Product profile

Assays were performed on purified wild-type and Trp387Phe variant murine COX-2 protein. Protein samples (750 nM) in 190 μl Tris-HCl buffer (100 mM, pH 8.0; 500 μM phenol at 37 °C) were treated with 100 μM [1-^{14}C]arachidonic acid (in 10 μl ethanol) for either 10 min or 1 h at 25 °C. Incubations were terminated by addition of 400 μl stop solution (80.6 μl ether, 11.4 μl methanol, 2.8 μl 1 M citric acid) after 1–3 h. The organic phase was removed and dried under nitrogen. Samples were then purged with argon and stored at −20 °C. To determine the products synthesized in the test samples, 5-, 11-, 12- and 15-HETE standard elution profiles were obtained. 1.6 μg of each standard was extracted and dried under nitrogen in the manner described above, resuspended in 500 μl methanol and placed in eppendorf tubes in 50 μl aliquots before purging with argon. HPLC conditions were modified from previous work[30]; we used a 5-mm Beckman Ultrasphere ODS C_{18} column (4.6 mm × 25 cm) run under reverse phase conditions at 1 ml min^{-1} with a mixture of the following buffers: buffer A, 0.1% v/v glacial acetic acid in water; buffer B, 0.1% v/v glacial acetic acid in acetonitrile. Initial conditions used were 70% A/30% B for 5 min then the gradient was changed linearly to 40% A/60% B over 30 min. After a 10-min isocratic period, the gradient was changed to 25% A/75% B in a linear manner over 15 min. This was followed by a 10-min isocratic period before changing the gradient to 100% B linearly over 10 min followed by a 5-min isocratic period. Product quantification was determined by use of an online liquid scintillation counter (IN/US Systems, β-RAM 2B) and a Varian 2050 detector at 235 nm to detect product containing a conjugated double bond.

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Correspondence and requests for materials should be addressed to R.G.K. (e-mail: ravi.g.kurumbail@monsanto.com). Coordinates for structures and models have been deposited in the Protein Data Bank under accession codes 1CVU, 1DCX, 1DD0 and 1DDX.

erratum

The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes

Yin-Long Qiu, Jungho Lee, Fabiana Bernasconi-Quadroni, Douglas E. Soltis, Pamela S. Soltis, Michael Zanis, Elizabeth A. Zimmer, Zhiduan Chen, Vincent Savolainen & Mark W. Chase

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Correspondence and requests for material should be addressed to P.S.S. (e-mail: psoltis@wsu.edu).

The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes

Yin-Long Qiu*, Jungho Lee*, Fabiana Bernasconi-Quadroni*, Douglas E. Soltis†, Pamela S. Soltis†, Michael Zanis†, Elizabeth A. Zimmer‡, Zhiduan Chen*§, Vincent Savolainen‖, Mark W. Chase‖

* *Institute of Systematic Botany, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland*
† *School of Biological Sciences, Washington State University, Pullman, Washington 99164-4236, USA*
‡ *Laboratory of Molecular Systematics, Smithsonian Institution, Washington DC 20560, USA*
‖ *Jodrell Laboratory Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK*

Angiosperms have dominated the Earth's vegetation since the mid-Cretaceous (90 million years ago)[1], providing much of our food, fibre, medicine and timber, yet their origin and early evolution have remained enigmatic for over a century[2–8]. One part of the enigma lies in the difficulty of identifying the earliest angiosperms; the other involves the uncertainty regarding the sister group of angiosperms among extant and fossil gymnosperms. Here we report a phylogenetic analysis of DNA sequences of five mitochondrial, plastid and nuclear genes (total aligned length 8,733 base pairs), from all basal angiosperm and gymnosperm lineages (105 species, 103 genera and 63 families). Our study demonstrates that *Amborella*, Nymphaeales and Illiciales-Trimeniaceae-*Austrobaileya* represent the first stage of angiosperm evolution, with *Amborella* being sister to all other angiosperms. We also show that Gnetales are related to the conifers and are not sister to the angiosperms, thus refuting the Anthophyte Hypothesis[1]. These results have far-reaching implications for our understanding of diversification, adaptation, genome evolution and development of the angiosperms.

Difficulty in identifying the earliest angiosperms is the result of three problems that characterize diversification of most major clades. First, the great divergence between gymnosperms and angiosperms makes assessment of character homology difficult and thus renders the otherwise powerful outgroup-approach problematic in morphological cladistic analyses[1,9]. Second, extinction, which is partly responsible for this divergence, has almost certainly occurred in both groups[1,10–16], and highlights the need of extensive taxon sampling when relying on the living diversity. Last, the fossil evidence indicates that the early angiosperms went through an explosive radiation[1,10–16], which to resolve requires the sampling of a large number of characters. Previous molecular analyses have had some success in resolving relationships among basal angiosperms[17–21]; however, their results are only weakly supported, and worse, are often contradictory because of evolutionary rate heterogeneity among lineages of the particular gene used, weak phylogenetic signal in single genes, and insufficient taxon sampling. From both theoretical and empirical studies, it is becoming increasingly clear that to address such a difficult issue as basal angiosperm phylogeny, extensive sampling in both dimensions of taxa and characters (genes) is necessary[22–24].

We obtained sequences of five genes from all three plant genomes: mitochondrial *atp1* and *matR*, plastid *atpB* and *rbcL* and nuclear 18S rDNA. They encode products involved in energy metabolism, carbohydrate synthesis and information processing. Thus, our character sampling strategy of taking multiple genes of different functions from all three genomes is designed to reduce homoplasy generated by gene-, function- and genome-specific molecular evolutionary phenomena such as rate heterogeneity, GC content bias, RNA editing and protein structural constraints[25,26]. To optimize the performance of phylogenetic methods in analysing complex diversification patterns in early angiosperms[22,23], we included 97 species, 95 genera and 55 families of basal angiosperms, essentially sampling all living families[5,8,11,20,21]. Eight gymnosperms from eight families were used as outgroups. The DNA sequences were analysed with parsimony methods; bootstrap (BS) and jackknife (JK) analyses were conducted to measure stability of phylogenetic patterns.

The same single most parsimonious tree was found in each of 1,000 random taxon-addition replicates in the analysis (Fig. 1). *Amborella*, a shrub of the monotypic New Caledonian family Amborellaceae, is sister to all other angiosperms, which are strongly (90% BS and 92% JK) supported as a monophyletic group. The next diverging lineage corresponds to Nymphaeales, the water lilies; its sister clade of the remaining angiosperms receives 98% BS and 99% JK support. The third clade consists of two small Australasian families, Austrobaileyaceae and Trimeniaceae, and two small eastern Asia-eastern North America disjunct families, Illiciaceae and Schisandraceae (Illiciales). All remaining angiosperms (euangiosperms) make up a strongly supported large clade (97% BS and 99% JK). The relationships among lineages within euangiosperms are resolved in the shortest tree but generally receive less than 50% BS support. All major lineages, however, are strongly supported; these agree with previous classifications[5,8,11] and results of cladistic analyses of morphological and molecular data[9,20,21,27]. Among gymnosperms, two gnetalean genera, *Gnetum* and *Welwitschia*, are not sister to angiosperms as suggested by the Anthophyte Hypothesis[1], but fall close to the conifers.

We observed one INDEL (insertion/deletion) in *matR* that supports the basal position of *Amborella*, Nymphaeales and Illiciales-Trimeniaceae-*Austrobaileya* (ANITA) in angiosperms: an 18-base-pair (bp) deletion in all euangiosperms but not in ANITA or gymnosperms, some of which have 6–15-bp deletions (Fig. 2). Although we cannot rule out the possibility that the sequence in the INDEL region of ANITA and gymnosperms results from independent insertions, two lines of evidence suggest that this scenario is unlikely. The sequences are found in all three ANITA lineages and all four gymnosperm lineages. Furthermore, there are identical or similar codons shared by ANITA and gymnosperms in the INDEL.

Reconstruction of deep phylogenies using DNA sequences has been plagued by problems caused by rate heterogeneity, weak phylogenetic signal in single genes, insufficient taxon sampling,

§ Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, 100093, China.

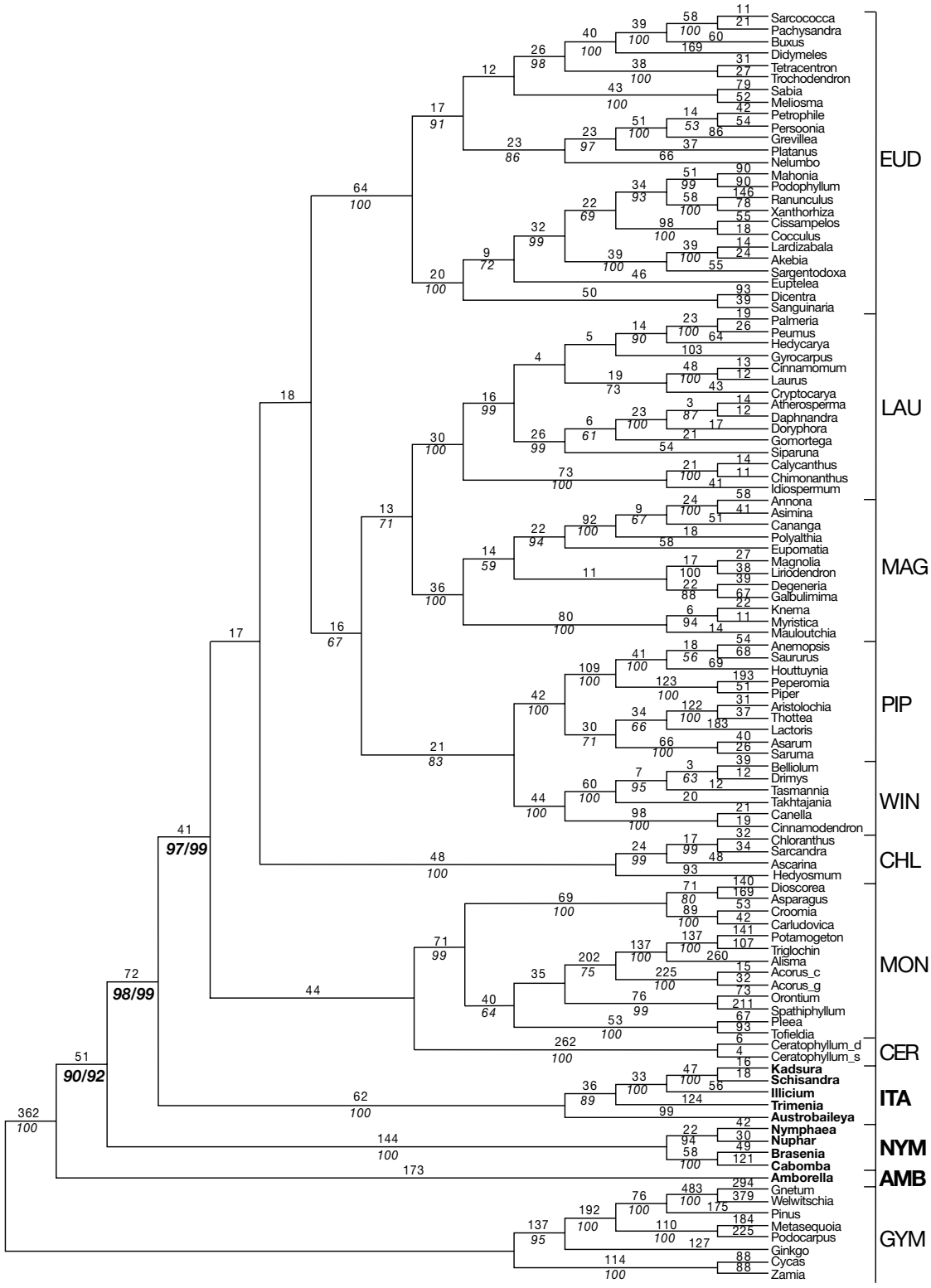

Figure 1 The single most parsimonious tree found in the five-gene DNA sequence analysis (tree length, 13,240 steps; consistency index, 0.413; retention index, 0.604). Numbers above branches are branch lengths (ACCTRAN optimization); those below in italics are bootstrap values (only those above 50% are shown; for branches related to ANITA (bold type), numbers below branches before the slash are bootstrap values and those after are jackknife values). GYM, gymnosperms; AMB, *Amborella*; NYM, Nymphaeales; ITA, Illiciales, Trimeniaceae and *Austrobaileya*; CER, *Ceratophyllum*; MON, monocots; CHL, Chloranthaceae; WIN, Winterales; PIP, Piperales; MAG, Magnoliales; LAU, Laurales; EUD, eudicots; *Acorus_c*, *A. calamus*; *Acorus_g*, *A. gramineus*; *Ceratophyllum_d*, *C. demersum*; *Ceratophyllum_s*, *C. submersum*.

explosive radiation, extinction and protein structural constraints[25,26]. In retrospect, our earlier studies using single genes suffered from some of these problems when *Ceratophyllum* was found to be sister to all other angiosperms[20,21]. The same concern could still be raised about our results presented here; however, the use of five genes with different functions from all three genomes and the sampling of almost all basal angiosperm families is likely to have reduced considerably the effect of these problems. Five lines of

```
Arabidopsis      AAT AAT AAT --- --- --- --- --- --- TGG --- GCC
Oenothera        ... ... ... --- --- --- --- --- --- ... --- ...
Vicia            ... ... ... --- --- --- --- --- --- .T. --- ...
Solanum          ... ... ... --- --- --- --- --- --- ... --- ...
Triticum         ... ..G ... --- --- --- --- --- --- .T. --- ...
Sarcococca       ... ... ... --- --- --- --- --- --- ... --- ...
Pachysandra      ... ... ... --- --- --- --- --- --- ... --- ...
Buxus            ... ... ... --- --- --- --- --- --- ... --- ...
Didymeles        ... --- ... --- --- --- --- --- --- G.. --- ...
Tetracentron     ... ... ... --- --- --- --- --- --- ... --- ...
Trochodendron    ... ... ... --- --- --- --- --- --- ... --- ...
Sabia            ... ... ... --- --- --- --- --- --- ... --- ...
Petrophile       ... ... ... --- --- --- --- --- --- ... --- ...
Persoonia        ... ... ... --- --- --- --- --- --- ... --- ...
Grevillea        ... ... ... --- --- --- --- --- --- ... --- ...
Platanus         ... ... ... --- --- --- --- --- --- ... --- ...
Nelumbo          ... ... ... --- --- --- --- --- --- ... --- ...
Mahonia          ... ... ... --- --- --- --- --- --- ... --- ...
Podophyllum      ... ... ... --- --- --- --- --- --- ... --- ...
Ranunculus       ... ... ... --- --- --- --- --- --- ... --- ...
Xanthorhiza      ... ... ... --- --- --- --- --- --- ... --- ...
Cissampelos      T.. ... ... --- --- --- --- --- --- ... --- ...
Cocculus         ... ... ... --- --- --- --- --- --- ... --- ...
Lardizabala      ... ... ... --- --- --- --- --- --- ... --- ...
Akebia           ... ... ... --- --- --- --- --- --- ... --- ...
Sargentodoxa     ... ... ... --- --- --- --- --- --- ... --- ...
Euptelea         ... ... ... --- --- --- --- --- --- ... --- ...
Dicentra         ... ... ... --- --- --- --- --- --- ... --- ...
Sanguinaria      ... ... ... --- --- --- --- --- --- ... --- ...
Palmeria         ... ... ... --- --- --- --- --- --- .T. --- ...
Peumus           ... ... ... --- --- --- --- --- --- ... --- ...
Hedycarya        ... ... ... --- --- --- --- --- --- ... --- ...
Gyrocarpus       ... ... ... --- --- --- --- --- --- ... --- ...
Cinnamomum       ... ... ... --- --- --- --- --- --- ... --- ...
Laurus           ... ... ... --- --- --- --- --- --- ... --- ...
Cryptocarya      ... ... ... --- --- --- --- --- --- G.. --- ...
Atherosperma     ... ... ... --- --- --- --- --- --- ... --- ...
Daphnandra       ... ... ... --- --- --- --- --- --- ... --- ...
Doryphora        ... ... ... --- --- --- --- --- --- ... --- ...
Siparuna         ... ... ... --- --- --- --- --- --- ... --- ...
Calycanthus      ... .T. ... --- --- --- --- --- --- ... --- ...
Chimonanthus     ... .T. ... --- --- --- --- --- --- ... --- ...
Idiospermum      ... ..A C.. --- --- --- --- --- --- ... --- ...
Annona           C.. ..G ... --- --- --- --- --- --- .TC --- ...
Asimina          ... ..G ... --- --- --- --- --- --- .TA --- ...
Cananga          ... ..G ... --- --- --- --- --- --- .TA --- ...
Polyalthia       ... ..G ... --- --- --- --- --- --- .TA --- ...
Eupomatia        ... ..G ... --- --- --- --- --- --- .TA --- ...
Magnolia         ... ... ... --- --- --- --- --- --- ... --- ...
Liriodendron     ... ... ... --- --- --- --- --- --- ... --- ...
Degeneria        ... ... ..A --- --- --- --- --- --- .T. --- ...
Galbulimima      ... ..G ... --- --- --- --- --- --- .TA --- ...
Knema            ... ..G ... --- --- --- --- --- --- ..T --- T..
Myristica        ... ..G ... --- --- --- --- --- --- .TT --- ...
Mauloutchia      ... ..G ... --- --- --- --- --- --- .TA --- ...
Anemopsis        ... ... ... --- --- --- --- --- --- ... --- A..
Saururus         ... ... ... --- --- --- --- --- --- .T. --- A..
Houttuynia       ... ... ... --- --- --- --- --- --- ... --- A..
Peperomia        ... ... ..G --- --- --- --- --- --- A.. --- A..
Piper            ... ... ... --- --- --- --- --- --- ... --- A..
Aristolochia     ... ... ... --- --- --- --- --- --- ... --- ...
Thottea          ... ... ... --- --- --- --- --- --- ... --- ...
Lactoris         C.. ... ... --- --- --- --- --- --- ... --- ...
Asarum           ... ... ... --- --- --- --- --- --- ... --- ...
Saruma           ... ... ... --- --- --- --- --- --- ... --- ...
Belliolum        ... ... ... --- --- --- --- --- --- ... --- ...
Drimys           ... ... ... --- --- --- --- --- --- ... --- ...
Tasmannia        ... ... ... --- --- --- --- --- --- ... --- ...
Canella          C.. ... ... --- --- --- --- --- --- ... --- ...
Cinnamodendron   C.. ... ... --- --- --- --- --- --- ... --- ...
Chloranthus      C.. ... ... --- --- --- --- --- --- ... --- ...
Sarcandra        C.. ... ... --- --- --- --- --- --- ... --- ...
Ascarina         C.. ... ... --- --- --- --- --- --- ... --- ...
Hedyosmum        C.. ... ... --- --- --- --- --- --- ... --- ...
Dioscorea        ... ..G ... --- --- --- --- --- --- .T. --- ...
Asparagus        ... ..G ... --- --- --- --- --- --- .T. --- ...
Croomia          ... ..G ... --- --- --- --- --- --- .T. --- ...
Carludovica      ... ..G ... --- --- --- --- --- --- .T. --- ...
Potamogeton      G.G ..G ... --- --- --- --- --- --- .T. --- ...
Triglochin       G.G ..G ... --- --- --- --- --- --- .T. --- ...
Alisma           G.G ..G ... --- --- --- --- --- --- .T. --- ...
Orontium         ... ..G ... --- --- --- --- --- --- .T. --- ...
Spathiphyllum    ... ..A ... --- --- --- --- --- --- .T. --- ...
Pleea            ... ..G ... --- --- --- --- --- --- .T. --- ...
Tofieldia        ..G ..G ... --- --- --- --- --- --- .T. --- ...
Ceratophyllum_d  T.. C.A ..A --- --- --- --- --- --- G.. --- ...
Ceratophyllum_s  T.. C.A ..A --- --- --- --- --- --- G.. --- ...

Kadsura          T.. .TG T.. ATA TAT ATA TAT ACA TAT G.. --- ...
Schisandra       T.. .TG T.. ATA TAT ATA TAT ACA TAT G.. --- ...
Illicium         T.. .TA T.. ACA TAT --- --- --- --- G.. --- ...
Trimenia         T.. .TA T.. ATA TAT ATA TAT --- --- G.. --- ...
Austrobaileya    G.. .TA T.. ATA TAT AGA TAT --- --- G.. --- ...
Nymphaea         ... ... ... AAT --- --- --- --- --- G.. --- ...
Nuphar           ... ... ... AAT --- --- --- --- --- G.. --- ...
Brasenia         ... C.G ... TAT --- --- --- --- --- GA. --- ...
Cabomba          ... C.G CT. ACA GTA ACG CAA CCA GTA A.T --- ...
Amborella        ... ... TCA AAT --- --- --- --- --- G.. --- ...

Gnetum           T.C T.. ... ACT ACC CGG --- --- --- G.. GGC ATA
Welwitschia      T.. ... .CC CAG CAT AAT --- --- AGT G.. GAC ATT
Pinus            ... ... ... AAT AAT GGG --- --- GTA GC. TTC T..
Ginkgo           ... ..G ... GAT AAT AGG --- --- GTA GC. TTC T..
Cycas            ... ..G ..G AAG AAC AGG --- --- GTA GC. TTC T..
Zamia            ... ..G G.G GAT AAC AGG --- --- GTA GC. TTC T..
```

Figure 2 The portion of the aligned matrix from mitochondrial *matR* showing the INDEL that distinguishes euangiosperms (top block) from ANITA (middle block) and gymnosperms (bottom block). Dots indicate nucleotides identical to the top sequence, and dashes indicate gaps. Sequences of *Arabidopsis*, *Oenothera*, *Vicia*, *Solanum* and *Triticum* (from GenBank; not used in the phylogenetic analysis) are included here to show the INDEL status in derived eudicots and monocots. The codon grouping shown is the correct reading frame. Abbreviations as in Fig. 1.

independent evidence support our identification of ANITA as the earliest angiosperms from the DNA sequence analysis. First, we carried out five single-gene analyses. Three of them, *atp1*, *matR* and *atpB*, placed ANITA at the base of angiosperms, with the exception that in the *atp1* analysis, *Amborella* fell into eudicots because of its divergent sequence. This example, together with that of *Ceratophyllum* in *rbcL* analyses[20,21], exposes the weakness of single-gene analysis, the very reason we conducted this multigene analysis. The 18S rDNA analysis yielded a polytomy among basal angiosperms (but an earlier study of 18S rDNA sampling 223 seed plants identified ANITA as the earliest angiosperms[19]), whereas analysis of *rbcL* still rooted the angiosperm tree at *Ceratophyllum*, consistent with earlier results[20,21] (removal of *Ceratophyllum*, however, changed the root to ANITA). We also analysed amino-acid sequences of the four protein-coding genes and still found ANITA at the base of angiosperms.

Second, the INDEL in *matR*, being a non-point-mutation character and thus less prone to convergence than nucleotide substitutions, clearly separates ANITA from euangiosperms and places them closer to the gymnosperms. Third, a study using duplicated phytochrome genes to root the angiosperm phylogeny and two analyses of multiple genes with different taxon sampling have independently corroborated that ANITA are the earliest angiosperms[27,28,31]. Fourth, all ANITA members (except Illiciaceae) share one morphological feature: carpel closure at anthesis through occlusion of the inner space by secretion. This feature is rare in euangiosperms and is probably a primitive condition in the earliest angiosperms[29]. Last, fossil evidence, although awaiting further discovery, is beginning to show a pattern consistent with our topology. Many floral structures from the Early Cretaceous show similarities to those of ANITA[1,12,13]. Taken together, these lines of evidence provide strong and clear support for this deep split of angiosperms.

Identifying the earliest angiosperms solves one of the two critical pieces of the angiosperm origin mystery[2], and will contribute to solving the other piece: determining the sister group of angiosperms among extant and fossil gymnosperms. Our analyses indicate that the prevalent view of the past two decades that Gnetales are sister to angiosperms is incorrect. These results will also help elucidate the evolution of several features that contributed to the rise and ultimate dominance of angiosperms in modern terrestrial ecosystems, for example, floral development, carpel closure, insect pollination and double fertilization. Finally, these findings will allow selection of appropriate model organisms to investigate critical changes in genomic evolution and developmental programmes, which have led to the overwhelming diversity we see today.

This study also has important implications for future work on reconstructing ancient phylogenies. DNA sequence analyses, although proven to be powerful in reconstructing recent evolutionary histories, have often failed to resolve difficult, long-standing controversies in organismal diversification patterns[25]. Although many problems may be responsible for this failure, one obvious aspect of experimental design has been paradoxically overlooked: collection of enough data to reduce sampling error. Several studies experimenting with extensive sampling of either taxa or characters have been published[17,19,20,26], but few with both (see ref. 27). Our analysis demonstrates that extensive sampling of both taxa and characters is important for resolving difficult phylogenetic issues. □

Methods

Gene sequencing

Total cellular DNA was extracted from fresh or silica-gel-dried leaves using the standard CTAB method (see ref. 21). The genes were amplified by conventional PCR and sequenced using an ABI-PRISM 377 DNA sequencer (PE Applied Biosystems) according to manufacturer's protocols with modifications. The five genes were aligned individually, using XPILEUP from the GCG package (Genetics Computer Group, Inc.) with different settings for gap-creation penalty and gap-extension penalty. Minor manual adjustments were made after computer alignment.

Phylogenetic analyses

For the taxa analysed, all 105 species had *rbcL* sequences, and 93, 88, 98 and 101 species had *atpB*, 18S rDNA, *matR* and *atp1* sequences, respectively (missing data for critical taxa: *Kadsura*: 18S rDNA, *Trimenia*: *atp1*, *Cycas* and *Zamia*: *atpB*, and *Metasequoia* and *Podocarpus*: *matR*). Each taxon had data for at least three out of the five genes. Parsimony (equal weighting) analyses were carried out using PAUP*4.0b2 (ref. 30). To search for islands of shortest trees, a heuristic search was conducted using 1,000 random taxon-addition replicates, one tree held at each step during stepwise addition, TBR branch swapping, steepest descent option in effect. MulTrees option in effect and no upper limit of MaxTrees. Both bootstrap and jackknife (50% character deletion) analyses were conducted using 1,000 resampling replicates and the same tree search procedure as described above except with simple taxon addition. The data matrix is available as Supplementary Information at http://www.nature.com.

All *atp1* and *matR*, and some *atpB*, *rbcL* and 18S rDNA sequences were generated in this study, deposited in GenBank under accession numbers AF197576-AF197815; remaining sequences were from GenBank and ref. 27.

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Correspondence and requests for materials should be addressed to Y.-L.Q. (e-mail: yqiu@systbot.unizh.ch; or after February 2000, yqiu@bio.umass.edu).

Biodiversity of plankton by species oscillations and chaos

Jef Huisman*†‡ & Franz J. Weissing§

* *Biological Sciences, Stanford University, Stanford, California 94305-5020, USA*
† *Center for Estuarine and Marine Ecology, CEMO-NIOO, PO Box 140, 4400 AC Yerseke, The Netherlands*
§ *Department of Genetics, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands*

Biodiversity has both fascinated and puzzled biologists[1]. In aquatic ecosystems, the biodiversity puzzle is particularly troublesome, and known as the 'paradox of the plankton'[2]. Competition theory predicts that, at equilibrium, the number of coexisting species cannot exceed the number of limiting resources[3–6]. For phytoplankton, only a few resources are potentially limiting: nitrogen, phosphorus, silicon, iron, light, inorganic carbon, and sometimes a few trace metals or vitamins. However, in natural waters dozens of phytoplankton species coexist[2]. Here we offer a solution to the plankton paradox. First, we show that resource competition models[6–10] can generate oscillations and chaos when species compete for three or more resources. Second, we show that these oscillations and chaotic fluctuations in species abundances allow the coexistence of many species on a handful of resources. This model of planktonic biodiversity may be broadly applicable to the biodiversity of many ecosystems.

We consider a well-known resource competition model[6–10] that has been tested and verified extensively using competition experiments with phytoplankton species[8,11–16]. Consider n species and k resources. Let N_i denote the population abundance of species i, and let R_j denote the availability of resource j. The dynamics of the species depend on the availabilities of the resources. The resource availabilities, in turn, depend on the rates of resource supply and the amount of resources consumed by the phytoplankton species. This gives the following model[6–9]:

$$\frac{dN_i}{dt} = N_i(\mu_i(R_1, \ldots, R_k) - m_i) \quad i = 1, \ldots, n \tag{1}$$

$$\frac{dR_j}{dt} = D(S_j - R_j) - \sum_{i=1}^{n} c_{ji}\mu_i(R_1, \ldots, R_k)N_i \quad j = 1, \ldots, k \tag{2}$$

Here $\mu_i(R_1, \ldots, R_k)$ is the specific growth rate of species i as a function of the resource availabilities; m_i is the specific mortality rate of species i; D is the system's turnover rate; S_j is the supply concentration of resource j; and c_{ji} is the content of resource j in species i. We assume that the specific growth rates follow the Monod equation[17], and are determined by the resource that is most limiting according to Liebig's 'law of the minimum'[18]:

$$\mu_i(R_1, \ldots, R_k) = \min\left(\frac{r_i R_1}{K_{1i} + R_1}, \ldots, \frac{r_i R_k}{K_{ki} + R_k}\right) \tag{3}$$

where r_i is the maximum specific growth rate of species i, K_{ji} is the half-saturation constant for resource j of species i, and min is the minimum function. This is a standard formulation used in numerous phytoplankton competition models[6–10].

When solved for equilibrium, this competition model predicts that the number of species cannot exceed the number of limiting resources. More precisely, there are k unknown resource availabilities in equation (1). Hence, in the generic case, the number of equilibrium solutions that satisfy equation (1) with $N_i > 0$ cannot

‡ Present address: Laboratory for Microbiology, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands.