

Phylogeny of Horsetails (*Equisetum*) based on the Chloroplast *rps4* Gene and Adjacent Noncoding Sequences

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ABSTRACT. *Equisetum* is a genus of 15 extant species that are the sole surviving representatives of the class Sphenopsida. Chloroplast DNA sequence data were used to examine the monophyly of the two accepted subgenera (*Equisetum* and *Hippochaete*) and the putative basal position of *Equisetum giganteum*. A plastid DNA region that includes *rps4* was sequenced for all species of *Equisetum*. Phylogenetic analyses using parsimony, likelihood or posterior probability criteria, all support the following inferences: (1) *Equisetum bogotense* is basal within the genus; (2) all other species group in two major sister clades: (i) the rest of subgenus *Equisetum* (7 species) and (ii) subgenus *Hippochaete* (7 species). On the basis of the present phylogeny, bisexuality would not be the ancestral state in the *Equisetum* genus, but characters shared among species of the subgenus *Equisetum*, such as superficial stomata and protruding antheridia, could be ancestral in the genus.

Horsetails (*Equisetum* L.) are the only survivors of the formerly more diverse Sphenopsida, free-sporing plants characterized by articulate stems bearing whorls of leaves at each node. According to the fossil record, primitive forms possibly assignable to Sphenopsida appeared in the late Devonian (Stewart and Rothwell 1993; Taylor and Taylor 1993). Sphenopsida then reached maximum diversity during the Carboniferous. Afterwards, major extinction episodes took place during the early Permian and the late Jurassic. Since the beginning of the Cenozoic, all known Sphenopsida have been herbaceous forms that are indistinguishable from living horsetails (Brown 1975; McIver and Basinger 1989; Stewart and Rothwell 1993). Fossils attributed to the genus *Equisetites* that closely resemble modern *Equisetum* date from the Permian (Boureau 1964; Stewart and Rothwell 1993), and possibly even from the Carboniferous (Emberger 1968; Taylor and Taylor 1993). On account of the similarity between *Equisetites* fossils and living *Equisetum* species, the distinction made between these two genera has been questioned to the point that Arnold (1947) proposed that *Equisetum* might be regarded as the oldest surviving vascular plant genus in the world.

Morphological variability within living species and extensive hybridization among them has long obscured the taxonomy of the genus *Equisetum*. Since the work of Hauke (1963, 1978), which significantly clarified the picture, 15 species of *Equisetum* have been widely accepted. The taxonomy of the genus *Equisetum* recognizes two subgenera (*Equisetum* and *Hippochaete*, Milde 1861), on the basis of stomatal position: superficial in *Equisetum* (*E. arvense*, *E. bogotense*, *E. diffusum*, *E. fluviatile*, *E. palustre*, *E. pratense*, *E. sylvaticum*, and *E. telmateia*), but sunken below the epidermal surface in *Hippochaete* (*E. giganteum*, *E. hyemale*, *E. laevigatum*, *E. myriochaetum*, *E. ramosissimum*, *E. scirpoides*, and *E. variegatum*). Other characters, such as the morphology of

gametophytes, also show a clear-cut distinction between the two subgenera (Table 1; Duckett 1973, 1979a).

Most living species of horsetails have very broad circumboreal distributions, their latitude ranging between 40° and 60° north (Hauke 1963, 1978). The exceptions are *E. bogotense*, *E. giganteum*, and *E. myriochaetum*, from Central and South America, *E. laevigatum*, which is restricted to North America, *E. diffusum*, endemic of the Himalayas, and *E. ramosissimum*, which ranges from Europe, Africa, and Asia to some Oceanic Islands. On account of overlapping distributions, many interspecific hybrids, involving all species except *E. bogotense*, are found in the wild. These hybrids are considered to be sterile (but see Krahulec et al. 1996) and to rely on vegetative reproduction for persistence and growth. Most of the hybrids have also been experimentally synthesized by controlled crosses (Duckett 1979b). Notably, the pattern of hybridization in the genus *Equisetum* can be used to distinguish two distinct groups of species. Within each group, species are connected by hybridization, whereas hybridization cannot occur between groups. This division based on hybrid occurrence perfectly matches with the taxonomic division of the genus into the two subgenera *Equisetum* and *Hippochaete* (Duckett 1979b).

The sectional classification of the subgenera *Equisetum* and *Hippochaete* is more controversial. Different divisions of the subgenus *Equisetum* have been proposed, depending on the characters prioritized for taxonomy. These characters include (i) stem dimorphism (Braun 1839), (ii) endodermal patterns (Pfitzer 1867), (iii) surface morphology of silica deposits (Page 1972), and (iv) antheridium morphology (Duckett 1973). Although there was some correlation between Duckett's observations concerning antheridium morphology and the classification proposed by Page (1972), Hauke (1974) considered that the available information was

TABLE 1. Characters differing between the two subgenera *Equisetum* and *Hippochaete*. ¹ except *E. bogotense* and *E. diffusum*. ² except *E. laevigatum*, and *E. ramosissimum* in colder climates. ³ except *E. ramosissimum*, *E. myriochaetum* and *E. giganteum*. ⁴ except *E. myriochaetum* and *E. laevigatum*. Data from Duckett (1973, 1979a), Hauke (1963, 1968, 1969, 1974, 1978) and Page (1972).

Characters	<i>Equisetum</i>	<i>Hippochaete</i>
Sporophyte		
stem		
persistence	annual ¹	perennial ²
morphology	branched	unbranched ³
cone apex	rounded	pointed ⁴
silica deposits	ornamented	amorphous
position of stomata	superficial	sunken
Gametophyte		
lamellae morphology		
antheridia	unistratose	column
position	protruding	sunken
cover cell number	usually >2	usually 2
Chromosome size		
	small	large

insufficient to formulate a sectional classification of the subgenus *Equisetum*. Later, Hauke (1978) constructed a phenetic dendrogram based on pairwise comparisons of species for many characters. According to Hauke's results, only one group is worth recognizing; this includes *E. arvense*, *E. pratense*, and *E. sylvaticum*. In contrast, from the pattern of hybridization within the subgenus *Equisetum*, Duckett (1979b) suggested that *E. pratense* and *E. sylvaticum* would form a unit separate from the remaining species.

A classification of the subgenus *Hippochaete* has also been proposed by Hauke (1963). Section *Incunabula* contains the regularly branched *E. giganteum*, section *Hippochaete* contains species with evergreen, unbranched aerial stems (*E. hyemale*, *E. variegatum*, and *E. scirpoides*), and the section *Ambigua* contains those species with intermediate morphology (*E. myriochaetum*, *E. ramosissimum*, and *E. laevigatum*). This division is partly supported by experimental data on hybridization (Duckett 1979b), but the morphology of *Hippochaete* gametophytes shows no clear discontinuities that fit the sectional division (Duckett 1979a). Moreover, the frequent occurrence of hybrids within subgenus *Hippochaete* suggests that the taxa are more closely interrelated than in subgenus *Equisetum*.

The phylogenetic position of horsetails has been investigated in several studies. Examination of early fossil record and morphological characters of land plants favors the grouping of horsetails with ferns in a monophyletic clade (Kenrick and Crane 1997). The same conclusion was reached with the study of characters derived from spermatogenesis, development, and morphology (Renzaglia et al. 2000). Recently, molecular phylogenies of extant vascular plants using either (i) mitochondrial small-subunit rDNA sequences (Duff

and Nickrent 1999), or (ii) both nuclear and mitochondrial small-subunit rDNA sequences (Renzaglia et al. 2000), or (iii) plastid *atpB*, *rbcL*, and *rps4* and nuclear small-subunit ribosomal DNA sequences (Pryer et al. 2001) also concluded that horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. The exact position of *Equisetum* with respect to ferns varies among the studies, some placing *Equisetum* as a sister clade to leptosporangiate ferns (Duff and Nickrent 1999; Renzaglia et al. 2000), some weakly supporting the grouping of *Equisetum* with Marattiopsida (Pryer et al. 2001), and some placing *Equisetum* as the sister group to all ferns (Kenrick and Crane 1997).

In contrast, phylogenetic relationships within the genus *Equisetum* have remained largely unexplored. Evolution in *Equisetum* was discussed by Schaffner (1925, 1930) and Hauke (1963), although a cladistic analysis of morphological characters has not yet been undertaken in horsetails. Considering that *E. giganteum* has large stems and persistent sheath teeth, as do most fossil members of this group, and because it has regular whorls of branches and stomata in many lines, as found throughout the subgenus *Equisetum*, both authors concluded that *E. giganteum* was the most primitive member of the genus. On the basis of the supposed primitive status of *E. giganteum*, the most advanced members of the subgenus *Hippochaete* were proposed to be *E. laevigatum* and *E. scirpoides* (Schaffner 1925; Hauke 1963).

Besides the evolution of sporophytic characters, the phylogenetic status of *E. giganteum* has implications for the evolution of sexuality in *Equisetum* gametophytes. Gametophytes of horsetails are usually unisexual: gametophytes first develop as male or female, with female gametophytes later producing antheridia, when not fertilized (Duckett 1970, 1972). Interestingly, gametophytes of *E. giganteum* are functionally bisexual (i.e., producing antheridia and archegonia simultaneously), a phenology that is unique in the genus (Hauke 1969, 1985). Because he considered *E. giganteum* to be primitive, Hauke hypothesized that bisexuality could have been the primitive condition in *Equisetum*. In contrast, *E. bogotense* gametophytes seem to be strictly unisexual (i.e., never showing sex reversal from archegonia to antheridia production), a phenology proposed to be the most advanced in the genus (Hauke 1968, 1969).

We here report a molecular phylogeny of the genus *Equisetum* using *rps4* chloroplast sequences. The objective of this study is to investigate the relationships among species of horsetails, and in particular to examine (i) the monophyly of the two subgenera and (ii) the basal position of *E. giganteum*, in order to explore the evolution of characters in this remarkable group of plants.

TABLE 2. Voucher and EMBL/GenBank information for specimens used in this study.

<i>Equisetum</i> L. <i>E. arvense</i> L. (Guillon 5, P-00271032; AJ583677).
<i>E. bogotense</i> Kunth (McAlpin s.n., UC-1733471; AJ583678).
<i>E. diffusum</i> D. Don (Gross 1, P-00271037; AJ583679).
<i>E. fluviatile</i> L. (Husby 5, P-00271028; AJ583680).
<i>E. giganteum</i> L. (Husby 7, P-00271026; AJ583681).
<i>E. hyemale</i> L. (Duckett s.n., CGG-19660161; AJ583682).
<i>E. laevigatum</i> A. Braun (Husby 2, P-00271030; AJ583683).
<i>E. myriochaetum</i> Cham. & Schlecht. (Armond 93, E-15013; AJ583684).
<i>E. palustre</i> L. (Guillon 4, P-00271033; AJ583685).
<i>E. pratense</i> Ehrh. (Page 1256, E-15005; AJ583686).
<i>E. ramosissimum</i> Desf. subsp. <i>debile</i> (Roxb.) Hauke (Walker 7714, E-15011; AJ583687).
<i>E. scirpoides</i> Michx. (Dunand s.n., G-452418; AJ583688).
<i>E. sylvaticum</i> L. (Page 7889, E-15001; AJ583689).
<i>E. telmateia</i> Ehrh. subsp. <i>braunii</i> (Milde) Hauke (Doran 22, CONN-122648; AJ583690).
<i>E. variegatum</i> Schleicher (Guillon 3, P-00271034; AJ583691).
Outgroups (sequences were obtained from GenBank/EMBL). <i>Angiopteris evecta</i> (J.R. Forst.) Hoffmann (AF313591).
<i>Danaea elliptica</i> Sm. (AF313589). <i>Ophioglossum reticulatum</i> L. (AF313594).
<i>Pteris plumula</i> Desv. (AF321694). <i>Salvinia molesta</i> Mitchell (AF313600).

MATERIALS AND METHODS

Sampling. All widely recognized extant species of *Equisetum* were represented in this analysis. The voucher and EMBL/GenBank accession details for specimens used in this study are listed in Table 2.

DNA Extraction, PCR, and Nucleotide Sequencing. Total genomic DNA was extracted from fresh or silica gel dried plant material using the DNAeasyTM Plant Mini Kit (Qiagen). The chloroplast region including the *rps4* gene and noncoding flanking sequences (approximately 1100 base pairs) was amplified using the universal primers S and T, as described by Demesure et al. (1995). Amplification mixtures (25 μ l) contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton[®] X-100, 2.5 mM MgCl₂, 200 μ M of each of the four dNTPs, 0.4 μ M of each primer, and 1 unit of Taq DNA polymerase (Promega). The PCR products were checked on agarose gel with ethidium bromide and purified by PEG precipitation (Rosenthal et al. 1993). Purified PCR products were sequenced using MWG Biotech custom sequencing service.

Outgroups, Sequence Alignment, and Indel Coding. Outgroups were chosen within ferns because recent phylogenetic studies agree on grouping horsetails and ferns in a monophyletic clade sister to seed plants (Kenrick and Crane 1997; Duff and Nickrent 1999; Renzaglia et al. 2000; Pryer et al. 2001). Outgroups were representative of major groups of ferns for which *rps4* sequence data are available: Filicopsida (*Pteris plumula* and *Salvinia molesta*), Marattiopsida (*Danaea elliptica* and *Angiopteris evecta*) and Ophioglossopsida (*Ophioglossum reticulatum*). Analyses also including (i) seed plant sequences or (ii) Lycopodiopsida and Isoetopsida sequences, in addition to fern sequences, gave identical results.

Automated alignment of sequences was performed using the ClustalX program (Thompson et al. 1994), and followed by refinement by eye. Sequence matrices are available from TreeBASE (study accession number S964, matrix accession number M1595-M1597).

Alignment of the complete *rps4* coding sequences was straightforward among *Equisetum* species, but only partial *rps4* sequences were available for outgroups and two regions proved difficult to align. Only unambiguously aligned sequences of outgroups were used in the matrix, bases of uncertain alignment being coded as missing. Thus, the number of characters was 624 and the percentage of cells scored as missing data was 4% in the *rps4* data set (TreeBASE matrix M1595).

For upstream and downstream noncoding DNA regions, only

one fern sequence was found in the data base, and this could not be aligned with *Equisetum* sequences. Thus, only *Equisetum* sequences were included in the alignment. These noncoding sequences were divided into conserved regions that could be aligned among all species (shown on a white background in Fig. 1), and blocks of variable regions (DNA insertions, deletions, repetitions, and stretches of polyT or polyA) that were present or absent depending on species (shown on a grey or black background in Fig. 1). Conserved noncoding DNA sequences were juxtaposed, small indels being coded as a single event, to yield the second matrix (308 characters, 1.4% of cells scored as missing; TreeBASE matrix M1596). Variable regions were treated separately because they result from large repetition, insertion or deletion events that are very different from single nucleotide substitutions. Therefore, a third matrix (15 characters, no missing data; TreeBASE matrix M1597) included blocks of variable regions, considered as multistate characters (i.e., coded as 1, 2 or 3, according to the color code in Figure 1, all possible transitions being equally weighted).

Phylogenetic Analyses. For maximum parsimony (MP) analyses of the first and second matrices, branch and bound searches were conducted using PAUP* 4.0b (Swofford 1998), saving all most parsimonious trees. Internal support for relationships was assessed using bootstrap analyses with 1,000 replicates. For MP analysis of the third matrix, a heuristic search was conducted using PAUP* 4.0b (Swofford 1998), starting from a tree constructed by stepwise addition, and branch swapping with tree-bisection-reconnection (TBR). A 1,000 replicate random stepwise addition sequence was specified, and all most parsimonious trees were saved.

Maximum likelihood (ML) analysis was conducted on the first matrix using PAUP* 4.0b (Swofford 1998). A general time reversible model of substitution with site-specific rates for first, second and third codon positions (GTR+SS) was chosen, based on Akaike Information Criterion. The MP tree with the greatest -ln score was used to estimate the model parameters. Heuristic search was then conducted, starting from a tree constructed by stepwise addition, and branch swapping with TBR. To avoid deleteriously restricting the search to a single island of trees, a random stepwise addition sequence was specified for 100 replicates. Resampling of codons was performed 1,000 times using the Bootstrap Codon program from John Huelsenbeck's laboratory. ML analyses were then conducted as above, recalculating the model parameters for each of the 1,000 bootstrap replicates, with 10 random-addition replicates per heuristic search.

Bayesian phylogenetic analyses were conducted on the first matrix using MrBayes version 2.0 (Huelsenbeck and Ronquist 2002). We used a GTR+SS model, starting with a random tree. Four Markov chains were run for 220,000 generations and sampled every 100 generations. Stationarity was reached at approximately generation 20,000, so the first 200 trees or "burn in" of the chain were discarded, and phylogenetic inferences are based on those trees sampled after generation 20,000.

RESULTS

Among the 624 characters present in the first matrix (*rps4* sequences), 291 sites (47%) were variable and 187 (30%) were phylogenetically informative. MP analysis of this data set yielded five equally parsimonious trees, which only differed in the positions of *E. palustre*, *E. pratense*, and *E. telmateia* (Fig. 2). *Equisetum bogotense* is sister to the rest of the genus (90% BS, DI = 5), divided into two clades: (i) the subgenus *Hippochaete* (91% BS, DI = 4) and (ii) the subgenus *Equisetum* without *E. bogotense* (86% BS, DI = 3). Support is lower for branches within each group, except for the following clades: (*E. arvense*, *E. diffusum*, *E. fluviatile*) (98% BS, DI = 5), and (*E. giganteum*, *E. hyemale*, *E. laevigatum*, *E.*

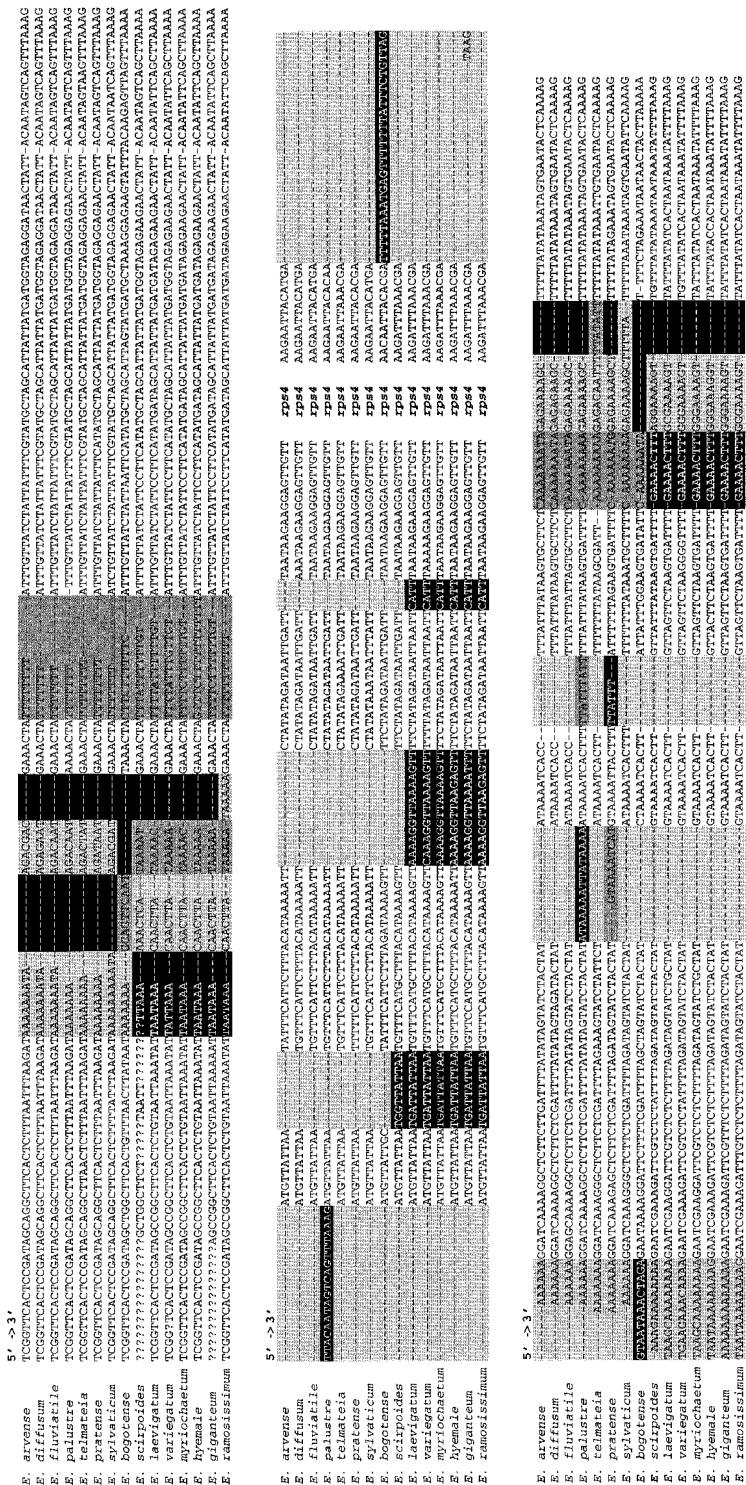


FIG. 1. Alignment and categorization of the noncoding sequences 5' and 3' to *rps4*. White background: sequences that can be aligned among all *Equisetum* species, juxtaposed in the second matrix. Black or grey background: stretches of Ts or As of variable length and indels depending on species, coded as multistate characters in the third matrix (at a given position, regions on a same color background were considered as homologous and coded as follows: black background = 1, grey background = 2, light grey background = 3). ? = missing data.

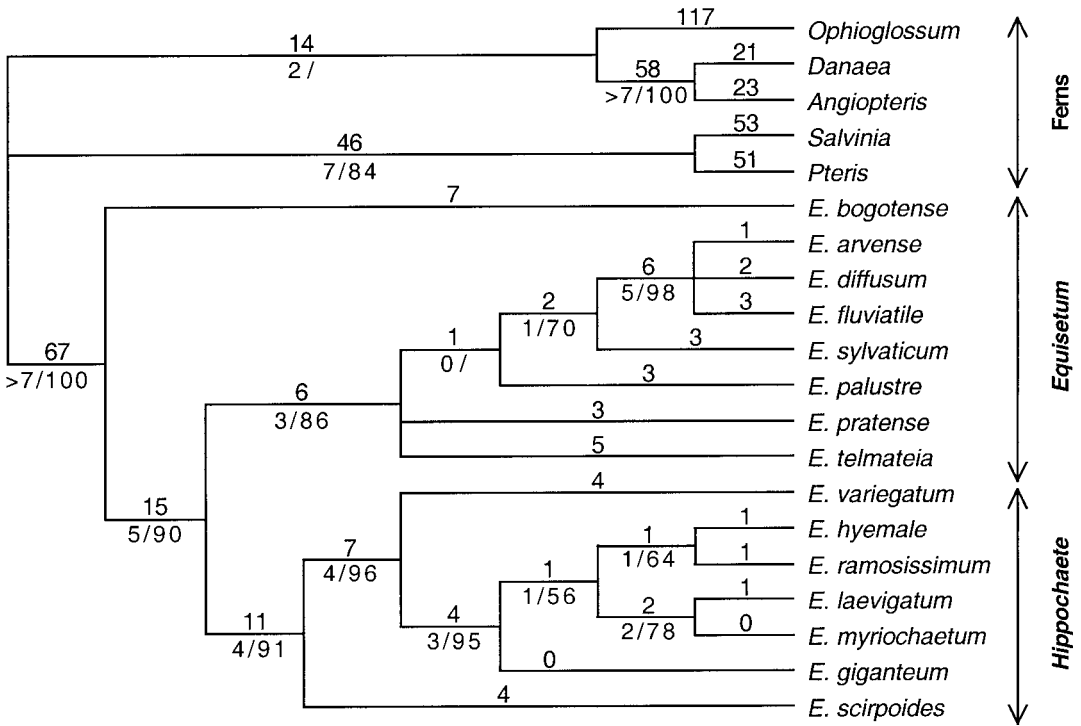


FIG. 2. One of five shortest trees obtained in a parsimony analysis of *rps4* sequence data (first matrix). Numbers above branches are branch lengths. Bremer decay indices (DI), followed by bootstrap percentages (based on 1,000 replicates), are given below branches. A null decay index means that the branch collapses in the strict consensus tree. Lines with no bootstrap values below denote nodes supported in <50% of the replications. Tree length = 508 steps; CI = 0.76; RI = 0.78.

myriochaetum, *E. ramosissimum*) (95% BS, DI = 3). *Equisetum scirpoides* is sister to a clade formed by the rest of the subgenus *Hippochaete* (96% BS, DI = 4).

For ML analysis of the first matrix, model parameter values were estimated from the MP tree with the greatest $-\ln$ score ($-\ln$ likelihood = 2913.36106). All 100 replicates of the heuristic search using the ML optimality criterion resulted in the same tree (Fig. 3). Both MP and ML trees are congruent, with a basal position of *E. bogotense*, and two major clades corresponding to the subgenera *Equisetum* (without *E. bogotense*) and *Hippochaete*. Bootstrap values are similar in both analyses yet with a higher support in ML for (i) (*E. arvense*, *E. diffusum*, *E. fluviatile*, *E. sylvaticum*) (84% BS), and (ii) (*E. laevigatum*, *E. myriochaetum*) (82% BS).

The Bayesian analysis of the first matrix yielded a consensus tree (Fig. 4) comparable to those obtained with MP and identical to that obtained with ML. Two clades weakly supported in MP and ML analyses are here recovered with 100% frequency: (*E. hyemale*, *E. laevigatum*, *E. myriochaetum*, *E. ramosissimum*) and (*E. hyemale*, *E. ramosissimum*).

Among the 308 characters present in the second matrix (noncoding sequences conserved among all *Equisetum* species, shown on a white background in Fig. 1), 99 sites (32%) were variable and 53 (17%) were phy-

logenetically informative. MP analysis of this data set yielded six equally parsimonious trees, which only differed in the positions of *E. palustre*, *E. telmateia*, and *E. giganteum* (Fig. 5). Strong bootstrap support is obtained for the same branches as with MP analysis of coding sequences, though with the caveat that the present trees are not rooted.

Among the 15 characters present in the third matrix (blocks of variable noncoding regions, shown on a grey or black background in Fig. 1), 14 were variable and 7 were phylogenetically informative. MP analysis of this data set yielded eight equally parsimonious trees (length = 18 steps; consistency index = 1.0; retention index = 1.0), which only differed in the positions of *E. palustre*, *E. pratense*, *E. telmateia*, and *E. sylvaticum* (result not shown). In these eight trees, subgenera *Equisetum* and *Hippochaete* are separated by five steps (DI = 5), and *E. scirpoides* is sister to the rest of the subgenus *Hippochaete* (DI = 2).

DISCUSSION

Trees resulting from the contrasting algorithmic analyses of *rps4* and adjacent noncoding 5' and 3' sequences are in good agreement. Throughout this discussion, only those clades that were supported in all

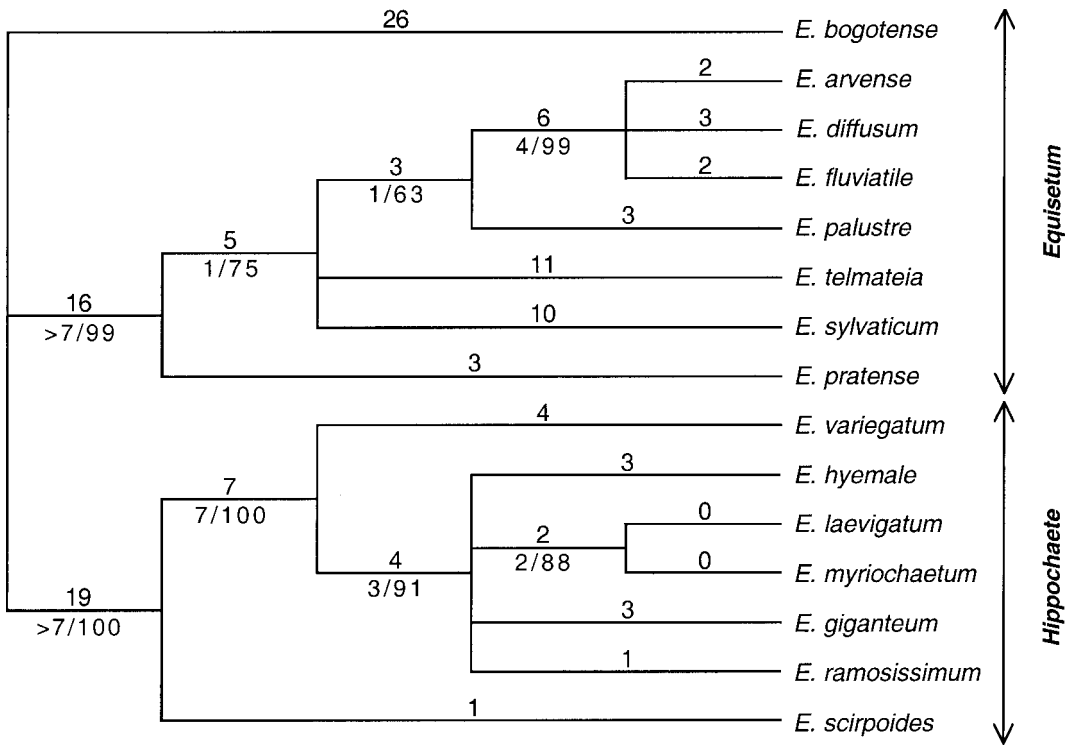


FIG. 5. One of 6 shortest trees obtained in a parsimony analysis of noncoding flanking sequences that can be aligned among all *Equisetum* species (shown on a white background in Fig. 1; second matrix). Numbers above branches are branch lengths. Bremer decay indices, followed by bootstrap percentages (based on 1,000 replicates) are given below branches. Lines with no bootstrap values below denote nodes supported in <50% of the replications. Tree length = 134 steps; CI = 0.90; RI = 0.94.

analyses are considered. Overall, the results support a basal position for the *E. bogotense* lineage, and then the divergence of two clades corresponding to subgenus *Equisetum* (without *E. bogotense*) and subgenus *Hippochaete*. Within the subgenus *Equisetum*, only *E. arvense*, *E. diffusum*, and *E. fluviatile* group in a consistently supported clade. Relationships are better resolved within the subgenus *Hippochaete*, with the successive divergences of the *E. scirpoides* and *E. variegatum* lineages, and a consistent sister group relationship between *E. laevigatum* and *E. myriochaetum*. From now, I shall designate *Euequisetum* the clade that includes *E. arvense*, *E. diffusum*, *E. fluviatile*, *E. palustre*, *E. pratense*, *E. sylvaticum*, and *E. telmateia* (i.e., subgenus *Equisetum* without *E. bogotense*).

The basal position of *E. bogotense* contrasts with previous opinions concerning the most ancestral lineage in *Equisetum*; *E. giganteum* was considered to be the most primitive member of the genus (Schaffner 1925, 1930; Hauke 1963), whereas *E. bogotense* gametophyte characters were thought to reflect an advanced position (Hauke 1968, 1969). Indeed, Hauke (1963) considered that large size, evergreen stems and bisexual gametophytes, as shown by *E. giganteum*, to be primitive states. Duckett (1979a, p. 179 and p. 201) already stressed that "neither gametophyte morphology nor

sexuality provide any definitive data to support the theory that *Hippochaete* contains the most primitive extant horsetails... it is difficult to attribute, with any confidence, specific morphological characters and features of the coning behavior to either advanced or primitive status." The present results substantiate this view, contradicting the widely held opinion that bisexuality, as found in *E. giganteum*, is the ancestral state in the genus *Equisetum*.

The basal position of *E. bogotense* has other interesting implications for the evolution of characters within the genus. Indeed, some distinctive features of *E. bogotense*, such as filamentous and strictly unisexual gametophytes with highly protruding archegonial necks and antheridia (Hauke 1968, 1969), may reflect an early divergence from other *Equisetum* lineages. On the other hand, characters shared among *Euequisetum* species and *E. bogotense* may be ancestral for horsetails. Among such characters are: (i) branched stems, (ii) a blunt cone, (iii) an outer endodermis common to all vascular bundles of the stem, (iv) superficial stomata, (v) unistratose lamellae in gametophytes, and (vi) protruding antheridia. This pattern of evolution is in keeping with the observation that *Equisetites* fossils from the Mesozoic show blunt cones and generally regular branching (Boureau 1964). Furthermore, it is congruent

with the timing of earliest appearances in the fossil record; the most ancient reported *Hippochaete* species dates from the early Cenozoic (Brown 1975), whereas *E. bryanii*, assigned to the subgenus *Equisetum*, dates from the middle Jurassic (Gould 1968).

During completion of the present work, Des Marais et al. (2003) also investigated the phylogenetic relationships among extant horsetails, using *rbcL* and *trnL-F* chloroplast DNA sequence data. Their MP analyses support the same basal position for *E. bogotense*, but their ML analysis places it as sister to subgenus *Hippochaete*. In either case, because subgenus *Hippochaete* would be nested within subgenus *Equisetum*, my conclusions regarding the evolution of characters in horsetails are equally valid. Future research focused on *E. bogotense* would be useful in order to confirm its position. Indeed, *E. bogotense* and *E. diffusum* have been the least studied horsetail species. Specifically, it would be interesting to perform hybridization experiments using *E. bogotense*. Natural hybrids of *E. bogotense* are unknown, but this may merely reflect the absence of interfertile sympatric species. In addition, cytological studies are needed to determine whether *E. bogotense* is also diploid and has the same chromosome number ($n = 108$) as all karyologically studied members of the genus (Hauke 1974).

In the light of the present phylogeny, various morphological characters appear to show some degree of homoplasy. Consider for example the shape of the cone apex; the most parsimonious scenario is the one assuming that (i) a blunt cone was the ancestral state for extant horsetails, (ii) a pointed apex later appeared in an ancestor of subgenus *Hippochaete*, and (iii) the apex reversed to a rounded state in an ancestor of the (*E. laevigatum*, *E. myriochaetum*) clade. Other examples of homoplastic traits are: (i) the coning behavior (heterophyadic stems found in *E. arvense* and *E. telmateia*), (ii) the stem branching pattern (branched throughout the subgenus *Equisetum* and in *E. ramosissimum*, *E. myriochaetum*, and *E. giganteum*), (iii) the endodermal pattern (with an individual endodermis found in both *E. fluviatile* and *E. giganteum*), and (iv) the shape of antheridial opercular cells and archegonial necks (most elongated in *E. bogotense* and *E. fluviatile*; Hauke 1963, 1968, 1978; Duckett 1973, 1979a).

Previous taxonomic divisions within both subgenera are not supported by the present phylogeny. Within *Hippochaete*, the only concordance is the grouping of *E. laevigatum* with *E. myriochaetum*, which was already present in Hauke's classification (1963). In contrast, the phylogeny supports a basal position within *Hippochaete* for *E. scirpoides*, which was formerly thought to possess advanced characters (small size, unbranched stems, outer common endodermis in both stems and rhizome, stomata arranged in single lines), instead of supposedly primitive *E. giganteum* (large size,

branched stems, bisexual gametophytes, stomata arranged in many lines). Within subgenus *Equisetum*, no previous classification proposed the grouping of *E. arvense*, *E. diffusum*, and *E. fluviatile*, which is highly supported in our analyses. Consistent with this clade are the observations that *E. arvense* hybridizes in the wild with both *E. diffusum* (*E. x wallichianum*) and *E. fluviatile* (*E. x litorale*), and that *E. x litorale* is the most common hybrid in the subgenus *Equisetum* (Hauke 1978; Duckett 1979b).

Since the sequences used in this study occur only in plastids, and since maternal transmission of plastids has been reported in the genus *Equisetum* (Guillon and Raquin 2000), the present phylogeny is in theory concerned only with maternal lineages. The gene tree may not exactly reflect species relationships if hybridization between differentiated *Equisetum* species has taken place in the past. Although hybrids between extant species are generally considered to be sterile, Krahulec et al. (1996) observed the development of gametophytes from *E. x moorei* (*ramosissimum x variegatum*) and *E. x meridionalis* (*hyemale x ramosissimum*). Unfortunately, the authors did not report whether antheridia or archegonia were expressed in these hybrid gametophytes. However, it is important to keep in mind Page's statement (1972, p. 367): "the lack of clear relationships between the living [horsetail] species and their reticulate pattern of linkage seem consistent with the view that hybridization has played a significant if not dominant role in their evolution." Future studies of *Equisetum* phylogeny should address this point by sequencing nuclear genes to compare with the existing plastid data.

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