis coursii* Humbert & Capuron)	AY236721	AY236747	AY236747	AY236844	*D. Ravelonarivo 1139* (MO), Madagascar
A. lucida Hook.f.	AY236722	AY236745	AY236745		*P. de Lange 3594* (AK), New Zealand, DNA aliquot from R. Olmstead
				AF238050	Graham et al. 2000
A. polystachya Forster	AY236732	AY237816	AY237816	AY236842	*T. Feild s.n.* (no voucher), Tahiti
A. rubricaulis Solms-Laub.	AY236731	AY236746	AY236746		*T. Feild s.n.* (no voucher), New Caledonia
				AF197592	Qiu et al. 2000, DNA aliquot: *L. Thien 500* (Tulane), New Caledonia, det. S. Renner
A. swamyana A. C. Smith		AY236748	AY236748	AY236843	*T. Feild s.n.* (no voucher), Fiji, Taveuni Island
Chloranthus Sw.:					
C. angustifolius Oliver	AY236724	AY236743		AY236839	*Kong 97701* (PE), China
			AF364600		Kong et al. 2002*a*, China
C. erectus (Buch.-Ham.) B. Verdcourt	AY236738			AY236834	Qiu, DNA aliquot 92017: *C. R. Parks s.n.*, Laguna, Philippines
		AF329949	AF329949		Kong et al. 2002*a*, China
C. fortunei (A. Gray) Solms-Laub.		AY236744		AY236840	*H. Takahashi 20779* (GIFU), Japan
			AF364601		Kong et al. 2002*a*, China
C. henryi Hemsl. (incl. *C. multistachys* Pei)	AY236735	AY236742		AY236837	Qiu, DNA aliquot 97097: Cult. at NA, originally from China, *K. Wurdack 2002–137* (US)
			AF364599		Kong et al. 2002*a*, China
C. japonicus Siebold	AY236723				*P. Lowry & P. Lôc 4943* (MO), Vietnam
		AF364603	AF364603		Kong et al. 2002*a*, China
				L12640	Qiu et al. 1993: Cult. by J. J. Wurdack, originally from Japan, *M. Chase 204* (NCU)
C. nervosus Collett et Hemsl.	AY236733			AY236841	*Kong 97120* (PE), China
		AF364602	AF364602		Kong et al. 2002*a*, China
C. oldhamii Solms-Laub.	AY236734			AY236838	*J. C. Wang s.n.*, China (Taiwan)
		AF364598	AF364598		Kong et al. 2002*a*, China (Taiwan)
C. serratus (Thunb.) Roem. & Schult.	AY236736			AY236836	*T. Feild s.n.*, Hong Kong
		AF364596	AF364596		Kong et al. 2002*a*, China
C. spicatus (Thunb.) Makino	AY236737	AY236741		AY236835	Qiu 99101, DNA aliquot: Cult. Hawaii, Honolulu, no voucher
			AF329950		Kong et al. 2002*a*, China

Hedyosmum Sw.:					
Subgen. *Hedyosmum* Solms-Laub.:					
H. orientale Merrill & Chun	AY236730	AY236749	AY236749	AY236848	*T. Feild & Li 23* (PE), China
Subgen. *Tafalla* (R. & P.) Solms-Laub.:					
H. arborescens Sw.	AY236720	AY236750	AY236750		Qiu, DNA aliquot: *M. Chase 338* (NCU), Puerto Rico
				L12649	Qiu et al. 1993
H. bonplandianum Kunth	AY236729		AY236751	AY236845	*T. Feild 1025* (voucher lost), Costa Rica
H. goudotianum Solms-Laub.		AY236754	AY236754		*G. McPherson 15898* (MO), Panama
H. sprucei Solms-Laub.	AY236719	AY236752	AY236752		L. Andersson, DNA aliquot: *G. Harling & L. Andersson 24138* (GB), Ecuador
				AY236846	L. Andersson (unpublished data)
H. translucidum Cuatrec.	AY236728		AY236753		L. Andersson, DNA aliquot: *G. Harling & L. Andersson 21980* (GB), Ecuador
				AY236847	L. Andersson (unpublished data)
Sarcandra Gardn.:					
S. chloranthoides Gardn.	AY236739	AY236740	AY236740		Qiu, DNA aliquot: *Qiu 92002* (NCU)
				L12663	Qiu et al. 1993, submitted to GenBank as *S.* "*grandifolia*" (Y. Qiu, personal communication, 2002)
Outgroups:					
Canellaceae:					
Canella winterana (L.) Gaertn.	AY236727				Cult. Munich Botanical Garden
		AY004152	AY004152		Karol et al. 2000
				AJ131928	Qiu et al. 1993
Magnoliaceae:					
Magnolia hypoleuca Sieb. & Zucc.	AY236725	AF012395	AY236755		Renner 1999 and this article: Cult. *Missouri BG 732556*
				X54345	Golenberg et al. 1990
Winteraceae:					
Drimys winteri J. R. Forster & G. Forster	AY236726				Cult. Munich Botanical Garden
		AY004143	AY004143		Karol et al. 2000
				AF093734	Hoot et al. 1999
Takhtajania perrieri (Capuron) M. Baranova & J.-F. Leroy		AY004146	AY004146		Karol et al. 2000
				AF206824	Soltis et al. 1999

Note. Vouchers are deposited in the following herbaria: AK, GB, GIFU (Gifu University, Japan), MO, NA, NCU, PE, and US.

Results

The aligned data sets comprised 1144 bp from the *trn*L intron and *trn*L-F spacer, 1403 bp of the *rbc*L gene, and 857 bp of the *rpl20-rps12* spacer region. Parsimony analysis of the concatenated data yielded 18 equally parsimonious trees (Consistency Index [CI] = 0.82, Retention Index [RI] = 0.91, with 209 mutations autapomorphic, 379 potentially synapomorphic). The trees differed only in the relationships found among *Ascarina coursii*, *Ascarina lucida*, and *Ascarina rubricaulis* and *Hedyosmum bonplandianum*, *Hedyosmum sprucei*, and *Hedyosmum translucidum*, which were not resolved with confidence. ML and Bayesian analysis (fig. 1) yielded the same topologies, again with the relationships within *Ascarina* and *Hedyosmum* poorly resolved.

For just the 24 *rbc*L sequences, statistical comparison of the likelihoods of the three clock trees with the single nonclock tree barely rejected a molecular clock. With the long-branched *Canella* excluded, likelihoods of clock and nonclock trees became statistically indistinguishable ($\chi^2 = 24.616$, df = 21, $P < 0.1$). The *rbc*L-only data set yielded the generic relationships found with the combined data (fig. 1) but differed in species relationships within *Ascarina* and *Chloranthus* because *rbc*L contains insufficient information within these genera. Since the *rbc*L data justified the clock assumption, we opted for constraining the *rbc*L clock topology to agree with the

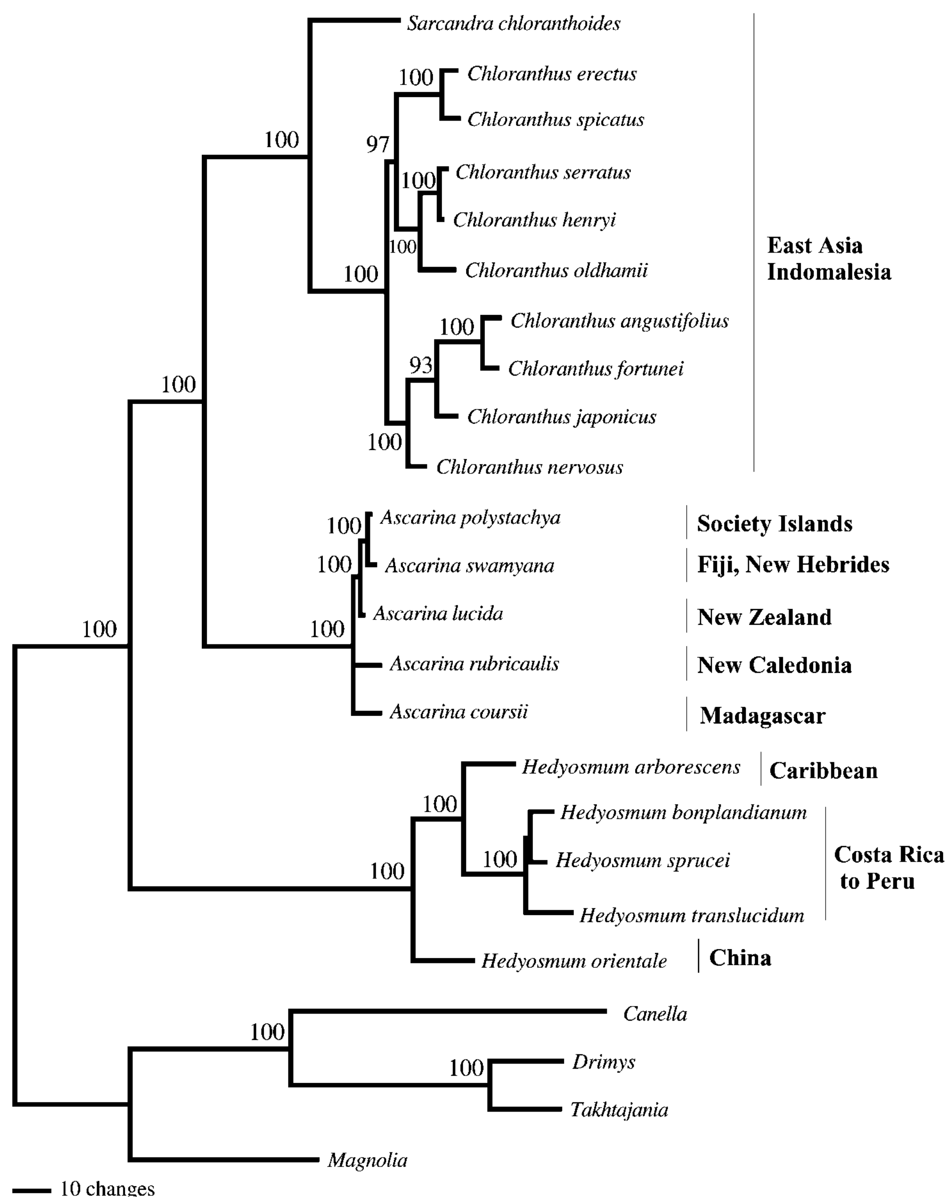

Fig. 1 Bayesian tree obtained from Chloranthaceae chloroplast *rbc*L, *rpl20*, *trn*L, and *trn*L-F sequences (3228 bp after elimination of most gapped characters) under the GTR + G model. Support values shown at nodes are posterior probabilities.

better-supported topology resulting from the combined *rbc*L + *trn*L + *trn*L − F + *rpl*20 data. Forcing one to agree with the other had little affect on branch lengths and rates. To calibrate genetic distances, we first set node A (fig. 2) to the age of the oldest *Hedyosmum*-like flowers, conservatively about 120 Ma (Friis et al. 1994, 1997). For an alternative calibration, node B (fig. 2) was set to 90 Ma on the basis of the oldest *Chloranthus*-like androecia (Herendeen et al. 1993).

With node A set to 120 Ma, substitution rates of 0.00018 or 0.00015 substitutions per site per million years can be calculated by dividing the genetic distances from node A to the tip by 120. This distance is 0.01847 in the *rbc*L tree found by heuristic searching under the clock model; it is 0.02183 in the *rbc*L tree constrained to agree with the combined topology (fig. 1). The first distance divided by 120 yields a rate of 0.00015, and the second yields a rate of 0.00018. Either rate yields a minimum age of 29 ± 11 Ma for node C, the onset of divergence among extant species of *Hedyosmum*. The distance from node C to the tip is 0.00441 in the unconstrained *rbc*L topology and 0.00526 in the constrained topology. The age of node D, the crown group of *Chloranthus*, is estimated at 11 Ma when the *rbc*L topology is constrained to agree with the combined tree and at 12 Ma if the *rbc*L-only topology is used. The distance from node D to the tip is 0.00197 in the constrained tree and 0.00180 in the unconstrained tree. The age of node E, the onset of divergence among extant species of *Ascarina*, is 9 to 10 ± 6 Ma. The distance from node E to the tip is 0.00154 in the constrained tree, which divided by 0.00018 is 9. It is 0.00151 in the unconstrained tree, which divided by 0.00015 is 10.

Setting node B to 90 Ma, on the basis of the Turonian *Chloranthus*-like fossils, results in rates of 0.000088 or 0.00083 substitutions per site per million years. The distance from node B to the tips is 0.00796 in the constrained tree, which divided by 90 is 0.000088. It is 0.00745 in the unconstrained clock tree, which divided by 90 is 0.000083. These slower rates give correspondingly older ages, namely 53 or 63 ± 20 Ma (from the unconstrained and constrained clock topologies, respectively) for node C, 22 Ma for node D (whether the unconstrained or constrained topology is used for the calculation), and 17 or 18 ± 13 Ma for node E from the unconstrained and constrained topologies, respectively.

Fig. 2 Maximum likelihood tree obtained from Chloranthaceae *rbc*L sequences under the GTR + G + P_{inv} + clock model with the topology constrained to agree with that found with combined *rbc*L, *rpl20*, *trn*L, and *trn*L-F data (fig. 1). Nodes *A* and *B* were used as alternative calibration points based either on *Hedyosmum*-like flowers from the Barremian-Aptian (120 Ma) or *Chloranthus*-like androecia from the Turonian (90 Ma). Node *C* denotes the onset of divergence among extant (crown group) *Hedyosmum*, node *D* denotes that among crown group *Chloranthus*, and node *E* denotes that among crown group *Ascarina*.

Discussion

Today, three of the four genera of Chloranthaceae are species poor and are more or less restricted in their distribution. *Chloranthus* and *Sarcandra*, with 10 and two species, are endemic in China, Japan, and Southeast Asia, while *Ascarina*, with 10 species, ranges from French Polynesia to New Zealand, New Caledonia, Borneo, New Guinea, and the Philippines, with a single species on Madagascar. Only *Hedyosmum* is species rich (40–50 species), and it is also the genus with the widest distribution. The fossil record, however, indicates that today's genera represent the survivors of a group that during the Early Cretaceous comprised many members in both Laurasia and Gondwana. *Clavatipollenites*, associated with the stem lineage of Chloranthaceae, and *Asteropollis*, associated with *Hedyosmum*, are among the oldest conspicuous angiosperm pollen types in Laurasia, southern Argentina, southwestern Africa, and Australia (Doyle et al. 2003 and references therein). This implies that Chloranthaceae have suffered widespread extinction, and great care is indicated when extrapolating from their reduced morphological and genetic diversity back in time. Widespread extinction leaves living taxa that are isolated from each other by numerous unique characters, causing problems for both molecular and morphological phylogenetic reconstruction. This probably explains why phylogenetic placement of Chloranthaceae has been so difficult. However, "long branches" caused by highly isolated taxa do not seem to be a problem within Chloranthaceae. At least for the *rbc*L gene, substitution accumulation in Chloranthaceae can be modeled as clocklike, and visual inspection of the combined intron, spacer, and gene data (fig. 1) also suggests no obvious outliers except for one of the outgroups, *Canella*.

In our data, the effects of alternative calibration fix points far outweighed the effects of topology. Depending on the calibration point used, we obtained Paleocene (60 Ma) or Oligocene (29 Ma) ages of crown group *Hedyosmum*, and Middle to Late Miocene ages for crown group *Chloranthus* and *Ascarina*, 22 or 11–12 Ma and 18 or 9–10 Ma, respectively. By contrast, constraining the *rbc*L-only topology to agree with the better-supported topology from the combined data had little effect on branch lengths. We are unaware of criteria by which to choose between alternative calibration points in data sets that satisfy the clock. Even where substitutions are clock-like and fossils are correctly identified and dated, estimates from the same tree could fail to overlap because of the stochastic nature of substitutions, rate variation (rates can vary considerably yet pass likelihood ratio tests), or because fossils assigned to nodes in fact represent the stem lineage. Few plant groups have fossil records and molecular data sets extensive enough to explore the effects of alternative fossil calibrations, and, realistically, most fossils will probably connect to "places" in the tree before or after a gene branching event (Hillis et al. 1996, fig. 10; Renner et al. 2000, 2001; Sanderson and Doyle 2001). One solution is to take the midpoint as a reasonable estimate, in our case an age of 45 Ma for the initial divergence among species in the crown group of *Hedyosmum*, 17 Ma for crown group *Chloranthus*, and 14 Ma for the initial divergence among crown group *Ascarina*. These young ages are one of the most striking results reported here because the fossil record of these genera goes back so much further. The fossils, however, clearly represent stem lineages, not crown groups.

Based mainly on plesiomorphic (chartaceous not fleshy) bracts, the single Asian species of *Hedyosmum*, *Hedyosmum orientale*, has been placed in a subgenus together with four species from Cuba, Jamaica, and Hispaniola and one Central American species (Todzia 1988). The cladistic analysis of Doyle et al. (2003) also showed it clustering with species from Cuba, Jamaica, and Hispaniola. Sequencing is needed of the four Caribbean species and also the unusual *Hedyosmum mexicanum*, long placed in a subgenus by itself because of its thick, fleshy, fused bracts (Endress 1971; Todzia 1988), to further test the phylogenetic position of *H. orientale*. Current results certainly do not reject Raven and Axelrod's hypothesis (1974, p. 564) that crown group *Hedyosmum* entered South America from the North. This possibly occurred via islands of the proto-Antilles arc as stepping stones as hypothesized for subclades of Melastomataceae and Lauraceae that also appear to have entered South America long before the closing of the Isthmus of Panama (Chanderbali et al. 2001; Renner et al. 2001).

Molecular data (Qiu et al. 1999, 2000; this article) and morphological data (Doyle and Endress 2000; Doyle et al. 2003) agree that the deepest split in the Chloranthaceae is between *Hedyosmum* and the remaining genera and that *Ascarina* is sister to *Sarcandra* and *Chloranthus*. There is also nearly complete agreement between the topology found for *Chloranthus* on the basis of combined chloroplast and nuclear (ITS) data (Kong et al. 2002*a*) and the one found here, which, however, is based on many of the same *trn*L intron and spacer sequences (table 1). The single weakly supported node (70% bootstrap support) in Kong et al.'s tree involved two species of *Chloranthus*, *Chloranthus nervosus* and *Chloranthus japonicus*, which were placed as sisters. Our data place *C. nervosus* basal to *Chloranthus japonicus*, *Chloranthus angustifolius*, and *Chloranthus fortunei*, but this, too, has limited support (70% bootstrap, 93% posterior probability). These four species are characterized by apically expanded stamen lobes, while their sister clade comprises species with shorter and variously lobed stamens. The least lobed androecia are found in the sister species *Chloranthus erectus* and *Chloranthus spicatus*.

The estimated divergence times of only 18–9 Ma for extant species of *Ascarina* in New Caledonia, New Zealand, French Polynesia, and Madagascar implies long-distance dispersal of the fruits. The Madagascan *Ascarina* (*Ascarinopsis*) *coursii* shows almost no sequence divergence from the Australasian species and long-distance dispersal between Madagascar, Australia, and Polynesia would indeed fit a pattern seen in many other fleshy-fruited groups disjunct between these areas (Thorne 1973; Renner and Meyer 2001; Renner et al. 2001; Malcomber 2002; S. S. Renner and L.-B. Zhang, unpublished data for Hernandiaceae). Ridley (1930, p. 465) mentions dispersal of the fleshy fruits of *Ascarina* by birds, and Raven and Axelrod (1974, pp. 612–613) went so far as to specify that *Ascarina* reached Madagascar from the east ("by long-distance dispersal from one of the other lands bordering the Indian Ocean"). Under their view, *A. coursii* should be embedded among Asian-Australasian-Polynesian species of *Ascarina*, which needs to be evaluated with longer or more variable DNA regions and denser sampling within *Ascarina*. The five species not yet sequenced are *Ascarina diffusa* A. C. Smith from the Solomon Islands to the Marquesas; *Ascarina marquesensis* A. C. Smith from the Marquesas; *Ascarina philippinensis* C. B. Rob. from Borneo, New Guinea, and the Philippines; *Ascarina subsessilis* Verd. from New Guinea; and *Ascarina solmsiana* Schltr. from New Caledonia. Addition of these species would permit full evaluation of the position of *A. coursii*. It is clear, however, that Raven and Axelrod were right about the means of dispersal in *Ascarina*.

Acknowledgments

We are grateful to Jim Doyle, Helena Eklund, and Patrick Herendeen for letting us see their article on Chloranthaceae before publication; Jim Doyle and Lennart Andersson for comments on the manuscript; Taylor Feild, Hiroshi Takahashi, Yin-Long Qiu, Lennart Andersson, Richard Olmstead, Hong-Zhi Kong, and Jenn-Che Wang for leaf material or aliquots; Lennart Anderson for unpublished *rbc*L sequences of *Hedyosmum*; Lennart Thien for color pictures of an *Ascarina rubricaulis* voucher specimen; Ken Wurdack for color pictures of a *Chloranthus multistachys* voucher; and Peter Linder, Elena Conti, and the Institute of Systematic Botany, University of Zurich, for inviting us to contribute a paper to the symposium honoring Peter Endress's contribution to the study of flower evolution and function.

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