more humid conditions. At such times it is also probable that tributaries (now dry), draining extensive areas and coming in from the desert along the western flank of the Main Nile, yielded more amphibole than pyroxene (D. A. Livingstone, personal communication). On the other hand, increased proportions of pyroxene transported to the Main Nile (that is, a decrease in *I*Amph values) would probably indicate a reduction of vegetation cover, accentuated erosion of the Ethiopian plateau and thus more arid conditions. Moreover, some fluctuations recorded in cores probably reflect temperature and rainfall oscillations. Lower temperatures may cause an increase in loads contributed by the Atbara and Blue Nile as a result of solifluction, lowered tree-lines and increased hillside-slope instability (M. A. J. Williams, personal communication).

These interpretations can be evaluated in the light of late Quaternary palaeoclimatological studies that use other techniques, such as the measurement of African lake levels (Fig. 3). The temporal pattern of the *I*Amph index in core S7 correlates, for example, with changes of level of Lake Abhe east of the Ethiopian plateau[23], and lakes in the Ziway-Shala Basin in the Ethiopian Rift[24] (Figs 1*b* and 3). High *I*Amph values in units IV and III (~40,000-20,000 yr BP) correspond to periods of high lake levels recorded before about 20,000-17,000 yr BP. Low *I*Amph values in unit II (~20,000 to 12,000-10,000 yr BP) correlate with low lake levels from about 20,000-17,000 to 14,000-10,000 yr BP. *I*Amph values which increase and then decrease upward in unit I, between 10 and 5.5 m from the core top, span a period of high lake levels between 7,000 and 4,000 yr BP. The increased *I*Amph values at ~3.4 m may be related to the climatic phases that induced high lake levels found for ~1,500 yr BP.

In summary, heavy minerals suites in dated Quaternary Nile delta deposits serve not only as provenance markers[11,19] but also as valuable indicators of East African palaeoclimatic oscillations. Pronounced time-related changes in proportions of pyroxene are directly related to climatic oscillations over the Ethiopian plateau, and to very large changes in sediment loads of major Nile tributaries. These changes were probably associated with larger-scale north-south displacements of climatic belts across extensive sectors of the African continent. This is supported by correlations of Nile delta compositional data with variations of lake levels in distant regions, such as Lake Chad[25] (Fig. 3) located at about the same latitude and about 3,000 km west of the Ethiopian plateau (Fig. 1*a*). Study of heavy minerals in dated sedimentary sequences holds promise as an independent criterion to be used in conjunction with other methods to interpret regional palaeoclimatic oscillations. □

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1. Stanley, D. J. *Science* **240,** 497-500 (1988).
2. Said, R. *The Geological Evolution of the River Nile* (Springer, New York, 1981).
3. Livingstone, D. A. *A. Rev. Ecol. Systematics* **6,** 249-280 (1975).
4. Williams, M. A. J. & Faure, H. (eds) *The Sahara and The Nile: Quaternary Environments and Prehistoric Occupation in Northern Africa* (Balkema, Rotterdam, 1980).
5. Hamilton, A. *Environmental History of East Africa. A Study of the Quaternary* (Academic, London, 1982).
6. Williams, M. A. J. & Adamson, D. A. in *The Sahara and The Nile: Quaternary Environments and Prehistoric Occupation in Northern Africa.* (eds Williams, M. A. J. & Faure, H.) 281-304 (Balkema, Rotterdam, 1980).
7. Adamson, D. A., Gasse, F., Street, F. A. & Williams, M. A. J. *Nature* **288,** 50-55 (1980).
8. Stanley, D. J. & Maldonado, A. *Nature* **266,** 129-135 (1977).
9. Rossignol-Strick, M., Nesteroff, W., Olive, P. & Vergnaud-Grazzini, C. *Nature* **295,** 105-110 (1982).
10. Hurst, H. E. *The Nile: A General Account of the River and the Utilization of its Waters* 2nd edn (Constable, London, 1957).
11. Shukri, N. M. *Q. J. geol. Soc. Lond.* **105,** 511-534 (1950).
12. Griffiths, J. F. (ed.) *Climates of Africa: World Survey of Climatology* Vol. 10 (Elsevier, Amsterdam, 1972).
13. Van Chi-Bonnardel, R. *The Atlas of Africa* (The Free Press, New York, 1973).
14. UNESCO *Tectonics of Africa* (UNESCO, Paris, 1971).
15. Pettijohn, F. J. *Sedimentary Rocks* 2nd edn (Harper & Brothers, New York, 1957).
16. Hassan, F. A. *Quat. Res.* **6,** 425-443 (1976).
17. Coutellier, V. & Stanley, D. J. *Mar. Geol.* **77,** 257-275 (1987).
18. Hilmy, M. E. *J. sedim. Petrol.* **21** (2) 109-120 (1951).
19. Stanley, D. J., Sheng, H. & Pan, Y. *J. Afr. Earth Sci.* **7,** 735-741 (1988).
20. El Askary, M. A. & Frihy, O. E. *Bull. Fac. Sci. Alexandria Univ.* (in the press).
21. Livingstone, D. A. in *The Sahara and The Nile: Quaternary Environments and Prehistoric Occupation in Northern Africa.* (eds Williams, M. A. J. & Faure, H.) 339-359 (Balkema, Rotterdam, 1980).
22. Bonnefille, R. & Riollet, G. *Quat. Res.* **30,** 19-35 (1988).
23. Gasse, F. *Nature* **265,** 42-45 (1977).
24. Gasse, F. & Street, F. A. *Palaeogeog. Palaeoclimatol. Palaeoecol.* **34,** 279-325 (1978).
25. Servant, M. & Servant-Vildary, S. in *The Sahara and The Nile: Quaternary Environments and Prehistoric Occupation in Northern Africa.* (eds Williams, M. A. J. & Faure, H.) 133-162 (Balkema, Rotterdam, 1980).

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Molecular evidence for pre-Cretaceous angiosperm origins

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FLOWERING plants or angiosperms have dominated the Earth's flora since at least the late Cretaceous[1] and were already highly diversified by Barremian times, about 120 million years (Myr) ago. However, because of the paucity of fossilized angiosperm reproductive structures from lower Cretaceous sediments[2,3] and the absence of generally recognized angiosperm fossils from pre-Cretaceous strata[4,5], their origins and early evolution remain obscure. Similarly, attempts to understand pre-Cretaceous angiosperm evolution[4-11] have been impaired by difficulties in defining and interpreting angiospermous characters in fossil specimens[8,12]. We report here molecular evidence suggesting that angiosperm ancestors underwent diversification more than 300 Myr ago.

To obtain a molecular view of angiosperm origins we have determined nucleotide sequences for full-size complementary DNAs of a slowly evolving glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), from six flowering plants. The six nucleotide sequences from animals[13,14], one from yeast[15], and nine from plants (this study and refs 16-18) yielded, upon alignment, 332 codons for comparison in each of the 16 coding regions. Divergence was measured with the weighted pathway method[19]. Pairwise comparisons between organisms for which divergence times are roughly known reveal that the nonsynonymous substitution rate (K_a) of GAPDH has remained constant along separate eukaryotic lineages (Table 1). Were the nonsynonymous rate for GAPDH within angiosperms or their antecedents markedly accelerated relative to that in animals, we would expect values of K_a for comparisons involving plants to exceed those for animals versus yeast. Yet the yeast outgroup reveals that the nonsynonymous rate for GAPDH in plants may be slightly (7%) lower than that in animals. Eukaryotic GAPDH sequences are at compositional equilibrium[20] for first and second codon positions (data not shown); values of K_a in comparisons between angiosperm GAPDH sequences should thus provide reasonable estimates for the timescale of angiosperm evolution.

Numbers of nonsynonymous and synonymous substitutions per site (K_a and K_s, respectively) for comparisons between angiosperm GAPDH sequences are shown in Table 2. Included in the table are the corresponding values for comparisons of full-size complementary DNA sequences for chalcone synthase (CHS), the key enzyme of anthocyanin biosynthesis, from seven[21] of the nine angiosperms considered here. The parallel analysis of enzymes from primary (GAPDH) and secondary (CHS) metabolic pathways contributes significantly to the elimination of potential errors inherent in phylogenetic inferences based upon a single gene. For these two nuclear genes and the seven species from which both sequences have been determined, average divergence at synonymous and nonsynonymous sites between monocots and dicots consistently exceeds that within

dicots, congruent with observations for plant organellar DNA sequences[22,23]. These findings suggest that no comparisons between monocots and dicots for GAPDH or CHS involve genes which were duplicated before the monocot-dicot separation.

Divergence at nonsynonymous sites between angiosperm GAPDH and CHS cDNAs and the implications for the course of angiosperm evolution are summarized in Fig. 1. This general picture of angiosperm evolution is quite distinct from that for which the fossil record can provide clear evidence. The time of divergence indicated for the monocot-dicot separation pre-dates the appearance of generally recognized angiosperm fossil remains by roughly 200 Myr. The deepest branch of the topology does not separate *Magnolia* from the remaining angiosperms[24], but rather monocotyledons from dicotyledons.

Numbers of synonymous substitutions per synonymous site, K_s (ref. 19), were determined for GAPDH and CHS cDNAs (Table 2) and set in relation to the timescale in Fig. 1 for estimation of synonymous rates in these two plant nuclear genes. Based on this timescale, the average synonymous substitution rate for angiosperm GAPDH from 35 pairwise comparisons is 2.3×10^{-9} per site per year, which is very close to that for GAPDH in the mammalian comparison (2.4×10^{-9} per site per year). The synonymous rate for GAPDH of angiosperms and mammals is then lower than the average for mammalian genes, yet this is not surprising in light of the generally positive correlation found between synonymous and nonsynonymous rates[19]. The average synonymous rate obtained for chalcone synthase from 12 total pairwise comparisons in which the method could effectively correct for multiple substitutions is 4.9×10^{-9} per site per year. This value is in good agreement with the average synonymous rate[19] for mammalian genes (4.7×10^{-9}). Thus, if the timescale depicted in Fig. 1 is approximately correct, the present data would suggest that nuclear synonymous rates in angiosperms and mammals are very similar.

There are several alternative hypotheses to account for the sudden appearance of highly diversified angiosperm taxa in lower Cretaceous sediments[4–12,25–27]. Evidence supporting the

TABLE 1 Numbers of nonsynonymous substitutions in glycolytic GAPDH nucleotide sequences from eukaryotes

	n	K_a	s.e.	Rate
Plants-animals	54	0.266	(0.021)	$0{\cdot}133 \times 10^{-9}$
Plants-yeast	9	0.269	(0.022)	0.135×10^{-9}
Animals-yeast	6	0.287	(0.023)	0.144×10^{-9}
Drosophila-vertebrates	6	0.181	(0.017)	0.150×10^{-9}
Mammals-chicken	2	0.047	(0.008)	0.088×10^{-9}
Human-rat	1	0.030	(0.006)	0.179×10^{-9}

Numbers of nonsynonymous substitutions (K_a) in glycolytic[16] GAPDH nucleotide sequences from eukaryotes. Values of K_a represent the average of mean values[19] for the number of pairwise comparisons given in column *n*. The average of standard errors (s.e.) over comparisons are indicated in brackets. Rates are expressed as numbers of substitutions per site per year and were calculated with the following divergence times[13,28,29]: plants-animals-yeast: 1,000 Myr; *Drosophila* (two genes)-vertebrates: 600 (Myr); mammals-chicken: 270 Myr; human-rat: 85 Myr. The comparison between mammals and chicken gives the lowest estimate of nonsynonymous substitution rate for GAPDH, consistent with observations for cytochrome *c* and globins[28,29] in comparisons of these vertebrates. The highest rate (human-rat) is subject to the stochastic error of a single comparison involving a rather small number of total substitutions. Average values of K_a in interkingdom comparisons involving plants are lower than that for animals versus yeast, suggesting a slightly lower overall rate for the line leading to angiosperms. Using yeast as an outgroup, the relative rates test[30] reveals no differences in plant versus animal rates that are significant at $P = 0.05$ (data not shown). The average nonsynonymous substitution rate for GAPDH from all 78 pairwise comparisons is 0.135×10^{-9}. The average nonsynonymous rate for those 15 comparisons excluding plants is 0.141×10^{-9}. The latter average nonsynonymous rate was conservatively chosen to apply to values of K_a in comparisons between angiosperms (Fig. 1) because it provides minimum estimates of angiosperm age. Although cells representing eukaryotic algae appeared $1.4\text{–}1.5 \times 10^9$ years ago[31,32], the first photosynthetic eukaryotes were probably not the ancestors of higher plants[32]. Use of divergence times exceeding the estimate of 10^9 years for plant-animal-yeast separation would result in lower average values of nonsynonymous rate for GAPDH and thus imply ages for angiosperms greater than those indicated in Fig. 1.

TABLE 2 Numbers of nonsynonymous and synonymous substitutions for GAPDH and CHS of angiosperms

		GAPDH			CHS		
		n	$K_a \times 100$(s.e.)	$K_s \times 100$ (s.e.)	*n*	$K_a \times 100$ (s.e.)	$K_s \times 100$ (s.e.)
Dicots versus monocots							
Ranunculus	-monocots	2	9.02 (1.1)	156 (47)	4	10.6 (1.1)	NC
Sinapis	-monocots	2	7.24 (1.0)	173 (20)			
Magnolia	-monocots	2	7.16 (1.0)	142 (20)	2	10.5 (1.1)	NC
Petroselinum	-monocots	2	10.5 (1.2)	177* (48)	2	11.1 (1.2)	NC
Scrophulariales	-monocots	6	9.62 (1.2)	156 (25)	4	11.7 (1.2)	NC
Monocots versus monocots							
Hordeum	*-Zea*	1	2.90 (0.6)	56 (7)	1	6.5 (0.8)	86 (17)
Dicots versus dicots							
Ranunculus	-remaining dicots	6	7.14 (1.0)	107 (13)	10	10.1 (1.1)	281* (11)
Sinapis	-remaining dicots	6	6.37 (0.9)	136 (23)			
Magnolia	-remaining dicots	6	6.27 (1.0)	141 (22)	6	9.7 (1.1)	240* (55)
Petroselinum	-remaining dicots	6	8.53 (1.0)	128 (17)	6	10.1 (1.1)	261* (26)
Scroph'ales	-remaining dicots	12	6.86 (1.0)	123 (16)	12	10.6 (1.1)	289* (108)
Antirrhinum	-Solanaceae	2	5.74 (0.9)	101 (12)	2	6.3 (0.9)	NC
Nicotiana	*-Petunia*	1	5.60 (0.9)	114 (14)			

The table shows the numbers of nonsynonymous (K_a) and synonymous (K_s) substitutions for GAPDH and CHS of angiosperms, as determined by the weighted pathway method[19] with a program kindly provided by W.-H. Li. Values of K (K_a or K_s) represent the average of mean values from the number of pairwise comparisons given in column *n*. The average standard error (s.e.) over comparisons[22] is indicated in brackets. Both CHS cDNAs of *Ranunculus*[21] and a full-size cDNA from the second active CHS gene of *Petunia* (J. Reif; unpublished data) were included in the comparisons. Cases in which the method was unable to correct for multiple substitutions at identical sites are indicated by NC. An asterisk denotes that the method was unable to correct in all possible pairwise comparisons. Scrophulariales is the common order of *Antirrhinum*, *Petunia* and *Nicotiana*, the latter two being common members of the Solanaceae. Comparisons for both genes are based upon alignments which exclude amino- and carboxy-terminal regions of length heterogeneity. An average angiosperm GAPDH pair contains 776 nonsynonymous and 220 synonymous sites. The *Nicotiana* sequence is only nearly full size[18] and thus contains about 3 per cent fewer sites. An average CHS pair contains 903 nonsynonymous and 264 synonymous sites. GAPDH cDNA clones were identified by heterologous plaque hybridization to the 1.1-kb *Pst*I fragment of ps198 (ref. 17) subcloned into plasmid vectors, and sequenced by the chemical cleavage method[33]. The *Petroselinum* cDNA library was provided by W. Schulz, the remaining clones were isolated from libraries previously described[21]. The published[16–18] and six new angiosperm sequences (submitted to GenBank), their alignment to other eukaryotic GAPDH sequences, and the K_a matrix of pairwise eukaryotic comparisons are available from the authors upon request.

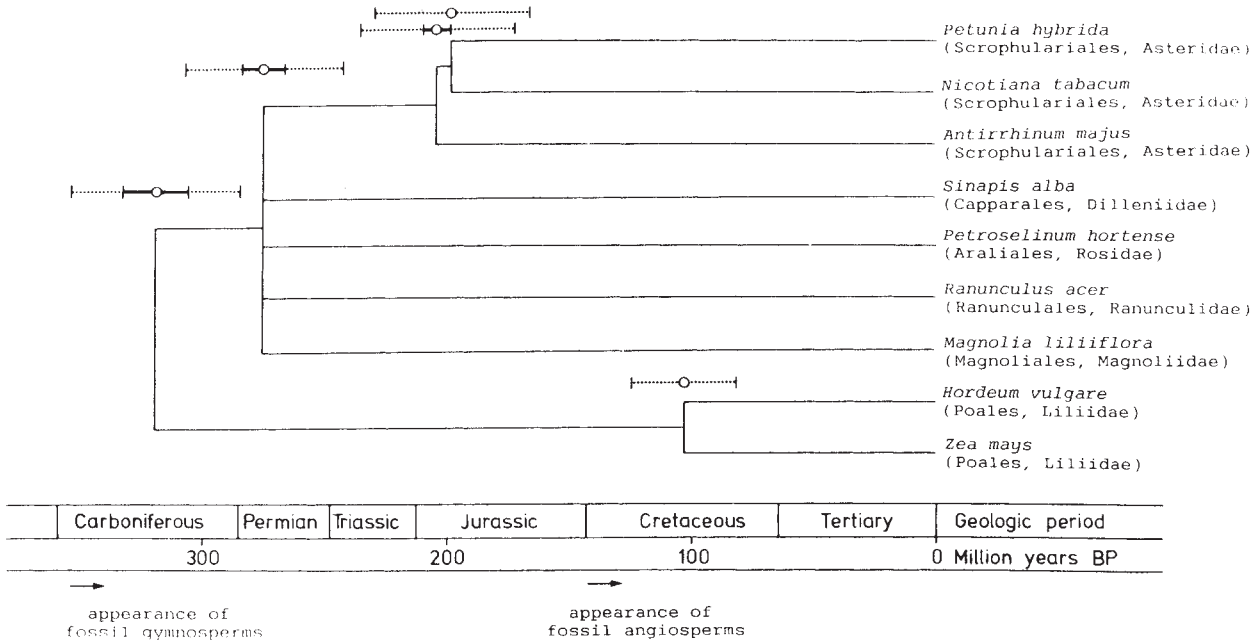

FIG. 1 A molecular view of angiosperm evolution based upon nonsynonymous substitutions in GAPDH and CHS. Order and subclass assignment[26] for each species is indicated in parentheses. The time of divergence between monocots and dicots was obtained by dividing the average value of K_a for GAPDH from all 14 pairwise monocot-dicot comparisons (0.0900 ± 0.0036) by twice the average nonsynonymous rate for GAPDH from Table 1 (0.141×10^{-9} per site per year). The time of subclass radiation for dicots was estimated by determining the ratios of Ka (dicots-dicots): K_a(dicots-monocots) for GAPDH and CHS for all five dicotyledonous subclasses represented (Table 1) and multiplying the average of these nine ratios (0.868 ± 0.027) with the derived divergence time for monocots-dicots. Divergence times within the Scrophulariales and for barley-maize were estimated solely on the basis of K_a and average rate for GAPDH. The geological timescale used is that of Thomas and Spicer[12]. Solid bars indicate the standard error for averages of mean K_a or, in the case of dicot subclass radiation, the standard error of monocot-dicot ratios. Dotted bars indicate the average standard error of K_a (ref. 19) across comparisons at a given node. Estimated divergence times shown are: *Zea–Hordeum* 103 ± 22 Myr; *Petunia–Nicotiana* 199 ± 32 Myr; *Antirrhinum*–Solanaceae 204 ± 32 Myr; dicotyledonous subclass radiation 276 ± 33 Myr; monocot-dicot separation 319 ± 35 Myr. Subclass assignments for these angiosperms are not identical in different classification schemes[10,26,34]. Molecular sequence (DNAML, DNAPENNY and DNABOOT of PHYLIP[35], version 3.0 provided by J. Felsenstein) and matrix (FITCH of PHYLIP, UPG and modified[36] UPG) tree-building algorithms did not yield consistent topologies for dicot subclasses, as reflected in the error bars in the figure.

view of Cretaceous angiosperm origins is of a negative nature in that lack of generally recognized angiosperm fossils from older strata is interpreted as nonexistence of the organisms in pre-Cretaceous time. The present molecular data may be interpreted as support for the theories of those who have argued in favour of a long pre-Cretaceous history for angiosperms. Palaeogeographic[4,25] and ecological[10] considerations have suggested that the lack of pre-Cretaceous angiosperm fossil remains could be explained through an adaptation of primitive angiosperms to restricted (upland) habitats, thus inhibiting fossilization up until their Cretaceous invasion of such lowland habitats as are conducive to sedimentation. The Permo-Triassic time of primary angiosperm radiation suggested by Axelrod[25] is easily reconciled with our results. Yet, using quite different arguments, proponents of polyphyletic angiosperm origins have also suggested that divergence between angiosperm lineages occurred in pre-Cretaceous times[7,27]. The temporal implications and early monocot-dicot separation shown in Fig. 1 do not exclude the hypothesis that some characters viewed as angiospermous[6] arose along independent ancestral routes. Recently, most parsimonous cladistic trees based upon morphological character states[9] have implicated Triassic angiosperm origins, an estimate of angiosperm age which would appear too conservative in light of the divergence between angiosperm GAPDH sequences. We conclude that the synthesis of molecular and palaeobotanical findings should result in the generation of experimentally testable hypotheses concerning angiosperm phylogeny. □

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1. Lidgard, S. & Crane, P. R. *Nature* **331,** 344–346 (1988).
2. Friis, E. M., Crane, P. R. & Pedersen, K. R. *Nature* **320,** 163–164 (1986).
3. Nishida, H. *Nature* **318,** 58–59 (1985).
4. Axelrod, D. I. *Evolution* **6,** 29–60 (1952).
5. Crane, P. R. *A. Mo. bot. Gdn* **72,** 716–793 (1985).
6. Krassilov, V. A. *A. Mo. bot. Gdn* **71,** 577–592 (1984).
7. Krassilov, V. A. *Bot. Rev.* **43,** 143–176 (1977).
8. Friis, E. M., Chaloner, W. G. & Crane, P. R. in *The Origins of Angiosperms and their Biological Consequences* (eds Friis, E. M., Chaloner, W. G. & Crane, P. R.) 1–15 (Cambridge Univ. Press, 1987).
9. Doyle, J. A. & Donoghue, M. J. *Bot. Rev.* **52,** 321–431 (1986).
10. Stebbins, G. L. *Flowering plants: Evolution Above the Species level* (Harvard University Press, Cambridge, 1974).
11. Hughes, G. L. *A. Mo. bot. Gdn* **71,** 593–598 (1984).
12. Thomas B. A. & Spicer, R. A. *Evolution and Paleobiology of Land Plants* (Croom Helm, London, 1987).
13. Tso, J. Y. *et al. Nucleic Acids. Res.* **13,** 2485–2502 (1985).
14. Yarbrough, P. O. *et al. Biochim. biophys. Acta* **908,** 21–33 (1987).
15. Holland, J. P. & Holland M. J. *J. biol. Chem.* **255,** 2596–2605 (1979).
16. Brinkmann, H. *et al. J. molec. Evol.* **26,** 320–328 (1987).
17. Martin, W. F. & Cerff, R. *Eur. J. Biochem.* **159,** 323–331 (1986).
18. Shih, M. C., Lazar, G. & Goodmann, H. M. *Cell* **47,** 73–80 (1986).
19. Li, W.-H., Wu, C.-I. & Luo, C.-C. *Molec. Biol. Evol.* **2,** 150–174 (1985).
20. Prager, E. M. & Wilson, A. C. *J. Molec. Evol.* **27,** 326–335 (1988).
21. Niesbach-Klösgen, U. *et al. J. molec. Evol.* **26,** 213–225 (1987).
22. Wolfe, K. H., Li, W.-H. & Sharp, P. M. *Proc. natn. Acad. Sci. U.S.A.* **84,** 9054–9058 (1987).
23. Ritland, K. & Clegg, M. T. *Am. Nat.* **130,** S74–S100 (1987).
24. Humphries, C. *Nature* **333,** 300–301 (1988).
25. Axelrod, D. I. *Bot. Rev.* **36,** 227–319 (1970).
26. Takhtajan, A. L. *Bot. Rev.* **46,** 225–359 (1980).
27. Meeuse, A. D. J. *All About Angiosperms* (Eburon, Delft, 1987).
28. Wilson, A. C., Carlson, S. S. & White, T. J. *A. Rev. Biochem.* **46,** 573–639 (1977).
29. Nei, M. *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York, 1987).
30. Li, W. H. & Tanimura, M. *Nature* **326,** 93–96 (1987).
31. Schopf, J. W. *Scient. Am.* **239,** 84–102 (1978).
32. van den Eynde, H. *et al. J. molec. Evol.* **27,** 126–132 (1988).
33. Maxam, A. M. & Gilbert, W. *Meth. in Enzym.* **65,** 499–560 (1980).
34. Cronquist, A. *An Integrated System of Classification of Flowering Plants* (Columbia University Press, New York, 1981).
35. Felsenstein, J. *Evolution* **39,** 783–791 (1985).
36. Li, W.-H. *Proc. natn. Acad. Sci. U.S.A.* **78,** 1085–1089 (1981).

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