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Molecular evolution of pteridophytes and their relationship to seed plants: evidence from complete 18S rRNA gene sequences

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Abstract: Complete 18S ribosomal RNA sequence data from representatives of all extant pteridophyte lineages together with RNA sequences from different seed plants were used to infer a molecular phylogeny of vascular plants that included all major land plant lineages. The molecular data indicate that lycopsids are monophyletic and are the earliest diverging group within the vascular land plants, whereas *Psilotum nudum* is more closely related to the seed plants than to other pteridophyte lineages. The phylogenetic trees based on maximum likelihood, parsimony and distance analyses show substantial agreement with the evolutionary relationships of land plants as interpreted from the fossil record.

The phylogenetic relationships between the different lineages of vascular plants are still not resolved. Various cladistic analyses (BREMER & al. 1987, GARBARY & al. 1993) and interpretations of the fossil record (NIKLAS & BANKS 1990, STEWART & ROTHWELL 1993) produced conflicting hypotheses about the phylogeny and the interrelationship of the major tracheophyte groups. For example, lycopsids are polyphyletic in analyses based upon comparisons of characters related to male gametogenesis (GARBARY & al. 1993), whereas palaeontological data support the monophyletic derivation of the *Lycopodiales* and the *Protolepidodendrales* from a common Devonian ancestor (NIKLAS & BANKS 1990). Furthermore, some authors suggest that the genus *Psilotum* is a direct descendant of the Silurian-Devonian *Rhyniopsida*, and therefore psilopsids are the earliest divergence in vascular plant evolution (MINKOFF 1983, BREMER & al. 1987). Others emphasize similarities to certain ferns (BIERHORST 1968, 1971) and instead prefer lycopsids to be the oldest extant group of pteridophytes (STEWART & ROTHWELL 1993, KENRICK 1994). Recent phylogenetic analyses of *rbc*L sequences could not resolve these problems because of the high level of homoplasy in the *rbc*L data (MANHART 1994).

Comparative analyses of small subunit ribosomal RNA have gained widespread acceptance for the inference of objective phylogenetic frameworks (WOESE 1987, WAINRIGHT & al. 1993). Therefore, we determined complete small subunit rRNA sequences for nine pteridophyte species to explore the phylogenetic posi-

tion of ferns and their allies. Additional published sequences of seed plants, bryophytes and two charophycean algae were used to infer an 18S rRNA phylogeny that included representatives of all major land plant lineages.

Material and methods

Taxa. The quill wort *Isoëtes durieui* BORY (*Isoëtales*; Genbank Accession No.: X83521) and the fern *Adiantum raddianum* PRESL (*Filicales*; X78889) were obtained from the Botanical Garden Heidelberg, Germany. The club mosses *Lycopodium taxifolium* SWARTZ (*Lycopodiales*; X83522) and *L. phlegmaria* L. (*Lycopodiales*; X81964), a spike moss *Selaginella umbrosa* LEMAIRE ex HIERON. (*Selaginellales*; X83520), the horsetail *Equisetum robustum* BRAUN [= *E. hyemale* L. var. *affine* (ENGELM.) EATON; *Equisetales*; X78890], the whisk fern *Psilotum nudum* L. (*Psilotales*; X81963) as well as the fern *Salvinia natans* (L.) ALLIONI (*Filicales*; X90413) were obtained from the Botanical Garden Erlangen, Germany. A second spike moss, *Selaginella vogelii* SPRING (*Selaginellales*; X75517), previously examined in our lab (KRANZ & al. 1995) was also included in the molecular analyses.

The sequence data of the following taxa were taken from the GenBank/EMBL databaes: *Zea mays* (K02202), *Oryza sativa* (X00755), *Glycine max* (X02623), *Arabidopsis thaliana* (X16077), *Zamia pumila* (M20017), *Taxus mairei* (D16445), *Pinus luchuensis* (D16446), *Podocarpus nakaii* (D16447), *Gingko biloba* (D16448), *Coleochaete scutata* (X68825), *Funaria hygrometrica* (X74114), *Klebsormidium flaccidum* (X75520), *Marchantia polymorpha* (X75521).

DNA isolation, DNA amplification and sequencing. DNA was isolated according to HUSS & al. (1986). The 18S rRNA genes were amplified with the polymerase chain reaction (PCR) as described previously (HUSS & SOGIN 1990). Both strands were sequenced using the Sequenase PCR Product Sequencing Kit (USB).

Sequence alignment and phylogenetic analyses. The 18S rRNA sequences were aligned manually, and only positions that could be aligned unambiguously were used in the phylogenetic analyses. Secondary structure models were constructed according to HUSS & SOGIN (1990) and NEEFS & DE WACHTER (1990) in order to optimize the alignment of homologous nucleotide positions. This procedure resulted in a total of 1717 aligned positions for the large dataset used for Fig. 1 A, and 1731 positions for the small dataset used for Figs. 2 and 3.

In both cases we did bootstrap analyses with the maximum likelihood method (100 replications; FELSENSTEIN 1981, 1985) using the fastDNAml program (OLSEN & al. 1994); fastDNAml calculations were done on a Sun SPARC station using the generalized two-parameter model of evolution (KISHINO & HASEGAWA 1989), empirical base frequencies, and a varying input order of taxa until the best log likelihood score was reached in three independent searches.

Maximum parsimony analyses were done with the computer program PAUP (SWOFFORD 1990). We did 500 bootstrap replications with the large dataset using the heuristic search strategy with the TBR branch swapping algorithm; the MULPARS option was in effect, and a random sequence addition was used with 5 repetitions. The six-species dataset was examined using an exhaustive search for the branching pattern and 100 bootstrap replications with a branch-and-bound algorithm for the calculation of the significance of the tree topology.

Different hypothetical arrangements of the pteridophyte lineages were compared using the Kishino-Hasegawa test in the DNAML and DNAPARS program of the PHYLIP-package (FELSENSTEIN 1993). This statistical test uses the mean and the variance of log-likelihood differences between trees, taken across sites. A tree is declared significantly worse, if the mean is more than 1.96 standard deviations different in comparison to the best tree.

Additional bootstrap analyses of the small dataset (100 replications) were performed using a distance-matrix least-square method (FITCH & MARGOLIASH 1967) with the programs SEQBOOT, DNADIST, FITCH, and CONSENSE from the PHYLIP package. The trees were rooted with the charophycean taxa *Coleochaete* and *Klebsormidium*, which have been shown to be closely related to land plants (KRANZ & al. 1995).

Fossil data. Fossil data were summarized from MINKOFF (1983), TAYLOR (1988), NIKLAS & BANKS (1990), INGROUILLE (1992), STEWART & ROTHWELL (1993), TAYLOR & TAYLOR (1993), and KENRICK (1994).

Results

The 18S rRNA base composition of all bryophytes, pteridophytes, gymnosperms, and dicots included in the analyses was between 48 and 50 mol% G + C; only the sequences from the charophytes (47 mol%) and the monocots (51 mol%) are slightly beyond this narrow range. Therefore, base composition was not expected to bias the phylogenetic analyses.

Phylogenetic trees inferred from both the maximum likelihood and the maximum parsimony analyses of the large dataset show an identical branching pattern (Fig. 1 A). The heuristic searches with the parsimony method resulted in a single shortest tree of 994 steps with a consistency index (CI) of 0.565 and a retention index (RI) of 0.648. The land plants form a monophyletic lineage with 96% bootstrap support in the maximum likelihood and 99% support in the parsimony analysis. Within this lineage, the liverwort branches first followed by the moss. The bryophytes are separated from the vascular plants with only moderate bootstrap support (67%/57%). A monophyletic origin of the homosporous (*Lycopodium*) and heterosporous lycopsid lineage (*Selaginella, Isoëtes*) is indicated in both analyses, but only the maximum likelihood analysis supports this results with a moderate bootstrap value of 80%. The lower support of the parsimony analysis (68%) may reflect the greater sensitivity of this method to unequal evolutionary rates within different lineages (FELSENSTEIN 1981). Longer branch lengths leading to both *Selaginella* species indicate an accelerated evolutionary rate compared with the other pteridophytes or bryophytes, and similar to the spermatophytes. Therefore, long branch attraction could be responsible for the topology given by a least squares method (FITCH) which shows *Selaginella* and *Isoëtes* as a sister group of the spermatophytes (54% bootstrap support; not shown).

The remaining vascular plants form a sister group to the lycopsids with high support in the maximum likelihood analysis (93%). The relationships between the seed plants, the ferns, *Psilotum*, and *Equisetum* are not resolved with the bootstrap analyses. While both analyses prefer a branching pattern with the ferns as the deepest branch and a position of *Psilotum* next to the spermatophytes, the short branch lengths as well as the low bootstrap values indicate a nearly simultaneous radiation of these groups.

The seed plants form a monophyletic group with two distinct lineages, the angiosperms and gymnosperms. Within the gymnosperms, *Ginkgo* branches first, followed by a cycad and the conifers, but only a monophyletic origin of the conifers is strongly supported by bootstrapping (100%). Similar values are obtained in support of a separation of monocots and dicots within the angiosperms.

A second dataset containing only one representative of the different pterido-

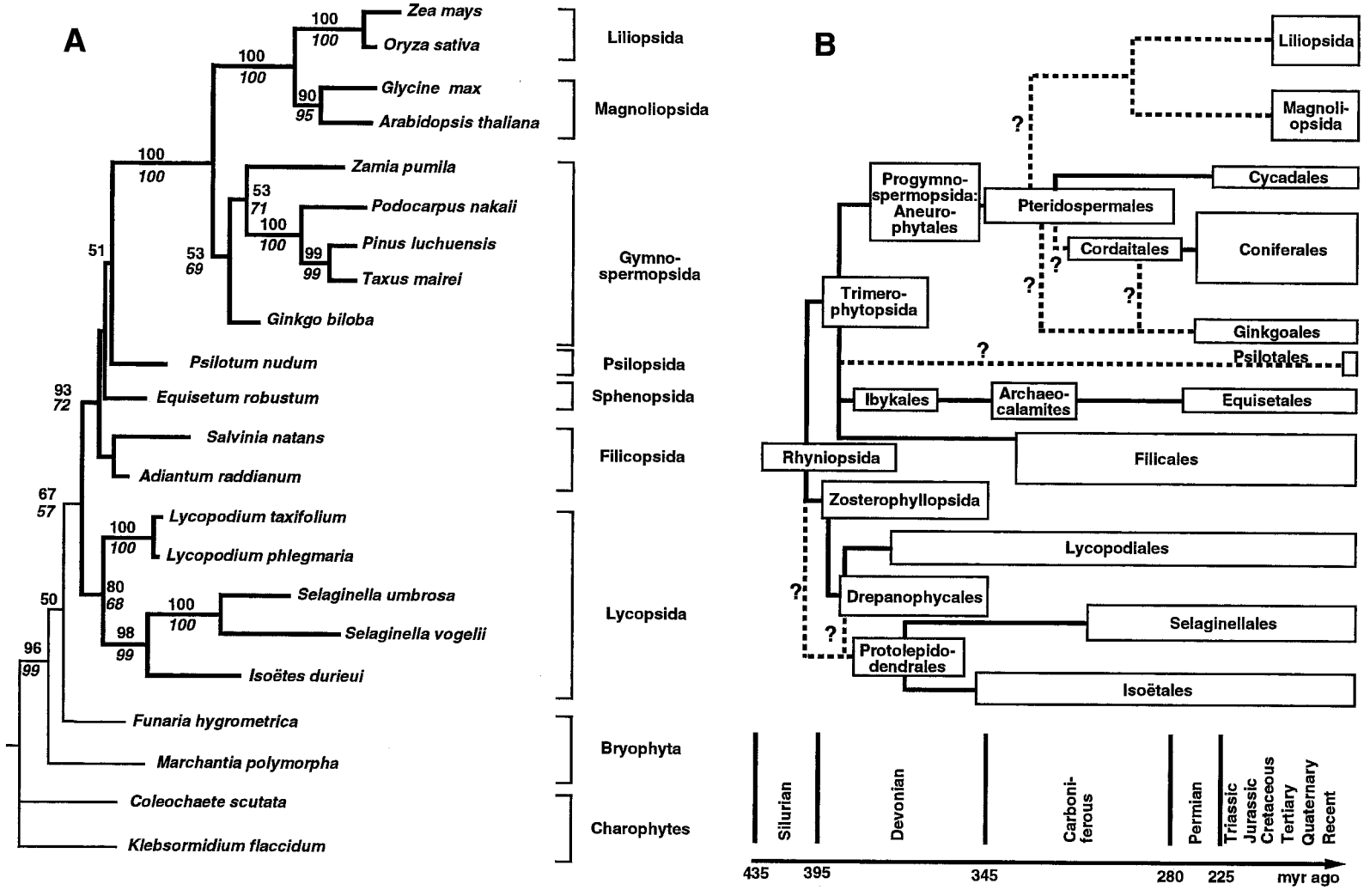

Fig. 1. Comparison between the molecular phylogeny of land plants inferred from 18S rRNA sequence data and the putative relationships of the different vascular plant lineages as suggested by the fossil record. *A* Phylogenetic tree generated by maximum likelihood analyses (ln likelihood = –8148.3972061) of 1717 unambiguously aligned sites. The percentage that corroborates topological elements in the bootstrap analysis (100 resamplings) is shown above the branches. Parsimony bootstrap values based on 500 resamplings with 5 heuristic searches each are shown in italics below the branches. Confidence levels below 50% are not indicated. *B* Suggested phylogenetic relationships of vascular plants and their distribution in geological time derived from the fossil record. Differing interpretations of the fossil data are indicated by dashed lines and question marks

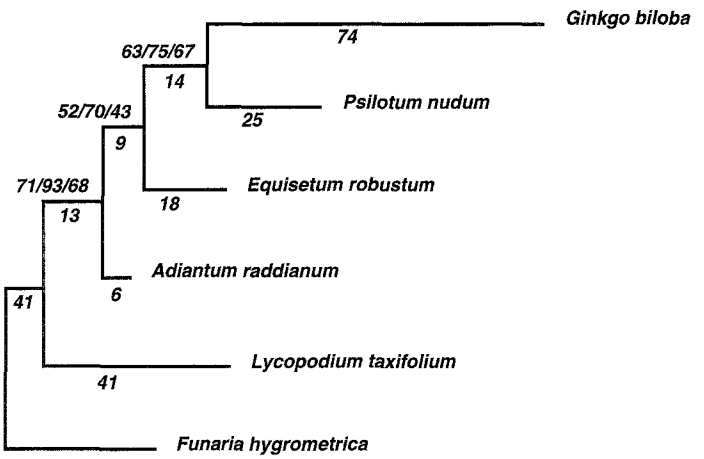

Fig. 2. Single most parsimonious tree of 241 steps inferred from 1731 aligned nucleotides of the 18S rRNA genes using an exhaustive search (CI = 0.855, RI = 0.348). The number of steps separating two nodes is shown below branches. Bootstrap values based on resamplings of parsimony (branch and bound), maximum likelihood and distance matrix searches (100 replications each) are shown above the internal nodes

phyte lineages (*Adiantum, Equisetum, Psilotum, Lycopodium*) together with one representative of the spermatophytes (*Ginkgo*) and the bryophytes (*Funaria*), was used in an attempt to resolve the polytomy obtained in Fig. 1 B. Omitting the slightly faster evolving heterosporous lycopsids and the spermatophytes, we were able to take 1731 positions of the alignment and run exhaustive parsimony searches with this dataset as well. A single most parsimonious tree of 241 steps was found in the parsimony exhaustive search with a high CI-value of 0.855 (RI = 0.348) and a topology identical to that resulting from the maximum likelihood and distance-matrix (least-square) analyses (Fig. 2). Bootstrapping (100 resamplings) supported the position of *Psilotum* next to the gymnosperm with 63%–75% followed by *Equisetum* (43%–70%) and *Adiantum* (68%–93%).

Several hypothetical arrangements of the four different lineages were tested using the Kishino-Hasegawa test in the maximum likelihood and parsimony analyses (Fig. 3). Each topology in which the fern or the horsetail were positioned as a sister group to the seed plant led to 7–10 additional steps in the parsimony analysis; however, in the statistical test, only the topologies C–F are significantly worse. Using the maximum likelihood algorithm, only tree D is declared significantly worse, but whenever *Psilotum* is not placed next to the gymnosperm, the resulting branch lengths are set as close to zero. The resulting tree topology has to be interpreted as a polytomy. As a consequence, the Kishino-Hasegawa test suggests either psilopsids and the gymnosperms as sister groups, or a multifurcation of all four lineages. A position of *Psilotum* either close to the fern (I, J) or as a primitive member of the pteridophytes (D, E) is not supported by our analyses. Instead, *Equisetum* rather than *Psilotum* is more closely related to *Adiantum* as indicated by the topologies K and L.

The phylogeny obtained from the 18S rRNA sequences is compared with an interpretation of the fossil record (Fig. 1 B) and discussed below.

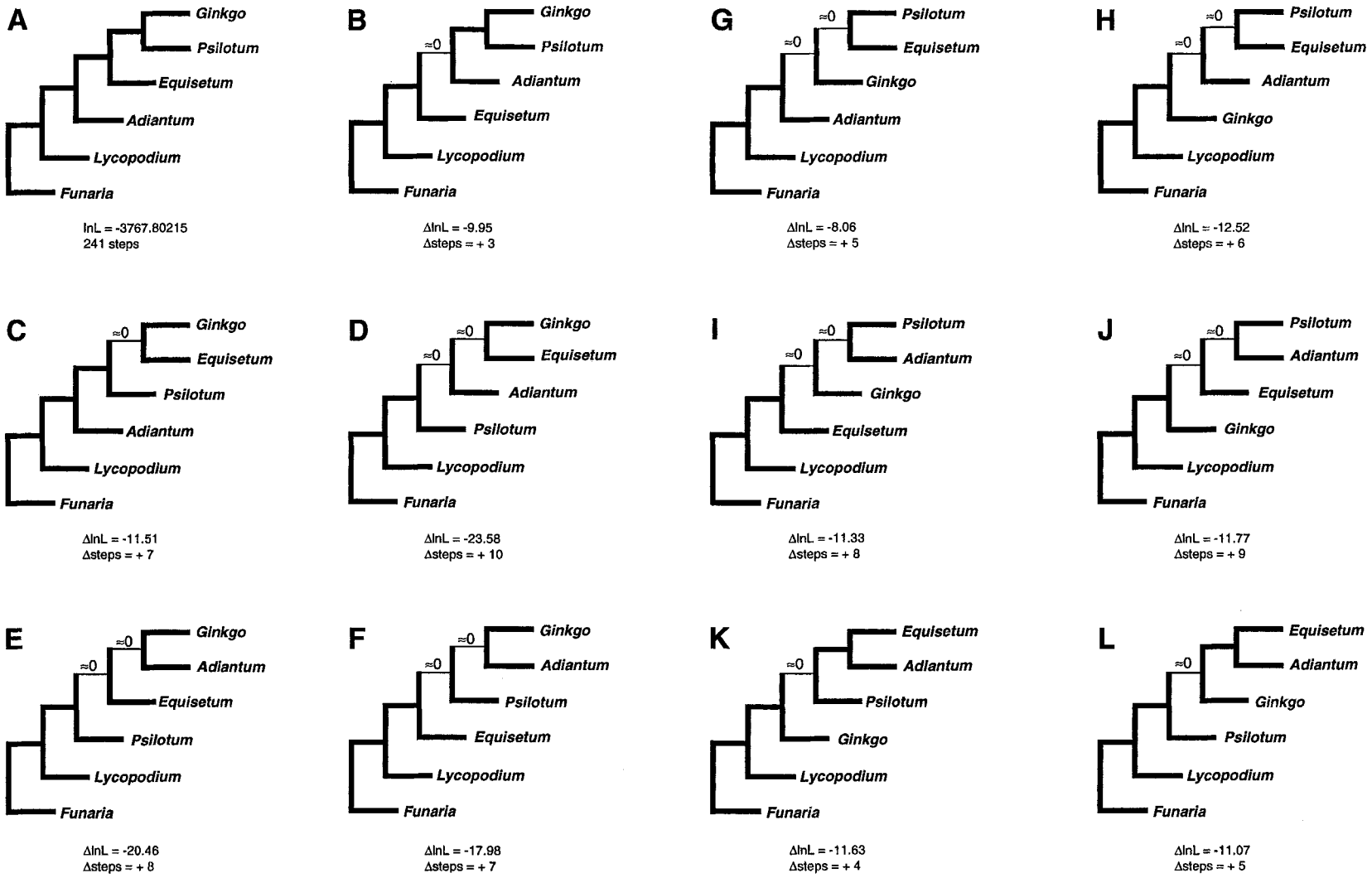

Fig. 3. Comparison of different hypotheses for the phylogenetic relationships between lycopsids, ferns, sphenopsids, psilopsids, and seed plants in the maximum likelihood and parsimony analyses with the Kishino-Hasegawa test. Branch lengths close to zero in the maximum likelihood analysis are indicated with thin lines and "≈0". The different hypotheses assume that either psilopsids (*A, B*), sphenopsids (*C, D*) or ferns (*E, F*) are the sister group to the seed plants. *G–L* denote all possible remaining arrangements of the six taxa with *Funaria* and *Lycopodium* always at the base of the tree topology. Tree *A* is declared best in the maximum likelihood analyses with a lnL value of –3767.80215 as well as in the parsimony analyses (241 steps). The differences in the lnL values and the number of the additional steps compared with topology *A* is indicated below each tree

Discussion

In the molecular analyses based on complete 18S rRNA gene sequences, tracheophytes share a most recent common ancestor with bryophytes (96%/99% bootstrap values; Fig. 1 A). Low bootstrap values (50% and 57%/67%) indicate a nearly simultaneous radiation of different bryophyte lineages and vascular plants. Fossil evidence indicates that primitive Lower Devonian vascular plants (rhyniophytes) had life cycles with alternation of isomorphic gametophytic and sporophytic phases. According to REMY (1982), some gametophytic characteristics of the rhyniophyte species *Lyonophyton* and *Sciadophyton* as well as sporophytic characteristics of *Aglaophyton major* and *Horneophyton* are also found in bryophytes. This might indicate that primitive land plants with an isomorphic life cycle were the ancestors of both bryophytes and tracheophytes.

Rhyniophytes are postulated to have given rise to zosterophylls (the proposed ancestors of lycopsids) and to trimerophytes, thought to be the ancestors of all other vascular land plant groups (STEWART & ROTHWELL 1993, NIKLAS & BANKS 1990). This early split is supported by chloroplast DNA gene order (RAUBESON & JANSEN 1992), the presence of similar introns in the *cox*III genes of *Lycopodium* and *Marchantia* (HIESEL & al. 1994), and by our analyses. The molecular data also indicate a monophyletic origin of the lycopods. This would favour the hypothesis of NIKLAS & BANKS (1990) that both Devonian lycopsid lineages (*Lycopodiales* and *Protolepidodendrales*) are derived from a common zosterophyll ancestor.

The branching order within the lycopsid lineage is also reflected in the fossil record (Fig. 1 B). The homosporous, eligulate *Lycopodiales* branch at the base of the vascular plant radiation and also appear first in the fossil record. Beginning with the Lower Devonian *Baragwanatia* there is a more-or-less continuous record of the *Lycopodiales* for the last 370 myr. A second homosporous lycopsid lineage, the *Protolepidodendrales*, appeared at about the same time in the Lower Devonian. The genus *Leclercqia*, reported from the mid-Devonian, is the only known homosporous lycopod possessing a ligule which is a typical attribute of the *Isoëtales* and *Selaginellales*. These two orders are heterosporous but ligulate, and are therefore thought to be derived from a protolepidodendrid ancestor.

The remaining vascular plants are combined by a bootstrap value of 93% in maximum likelihood analyses. This subtree is characterized by a poorly resolved polytomy leading to ferns, *Equisetum, Psilotum,* and seed plants. Because a sequential branching pattern of these groups is supported only by moderate bootstrap values in the small dataset, a separation of these groups within a short period of time has to be assumed. In the fossil record, the corresponding position of this polytomy is represented by the trimerophytes. The members of the class *Trimerophytopsida* are derived from the rhyniophytes, and are the putative ancestors of sphenopsids (horsetails and related taxa), ferns and progymnosperms (STEWART & ROTHWELL 1993). Thus, a nearly simultaneous radiation of ferns, horsetails and seed plants is indicated by both the fossil record and the 18S rRNA analyses.

The evolutionary position of the extant class *Psilopsida* with the genera *Psilotum* and *Tmesipteris* is still enigmatic. On the basis of morphology, *Psilotum* bears many resemblances to the fossil rhyniophytes: low degree of organ differentiation, absence of roots and vascularized leaves, and axes that branch in a three-dimen-

sional dichotomous manner. As in the *Rhyniopsida*, sporangia of *Psilotum* and *Tmesipteris* are eusporangiate and produce large numbers of isospores. Both genera differ from *Rhynia* in having laterally-borne instead of terminal sporangia. Because of the lack of an intervening fossil record, it is impossible to decide whether psilopsids are direct descendants from the *Rhyniopsida* or not. BIERHORST (1968, 1971) reported similar patterns of embryo development and gametophytic structures between *Psilotum* and primitive leptosporangiate ferns of the extant genus *Stromatopteris*, and therefore concluded that psilopsids are related to certain ferns. However, the distribution of flavonoid compounds in *Psilotum, Tmesipteris* and members of the *Stromatopteridaceae*, does not support a close relationship between both groups (COOPER-DRIVER 1977). The strongly developed mycotrophy of *Psilotum* is regarded as an evidence for a derived status of the psilopsids, but investigations of endomycorrhizal fungi now suggest that Rhynie chert plants from the Devonian might already have been associated with mycorrhizal fungi (PIROZYNSKI & DALPÉ 1989, SIMON & al. 1993).

The analyses of the small dataset shown in Fig. 2 favour a psilopsid lineage that is independent from the one leading to the ferns and places *Psilotum* with 75% bootstrap support in the maximum likelihood analysis as a sister group to the seed plants. While computer simulation studies showed that the maximum likelihood methods provide accurate tree topologies under conditions of equal and a broad range of unequal evolutionary rates (KUHNER & FELSENSTEIN 1994), the results of the statistical tests indicate that psilopsids, sphenopsids, ferns, and spermatophytes evolved within a short geological period of time. As horsetails, ferns and seed plants are derived from the Devonian trimerophytes, our analyses support an independent trimerophyte origin of the psilopsids as well.

Concerning the ferns included in the analyses, the homosporous genus *Adiantum* and the heterosporous aquatic fern *Salvinia* form a monophyletic group which is not supported by bootstrapping. Therefore, it will be necessary to include additional taxa in future analyses to resolve the relationship between homosporous and heterosporous ferns.

The supposed ancestors of seed plants are the fossil progymnosperms. These Middle and Lower Devonian plants possessed gymnospermous secondary wood, but reproduced by free-sporing, pteridophytic methods. A close relationship between trimerophytes and the progymnosperms is suggested because of reproductive characteristics like the fusiform sproangia. Although the progymnosperms are accepted as ancestors for all seed plants, there is less agreement about a monophyletic or polyphyletic origin of the gymnosperms. One hypothesis suggests an independent evolution of conifers and cycads from two progymnosperm lineages (BECK & WIGHT 1988). In contrast, ROTHWELL (1982) proposed that the progymnosperm order *Aneurophytales* gave rise to the Lower Carboniferous seed ferns (*Pteridospermales*), and that all modern gymnosperms evolved from this group. Our analyses support a monophyletic origin with low or moderate bootstrap values (53%/69%). A separation of the cycads from the conifer lineage within the Devonian progymnosperms rather than the Carboniferous seed ferns seems unlikely, because in the molecular phylogeny, the angiosperms diverge before the conifer/cycad split and therefore should have originated as early as in the Lower Devonian. This contrasts with results from phylogenetic studies assuming

molecular clocks, which indicate that the last common ancestor of extant seed plants should have existed in the Upper Carboniferous (SAVARD & al. 1994).

The phylogenetic position of the extant genus *Ginkgo* is also uncertain. Some authors suggest that the *Ginkgoales* are more closely related to the conifers and cordaites (DOYLE & DONOGHUE 1986), others emphasize the similarities in the reproductive structures of *Ginkgo* and cycads. The latter view would favour the hypothesis that *Ginkgoales* are derived from some Carboniferous seed fern group, and since that time evolved simultaneously with the pteridosperms, cordaites and conifers (MEYEN 1984). This scenario is reflected in the 18S rRNA phylogeny. Within the gymnosperms, *Ginkgo* branches first, followed by the cycad *Zamia* and the conifers. The bootstrap support for this topology is rather low, but a nearly simultaneous radiation of the ancestors of cycads and conifers, the *Ginkgoales*, and the angiosperms is much more likely than a close relationship between *Ginkgo* and the conifers.

Concerning the evolution of the angiosperms, both maximum likelihood and parsimony analyses support a monophyletic origin (100% bootstrap support) of this lineage. The monocots (*Liliopsida*) are clearly separated from the dicots (*Magnoliopsida*). Almost every group of vascular plants has at one time or another been implicated by palaeobotanists as the progenitor of the flowering plants. Since complete 18S rRNA sequences are not available from members of the *Gnetales* and from primitive angiosperms, a critical comparison between molecular and fossil data is not yet feasible.

An increasing amount of molecular data will enable us to compare molecular phylogenies with the large number of fossil informations in more detail. The synthesis of molecular and palaeobotanical data will help to test controversial interpretations of the fossil record. Because of the substantial agreement between both datasets, it will further be possible to identify the phylogenetic position of critical taxa, even when fossil data are lacking.

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