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PHYLOGENETIC ANALYSIS OF *ASPLENIUM* SUBGENUS *CETERACH* (PTERIDOPHYTA: ASPLENIACEAE) BASED ON PLASTID AND NUCLEAR RIBOSOMAL ITS DNA SEQUENCES[1]

CAROLINE J. VAN DEN HEEDE,[2] RONALD L. L. VIANE,[2,4] AND MARK W. CHASE[3]

[2]Pteridological Section, Department of Biology, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium; and
[3]Jodrell Laboratory, Royal Botanic Gardens, Richmond, Surrey, TW9 3DS, UK

Phylogenetic relationships among 20 taxa of the fern genus *Asplenium* subgenus *Ceterach* (Filicopsida, represented by 73 accessions) were investigated using DNA sequence data from the nuclear ribosomal internal transcribed spacers (ITS nDNA) and plastid *trnL-F* intergenic spacer. In addition, a single sample per taxon was used in an analysis of the plastid *rbcL* gene. Chromosome counts were determined for all the samples, and these demonstrated a range from diploid to octoploid. Analyses of the DNA sequence data indicated that *Asplenium* subgenus *Ceterach* is polyphyletic, implicating homoplasy in the characters previously used to circumscribe this taxon. Plastid *trnL-F* and *rbcL* analyses resulted in identical tree topologies. The trees produced from the separate plastid and nuclear matrices agree in (1) the recognition of identical groups of accessions corresponding to *A. dalhousiae*, *A. ceterach*, *A. aureum*, *A. cordatum*, *A. phillipsianum*, and *A. haughtonii*; (2) the division of *A.* subg. *Ceterach* into two subclades, a Eurasian-Macaronesian and a strictly African alliance; (3) the position of *A. dalhousiae* as a member of the former subclade; (4) the lack of genetic variation in *A. cordatum* despite its morphological variability; and (5) the clustering of each autopolyploid with their diploid ancestor. However, the plastid and nuclear trees differ in their placement of *A. haughtonii* and *A. dalhousiae*, which might be due to different evolutionary histories of nuclear and plastid genomes, and is possibly an indication of ancient hybridization. The analyses confirm the existence of several strictly African taxa. *Asplenium phillipsianum* and *A. cordatum* each form species complexes of diploid and autopolyploid taxa, from which a third, morphologically intermediate, allotetraploid species has originated. *Asplenium haughtonii* is a distinct endemic species from Saint Helena. The maternally inherited plastid sequences support the hypothesis that *A. aureum* is an ancestor of *A. lolegnamense* and of *A. octoploideum*. Because gene conversion did not eliminate divergent ITS alleles in the allopolyploids, their reticulate ancestry could be demonstrated. Biparentally inherited nrITS sequences support the allopolyploid status of *A. aureum*, *A. lolegnamense*, and *A. punjabense*, indicating they share the ancestral *A. javorkeanum* genome.

Key words: Africa; Aspleniaceae; *Asplenium* subgenus *Ceterach*; gene conversion; ITS; molecular phylogenetics; Pteridophyta; *rbcL*; *trnL-trnF* intergenic spacer.

Asplenium subgenus *Ceterach* (Willd.) Bir et al. is a small group of about 10 fern taxa within the large, subcosmopolitan genus *Asplenium* L. (720 species; Kramer and Viane, 1990). Although characterized by a variable gross morphology, Aspleniaceae are anatomically and cytologically homogeneous. The basic chromosome number (*x*) is 36. Both auto- and allopolyploidy are common driving forces of evolution in Aspleniaceae (Wagner, 1954; Lovis, 1977; Reichstein, 1981). Thus, in the well-known Holarctic fern flora, *Asplenium* consists of about 53% (ancestral) diploids, 24% autopolyploids, 20% allopolyploids, and 3% apogamous taxa. Polyploidy is at least equally common in the tropical taxa, but their status and ancestry remain largely unexplored.

There is no modern monograph of Aspleniaceae including a phylogenetic assessment, and the monophyly of *A.* subgenus *Ceterach* was never investigated. The only worldwide overview of *Asplenium* is that of Mettenius (1859). Cytological studies have led to the present well-known phylogeny of northern temperate taxa (Viane et al., 1993; Wagner et al., 1993), but relationships among tropical and southern temperate taxa remain largely unknown. Chromosome numbers were compiled by Löve et al. (1977) and Reichstein (1981).

Asplenium subgenus *Ceterach* contains xerophytic rock ferns with the dorsal side of the lamina densely covered with scales (Fig. 1). The indusium is reduced or absent, and the veins are anastomosing (Eurasian group) or free (strictly African group). Ploidy ranges from diploid to octoploid. The number of species is still unclear and varies from five or seven to 14, depending on the weight given to certain morphological characters and whether different cytotypes are considered separate species (Van den heede et al., 2002). Most taxa are morphologically similar and difficult to distinguish in the field. Representatives of *Ceterach* occur in Europe, Macaronesia, throughout southwestern Asia to the Himalayas, and in the drier parts of North, East, and southern Africa. Islands like Madeira and Saint Helena have endemic species.

Rasbach et al. (1987) have shown that *A. ceterach* L. is an autotetraploid, which must have originated via chromosome doubling in the morphologically identical diploid, *A. javorkeanum* Vida (synonym: *A. ceterach* subsp. *bivalens*

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[4] Author for reprint requests (e-mail: ronnie.viane@rug.ac.be).

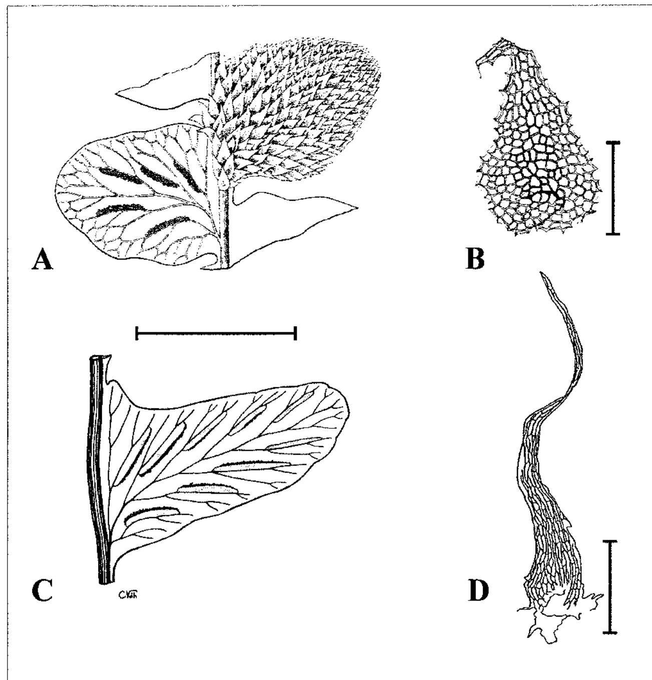

Fig. 1. Pinnae with venation pattern and sori (A, C), and dorsal laminal scales (B, D). *Asplenium ceterach* (A, B): left pinna with scales removed to show veins with marginal anastomoses and almost naked sori; scales with penta- or hexagonal cells. *Asplenium dalhousiae* (C, D): pinna with free veins and sori with distinct dorsal indusium; scales with elongated cells. Scale bar (A, C) = 1 cm, (B, D) = 0.5 cm. (A) from Luerssen, 1889.

(D.E.Mey.) Greuter and Burdet). *Asplenium* ×*mantoniae* Vár-óczy and Vida is the triploid hybrid between *A. javorkeanum* and *A. ceterach*. Within the same complex, the autohexaploid *A. cyprium* Viane and Van den heede described from Cyprus is also known from Greece and Sicily (Viane et al., 1996; Van den heede et al., 2002).

To date, three cytologically different endemic species are known from Macaronesia. *Asplenium aureum* Cav. was found to be tetraploid by Manton (1950) and suggested to be allotetraploid by Vida and Reichstein (Viane and Reichstein, 1992). *Asplenium octoploideum* Viane and Van den heede (synonym: *A. parvifolium* sensu Vida and Reichstein) is the allo-octoploid species that probably formed by chromosome doubling in the tetraploid hybrid between *A. aureum* and *A. ceterach* (Viane and Reichstein, 1992; Van den heede and Viane, 2002). Manton et al. (1986) and Gibby and Lovis (1989) suggested that the Madeiran allohexaploid *A. lolegnamense* (Gibby and Lovis) Viane originated by hybridization between *A. aureum* and *A. octoploideum*. Though at present their ancestors only occur together in this archipelago, there seems to be no conclusive molecular evidence that *A. octoploideum* and *A. lolegnamense* were formed on the Canary Islands, thus they may show a relict distribution pattern. *Asplenium* ×*chasmophilum* Van den heede and Viane is the natural, sterile hexaploid hybrid between *A. ceterach* and *A. octoploideum*, at present only known from its type locality on Tenerife (Van den heede and Viane, 2002). Chromosome pairing behavior in this hybrid confirms the allotetraploid status of *A. aureum* (C. J. Van den heede and R. L. L. Viane, unpublished data).

For the African *A. capense-cordatum-phillipsianum* complex, some chromosome counts were published by Bir et al. (1985), who reported one diploid and three tetraploid specimens without mentioning auto- or allopolyploidy. We were able to confirm (Bir et al., 1985) that *Asplenium haughtonii* (Hook.) Bir et al. from Saint Helena is tetraploid.

Within Aspleniaceae, *A. dalhousiae* Hook., sometimes put into subg. *Ceterachopsis* (J.Sm. ex Ching) Bir on the sole basis of its frond morphology, represents the putatively closest relative of *A.* subg. *Ceterach*. Copeland (1947) already pointed out that it is impossible to define *Asplenium* so as to exclude the *Ceterachopsis* group. According to Pichi Sermolli (1979, p. 191), "There are good grounds for thinking that *Ceterach* derived from *Asplenium* through the intermediate genus *Ceterachopsis*." Four characters distinguish *A. dalhousiae* from the European *Ceterach* group: absence of a dense layer of dorsal scales, structure of the laminal paleae, open venation pattern, and presence of a true, well-developed indusium (Fig. 1). In fact, these are all putative plesiomorphies within *Asplenium*, from which *Ceterachopsis* only differs by the single apomorphy: a pinnatipartite to pinnatisect (De Candolle, 1813; Pichi Sermolli, 1972) lamina. In most enzyme systems studied, *A. dalhousiae* shows unique zymograms different from those of the members of *A.* subg. *Ceterach* (C. J. Van den heede, unpublished data). *Asplenium dalhousiae* has a disjunct range with a wide distribution in southwestern Asia and smaller ones in Ethiopia and in southwestern North America. We investigated one specimen from each of these areas of distribution.

We also included *Asplenium punjabense* Bir et al., endemic to the western Himalayas, in the analysis. This fertile allohexaploid has been previously documented to have originated by chromosome doubling of a triploid hybrid between diploid *A. dalhousiae* and tetraploid *A. ceterach* (Bir et al., 1985) and can be used to evaluate which molecular method traces reticulate evolution best.

While preparing a biosystematic revision of *A.* subg. *Ceterach* (C. J. Van den heede, unpublished data), we generated hypotheses about possible relationships between members (and hybrids) of this complex based on (micro)morphological and cytological studies. We felt the need to obtain independent estimates for our phylogenetic ideas with molecular data, such as isozymes and DNA. During our fern studies for the *Flora of Tropical East Africa,* it also became evident that further research was necessary to find out how many *Ceterach* species exist in Africa. Schelpe (1970) and Schelpe and Anthony (1986) recognized only a single, variable species, *Ceterach cordatum* Sw., because they could not find characters to distinguish additional taxa.

The first DNA sequencing of pteridophytes was done by Hasebe et al. (1993), who analyzed nucleotide variation in the plastid *rbcL* gene from several leptosporangiate fern families. At present, most molecular analyses of Pteridophyta are based on *rbcL* sequences (e.g., Hasebe et al., 1994, 1995; Wolf et al., 1994, 1999; Murakami, 1995; Pryer et al., 1995; Murakami et al., 1998, 1999a, b; Yatabe et al., 1999; Schulze et al., 2001). Besides *rbcL,* some fern studies used sequences of other regions of plastid DNA, such as 16S rDNA (Manhart, 1995), internal transcribed spacers (plastid ITS; Pahnke et al., 1996) and *trnL-F* (Hauk et al., 1996; Haufler et al., 2000). The first nuclear gene used for phylogenetic research in pteridophytes was 18S rRNA (Raubeson and Stein, 1995; Wolf, 1995, 1996; Kranz and Huss, 1996), which is useful for examining relationships among the major groups of Pteridophyta, although the resulting trees are similar but less resolved than those for *rbcL.* Wolf (1996) also tried to sequence two other

nuclear regions of fern genomes, 26S and the internal transcribed spacers (ITS), but concluded that it was difficult or impossible to get ''clean'' sequences and that fine tuning of techniques and cloning might be necessary. Additionally, the few sequences he obtained were difficult to align because of the apparent rapid accumulation of insertions and deletions. Wolf (1996) suggested that nucleotide variation in nrITS could be taxonomically useful within fern genera, species, and populations. The first analysis of fern ITS regions was on cheilanthoid ferns by Gastony and Rollo (1998), who did not report any difficulties similar to those encountered by Wolf (1996).

In this study, three DNA sequence regions (plastid *trnL-trnF* intergenic spacer, the *rbcL* gene, and nrITS) are used to examine interspecific relationships within *A.* subgenera *Ceterach* and *Ceterachopsis*. We also compare sequences of specimens from different populations of the same species for *trnL-F* and ITS. Intraspecific variation in sequences of plastid DNA (reviewed by Harris and Ingram, 1991; Soltis et al., 1992; Fujii et al., 1997) and ITS of nrDNA have been reported (Baldwin et al., 1995; Fujii et al., 2001). According to Harris and Ingram (1991), intra-individual sampling should be considered for studies at the species level. Plastid DNA polymorphism is quite common in perennial taxa, and even in the absence of morphological variation they may show variation due to substitutions and insertions/deletions (indels). In wild taxa, plastid DNA variation may become established by hybridization and ''introgression'' and thus increase genetic diversity (Harris and Ingram, 1991).

The *trnL-F* region is the non-coding, intergenic spacer between the *trnL* (UAA)3′ exon and *trnF* (GAA) of plastid DNA, for which Taberlet et al. (1991) designed ''universal'' primers. Taberlet et al. (1991) suggested that sequences of intergenic spacers of plastid DNA might be useful for evolutionary studies of closely related species and populations of the same species. In Iridaceae, the *trnL-F* region evolves three times faster than *rbcL* (Soltis and Soltis, 1998). However, in other groups of plants, the *trnL-F* intergenic spacer is as, or more, conserved than *rbcL* (Asmussen and Chase, 2001). In Ophioglossaceae, the number of variable positions in *trnL-F* is 3–5 times higher than in *rbcL* despite the fact that the *trnL-F* spacer is about one-third the length of *rbcL*; Hauk et al. (1996) found that *rbcL* and *trnL-F* analyses produced nearly identical tree topologies.

Sequencing of the ITS region of 18S–26S nuclear ribosomal DNA has been useful for phylogenetic reconstruction at specific and generic levels in many angiosperms (reviewed in Baldwin et al., 1995). This region consists of two spacers, ITS1 and ITS2, separated by the conserved 5.8S gene. Plant ribosomal DNA consists of thousands of repeats and often paralogues (Soltis and Soltis, 1998). Due to concerted evolution, the sequence similarity between different, functional ribosomal copies within an organism is extremely high in most plant species (Dover, 1982; Arnheim, 1983; King, 1993; Baldwin et al., 1995). Concerted evolution is not well understood, but includes several mechanisms such as unequal crossing over, gene conversion, and transposition (Dover, 1982; Soltis and Soltis, 1998; Graur and Li, 2000). However, concerted evolution has not homogenized rDNA repeats in all taxa. Some plant genomes exhibit ribosomal DNA diversity including multiple functional ITS copies (divergent paralogues), nonfunctional ITS copies (''pseudogenes''), and recombinants (Buckler et al., 1997; Soltis and Soltis, 1998; Hershkovitz et al., 1999). According to Hershkovitz et al. (1999), the 5.8S gene provides an indicator of the functionality of cloned ITS copies. An aligned 5.8S sequence showing transversions and/or indels that are absent in the other sampled taxa might indicate that the particular rDNA copy is not expressed. Within-individual ITS polymorphism may result from mutation or hybridization between genomes with different, functional ITS sequences. Sang et al. (1995) were able to document reticulate evolution in *Paeonia* and detect hybridization using nrITS. Both parental ITS sequences have been maintained in allopolyploid species of *Krigia* and *Tragopogon* (Asteraceae), as well as in *Paeonia* species of about one million years of age (Kim and Jansen, 1994; Sang et al., 1995; Soltis and Soltis, 1998). Similarly, Campbell et al. (1997) uncovered extensive ITS sequence polymorphism within *Amelanchier* (Rosaceae) taxa, which are suspected to be relatively old. They postulated that this polymorphism is mainly created by hybridization and that polyploidy and/or agamospermy could retard concerted evolution.

Comparisons of plastid and nuclear DNA analyses can significantly improve understanding of the origin of polyploid species (Soltis and Soltis, 1991; Soltis et al., 1992; Wendel and Doyle, 1998). Because several of the ferns in this study were thought to be autopolyploid, it was necessary to know the exact ploidy of each individual to interpret the molecular results. Therefore we determined the cytological status of each accession used in this study. The main objectives of this study were to investigate the interspecific relationships within *A.* subg. *Ceterach*, evaluate earlier hypotheses about the phylogeny in this group, look for evidence of reticulate evolution, and determine how many *Ceterach* species occur in Africa.

MATERIALS AND METHODS

Taxon sampling—Apart from MO04926887, collected by A. Reina et al. (MO), vouchers are deposited at GENT (taxa, localities, vouchers, ploidies, genome formulae, and GenBank accession numbers have been archived at the Botanical Society of America website [http://ajbsupp.botany.org/v90/]). Localities of DNA vouchers are shown on the map of Fig. 2. Material for this study has been cultivated in Ghent Botanical Garden (Belgium) from spores collected by C. J. Van den heede and R. L. L. Viane in the wild or from herbarium specimens. From the living *Ceterach*(*opsis*) collection of about 1100 specimens, 73 accessions previously examined in chromosome and isozyme studies were selected for our DNA work (http://ajbsupp.botany.org/v90/). Because plant health influences isozyme results (Stebbins, 1989; Acquaah, 1992; Van den heede et al., 2002), we included only vigorously growing plants in this study.

Cytological preparation—For chromosome counts, immature spore mother cells were fixed in freshly prepared 3 : 1 absolute ethanol : glacial acetic acid and stored frozen until required. Acetocarmine squash preparations were manually made as described by Heitz (1925, 1950) and Manton (1950). Photographs were taken with an Olympus BH2 microscope. Preparations were made permanent by dehydrating the cover slip and slide in graded mixtures of acetic acid and absolute ethanol, followed by mounting in Euparal (Manton, 1950). All permanent preparations are retained in the Pteridological Section of the Department of Biology at Ghent University, Ghent, East Flanders, Belgium. Counted plants were used as standards to compare the nuclear DNA content of additional specimens by a flow cytometer (Partec PA-1), using the manufacturer's protocol (Partec, Münster, Nordrein-Westfalen, Germany).

DNA extraction—Total DNA was extracted from fresh leaf material (0.5–1.0 g) using the modified 2 × cetyltrimethyl ammonium bromide (CTAB) protocol of Doyle and Doyle (1987). The DNA was precipitated in cold (4°C) absolute ethanol for at least 5 d, pelleted, and purified via cesium chloride/

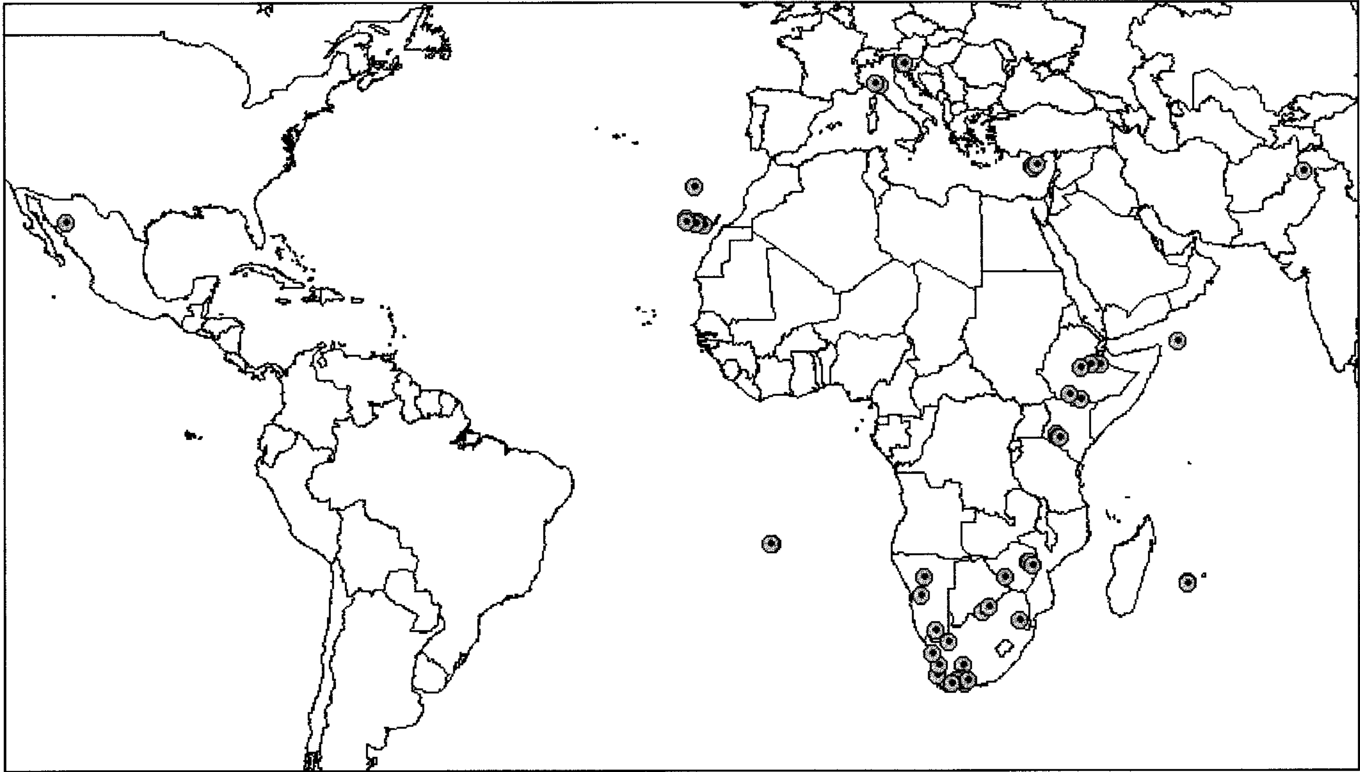

Fig. 2. Localities of vouchers used for DNA sequencing (see http://ajbsupp.botany.org/v90/).

ethidium bromide gradient (1.55 g/mL). Subsequently, CsCl and other residual salts were removed by dialysis. Ethidium bromide was removed with butanol.

Amplification and cloning—The *trnL-trnF* intergenic spacer was amplified using the universal primers e and f of Taberlet et al. (1991). Polymerase chain reaction (PCR) amplification of *rbcL* fragments followed Hasebe et al. (1994) using the same forward and reverse primers. The thermal cycling protocol for plastid DNA consisted of 28–30 cycles, each comprising 1 min denaturation at 94°C, 30 s annealing at 48°C, and 1 min chain extension at 72°C. The final extension was at 72°C for 7 min.

The ITS region (ITS1, the 5.8 gene, and ITS2) was amplified with a primer designed to exclude a frequent bryophyte contaminant (epiphyte) (5′-CCTGCGGAAGGATACTTGTCG-3′). We used the reverse primer, ITS4, described by White et al. (1990). Thermal cycling of ITS involved an initial premelt at 94°C for 2 min followed by 30 cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 2.5 min chain extension at 72°C. The final stage was a 7-min extension at 72°C. Addition of 2% dimethylsulfoxide (DMSO) greatly improved both PCR and cycle sequencing reactions of the guanine and cylosine (GC)-rich ITS region (Varadaraj and Skinner, 1994); in PCR its addition helped to guarantee that the amplified product contained mostly functional copies of ITS, whereas in cycle sequencing DMSO helped to overcome premature terminations caused by GC-rich regions.

The ITS PCR products of samples with multiple sequence signals were cloned using Promega's pGEM-T Easy Vector System (Crawley, West Sussex, UK). Ligations and transformations were prepared according to the technical manual provided by the manufacturer. Transformed *E. coli* cells were spread onto agar plates and incubated at 37°C for 17 h. Between 9 and 12 white colonies per plate (selected at random) were touched with a sterile 1 μL tip and subsequently used as template for reamplification with the same primers. Double-stranded PCR products were purified with CONCERT Rapid PCR Purification System using the manufacturer's protocols (Invitrogen Life Technologies, Paisley, Scotland, UK).

Sequencing and alignment—Cycle sequencing reactions of 10 μL were carried out using the ABI Taq Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, Cheshire, UK) with 1 μL of the dye mixture. The PCR amplification primers were used also as sequencing primers. For *trnL-F/rbcL,* both strands were sequenced on an ABI 377 automated sequencer (according to the manufacturer's protocols). Sequencing was usually straightforward with the exception of *A. phillipsianum* (Kümmerle) Bir et al. specimens due to a GC-rich area near the middle of the *trnL-F* intergenic spacer. All ITS clones from PCR products with heterogeneity were first sequenced with the forward primer. From identical sequences, we chose one clone for sequencing the reverse strand. Sequences were edited and assembled using Sequencher software (Gene Codes Corporation, Ann Arbor, Michigan, USA). All ITS sequence versions were submitted to BLAST search in GenBank to exclude contaminants (e.g., fungi; Soltis and Soltis, 1998), which resulted in complete nrITS sequences for 42 taxa. Putative allopolyploids showed two sequence types. Assembled sequences were manually aligned prior to analysis. No gaps were needed to align any of the *rbcL* and 5.8S sequences. Because ITS sequences for other ferns were not available in the GenBank, boundaries of ITS1 and ITS2 were determined by comparison with sequences of Asteraceae (Baldwin, 1992) and of *Nyssa ogeche* (Nyssaceae; Fan and Xiang, 2001). Complete sequences for the *trnL-F* intergenic spacer were obtained for 72 samples. For *rbcL,* we randomly selected a single accession from each species and produced 11 new *rbcL* sequences (http://ajbsupp.botany.org/v90/).

Data analysis—Based on *rbcL* evidence (Hasebe et al., 1995; Pryer et al., 1995), *trnL-F* sequences of the following leptosporangiate ferns were used as outgroups: a putatively more primitive species of *Dennstaedtia* (R. Cranfill, University of California, Berkeley, unpublished data); two *Polypodium* species (AF159183–159184) published by Haufler et al. (2000); and *A. nidus* L. (AF425118), *A. scolopendrium* L., and *A. unilaterale* Lam. sequences provided by R. Cranfill (unpublished data). For *rbcL,* sequences of 27 *Asplenium* species taken from GenBank (http://ajbsupp.botany.org/v90/) were included.

Dennstaedtia samoensis (Brack.) T. Moore (U18637) and *Microlepia strigosa* (Thunb.) C. Presl (U05931) from the more distantly related Dennstaedtiaceae were designated as the outgroup for the *rbcL* analysis.

Because *A. dalhousiae* came out as a member of the ingroup in our *trnL-F/rbcL* trees and due to the fact that no ITS sequences of other ferns were available in GenBank, we used midpoint rooting for the ITS tree. This procedure gave a similar pattern of relationships as the plastid trees, for which we could use outgroups. The matrices were analyzed with PAUP 4.0b10 (Swofford, 2002), using a heuristic search and maximum parsimony methods under the Fitch criterion (unordered and equally weighted characters; Fitch, 1971). Shortest trees were obtained with 100 replicates of random taxon addition and tree bisection-reconnection (TBR) branch swapping. MulTrees was on (saving multiple, equally parsimonious trees), and no tree limit was used. All other settings were the standard defaults. Gaps (hypothesized insertions/deletions [indels]) were treated as missing values and not separately scored; thus gap information was not included in the analyses (bootstrap percentages were already high without including them). Internal support was assessed using 1000 bootstrap replicates, saving five trees per replicate to reduce time spent in swapping large numbers of trees, and TBR swapping. Percentages of less than 50% are not reported here; 85–100% is considered as strong support. The trees of Figs. 3 and 5 are shown with branch lengths proportional to the number of estimated substitutions (ACCTRAN optimization). The sequences are deposited in GenBank (http://ajbsupp.botany.org/v90/). Alternative topologies among the equally most parsimonious trees identified affect only groupings of accessions within species, and such patterns are not issues in this study.

RESULTS

Cytology—Chromosomes were counted for 25 of the 73 accessions used in this study. These samples were subsequently used as standards to determine by flow cytometry the ploidy of the remaining 48 specimens (http://ajbsupp.botany.org/v90/).

Analysis of trnL-trnF intergenic spacer data—The length of the *trnL-trnF* intergenic spacer varied from 338 base pairs (bp) in *A. cordatum* (CV814a) to 404 bp in *A. dalhousiae* (TR7634). The aligned matrix was 525 bp long with the outgroups and 450 bp without. The matrix contained 238 variable sites of which 148 were potentially parsimony informative, whereas the *Ceterach* matrix (without the outgroups) included only 76 potentially parsimony informative characters (80 variable positions in all). Thirteen gaps varying from 1 to 20 bp in length were introduced to align the Eurasian-Macaronesian *Ceterach* group with the African ceterach group. All accessions of *Asplenium dalhousiae* differ from those of the other taxa in the possession of a unique duplication of 19 bp. Furthermore, *A. punjabense* (TR7675) and *A. ceterach* from Italy (CV494) share another unique duplication of 17 bp. Sequence alignment of the complete matrix including the outgroups required 10 additional gaps of 1–20 bp.

Analysis resulted in 100 most parsimonious trees of 327 steps with a consistency index (CI) = 0.88 and a retention index (RI) = 0.97. One of the shortest trees (selected at random) is presented in Fig. 3 with the branch lengths (i.e., the numbers of substitutions; ACCTRAN optimization) above and the bootstrap percentages below each branch (groups that received less than 50% are not indicated). There is little variation among the trees due to the fact that PAUP calculates the number of trees based on arbitrary dichotomies of accessions that have no differences in their sequences. A strict consensus tree of all equally most parsimonious trees is consistent with the interspecific relationships of the ingroup species shown in this single tree. In all shortest trees, *A.* subg. *Ceterach* is polyphyletic. Within Aspleniaceae, *A. unilaterale* is sister to all other *Asplenium* species (bootstrap percentage [BP] 100), which in turn are composed of two well-supported subgroups. The first group (BP 83) corresponds to *A. nidus*, *A. scolopendrium*, and the Eurasian *Ceterach* group including *A. dalhousiae*; the second group (BP 100) consists of the strictly African ceterach species as currently recognized by C. J. Van den heede and R. L. L. Viane. Within the first subclade, the relationships of *A. nidus*, *A. scolopendrium*, and the Eurasian *Ceterach* group are unresolved in the strict consensus tree (see arrow; Fig. 3).

The African ceterach clade is divided into an *A. cordatum* group (BP 90) and an *A. phillipsianum-haughtonii* group (BP 93). The first group (BP 90) clusters all *A. cordatum* samples ranging from diploid (2*x*) to hexaploid (6*x*). The second group (BP 87) bifurcates into the *A. phillipsianum* (2*x*–6*x*; BP 94) and *A. haughtonii* (4*x*–6*x*; BP 99) clades. Intermediate forms (labeled as *A.* sp.) between *A. cordatum* and *A. phillipsianum* are found in either the *A. cordatum* or *A. phillipsianum* clade.

The well-supported (BP 97) non-African *Ceterach* clade includes two strongly supported subclades. The first (BP 100) consists of *A. punjabense* and all specimens belonging to the *A. ceterach* complex from the Mediterranean (Cyprus, Italy, Slovenia) and Tenerife. All species (2*x,* 4*x,* 6*x*) and hybrids (3*x* and 6*x*) have identical *trnL-F* sequences except for the morphologically identical tetraploid CV494 (Italy), which differs by three substitutions. In the second subclade (BP 100), *A. dalhousiae* is sister to the *A. aureum* clade. The *A. dalhousiae* clade forms a well-supported group (BP 100) in which the Ethiopian and Pakistani specimens differ from the Mexican specimen by a single substitution. The *aureum* clade is poorly supported (BP 64) and groups three Macaronesian species, *A. aureum*, *A. lolegnamense*, and *A. octoploideum*.

Analysis of rbcL data—To evaluate the putative polyphyly of *A.* subg. *Ceterach* shown by the *trnL-F* results, we assembled an *rbcL* matrix (sequences of 11 ''*ceterach*'' taxa and 27 other *Asplenium* species) using *Dennstaedtia* and *Microlepia* as outgroups. This *rbcL* matrix had 1301 characters (no indels present), of which 391 were variable and 260 potentially parsimony informative. A heuristic search performed under the same conditions as for the *trnL-F* analysis resulted in four most parsimonious trees of 788 steps with CI = 0.60 and RI = 0.75. One of the shortest trees (selected at random), Fig. 4, is identical to that produced with just the *trnL-F* data.

Within Aspleniaceae, the *Hymenasplenium* group (Mitui et al., 1989; Kramer and Viane, 1990; Murakami and Moran, 1993; Murakami, 1995; Cheng and Murakami, 1998) is sister to the other *Asplenium* species (BP 99), which agrees with the results of Murakami (1995). Due to limited taxon sampling and low sequence divergence, several BP are low, and relationships of *A. marinum*, *A. hemionitis*, and the strictly African ceterach group are unresolved in the strict consensus tree. However, the polyphyly of *A.* subg. *Ceterach* is clearly supported by the *rbcL* data. Increased taxon sampling is needed to identify the sister group of the well-supported clade (BP 100) of strictly African ceterach taxa. Sister to the Eurasian *Ceterach* group (BP 99) is the strongly supported clade (BP 100) of Mediterranean *A. sagittatum* (DC.) Bange and Holarctic *A. scolopendrium*. Again, the non-African *Ceterach* group includes two fully supported subclades (BP 100). In the first, *A. dalhousiae* is sister to the strongly supported (BP 95) *A. aureum* clade clustering the three Macaronesian species. The

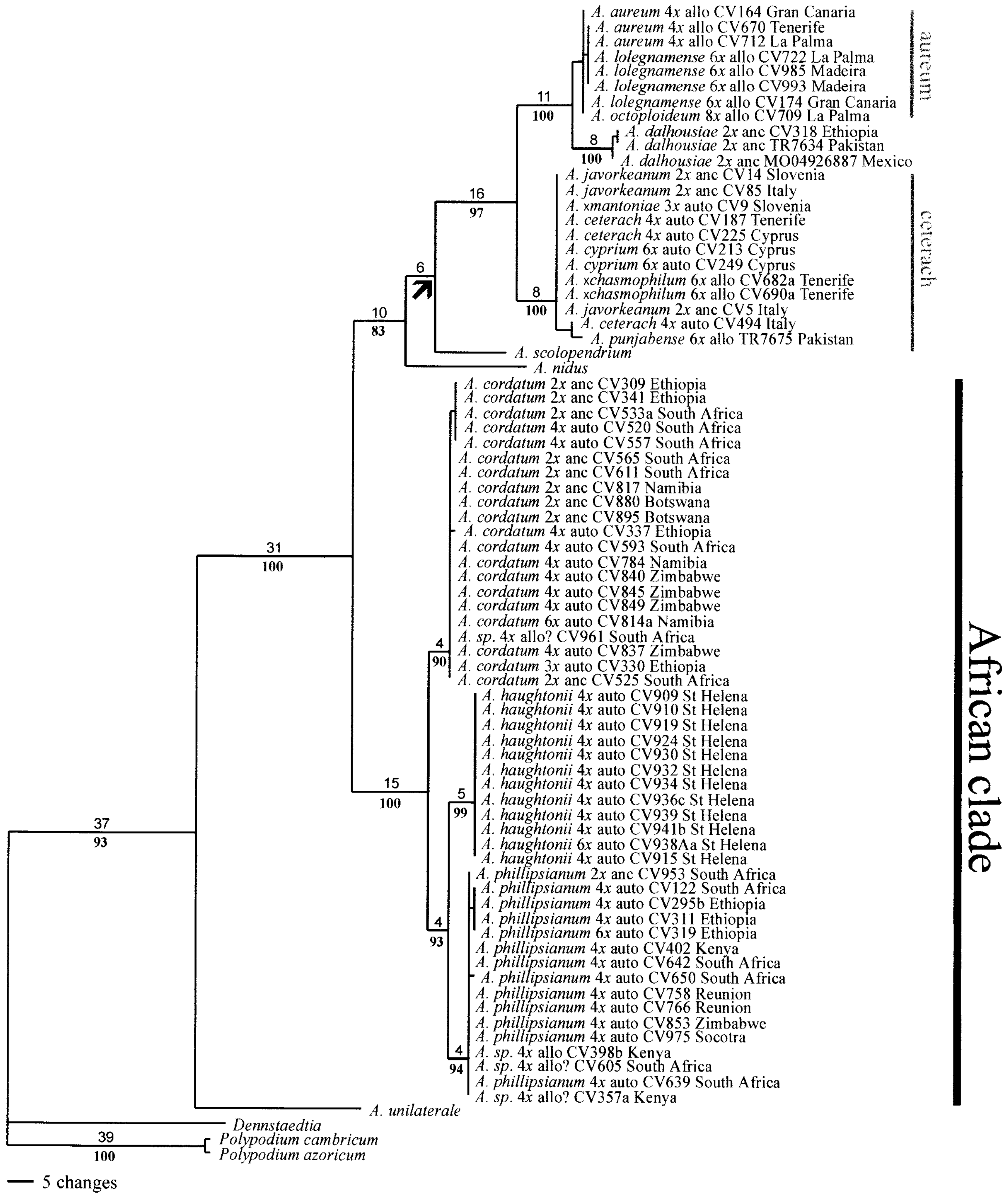

Fig. 3. One randomly selected tree of the 100 shortest trees of *Asplenium* subg. *Ceterach* and *A. dalhousiae* resulting from parsimony analysis of sequences of the *trnL-F* intergenic spacer; length = 446 steps, CI = 0.88, and RI = 0.97. Numbers above the branches are lengths (ACCTRAN optimization); those below the branches indicate bootstrap percentages greater than 50% (1000 replicates). *Dennstaedtia* was specified as outgroup. The arrow indicates the single node that collapses in the strict consensus of all shortest trees. Next to ploidy (2*x*, 3*x*, etc.), putative ancestral (anc), autoploid (auto), or alloploid (allo) status is indicated. The aureum, ceterach, and African clades are indicated by a vertical bar.

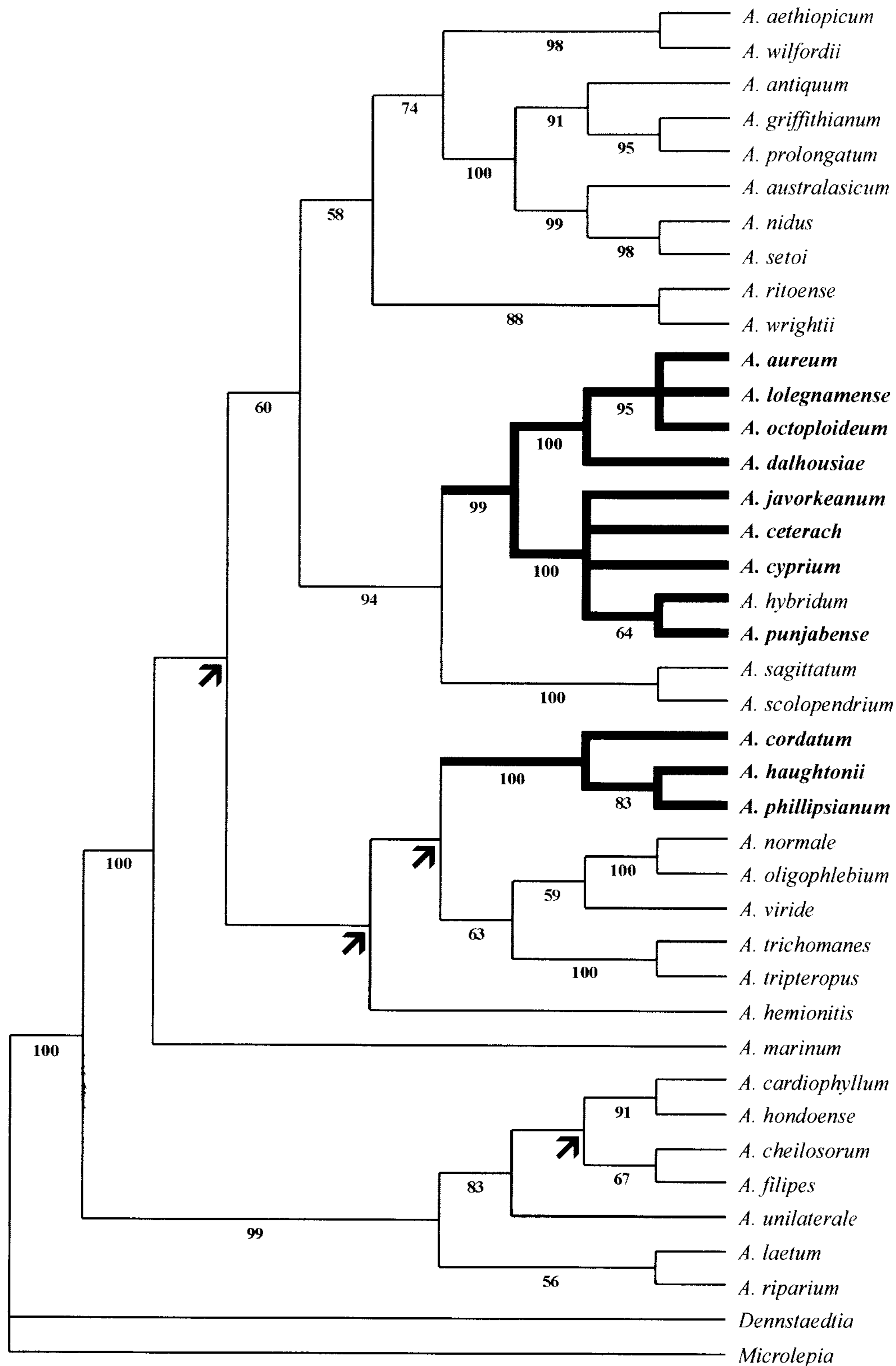

Fig. 4. One of four most parsimonious trees for Aspleniaceae based on our 11 *rbcL* sequences of taxa of *Asplenium* subg. *Ceterach* (bold) and 27 *rbcL* sequences of other *Asplenium* species presently available in GenBank (http://ajbsupp.botany.org/v90/); length = 788 steps, CI = 0.60, and RI = 0.75. *Dennstaedtia* and *Microlepia* were specified as outgroups. Numbers are bootstrap percentages greater than 50% (1000 replicates). Arrows indicate nodes that collapse in the strict consensus of all shortest trees. The polyphyly of *A.* subg. *Ceterach* (branches with thick lines) is confirmed by *rbcL* data.

second subclade groups all taxa of the *A. ceterach* complex, *A. hybridum* (Milde) Bange, and *A. punjabense*. *Asplenium hybridum*, endemic to the Dalmatian Islands (Croatia), is a fertile allotetraploid that originated by chromosome doubling in a diploid hybrid between diploid *A. javorkeanum* and diploid *A. sagittatum* (Vida, 1963; Reichstein, 1981). The position of *A. hybridum* indicates that *A. javorkeanum* served as the maternal parent in this event.

Analysis of ITS data—Internal transcribed spacer 1 ranged in length from 237 to 279 bp; ITS2 varied from 191 to 225 bp. The shortest ITS1 and ITS2 regions were found in *A. dalhousiae*. The GC content in ITS1 ranged from 47.7% in *A. haughtonii* (CV915) to 61.0% in *A. aureum* (Cav.) Buch (CV712) and for ITS2 from 50.5% in *A. haughtonii* (CV924) to 68.1% in *A. javorkeanum* (CV14). With its 164 bp, the 5.8S subunit was uniform in size although slightly variable in GC content (53.0–57.3%). Internal transcribed spacer 2 includes more (127) potentially parsimony informative characters than ITS1 (110). The ten variable positions of the 5.8S rDNA gene are all potentially parsimony-informative. In addition, the flanking portion of the 26S rDNA gene (48 bp) contains six informative sites, whereas the flanking part (only 2 bp) of the 18S gene is not informative. Sequence alignment required 23 and 15 gaps (varying from 1 to 40 bp in length) in ITS1 and ITS2, respectively. Indels were absent in the 5.8S rRNA gene.

The ITS1 + 5.8 S + ITS2 region had an aligned length of 878 bp. However, the total length of the region analyzed, including the short regions of 18S and 26S, consisted of 927 sites. Analysis resulted in 96 most parsimonious trees of 335 steps with CI = 0.85 and RI = 0.98. One of the shortest, midpoint-rooted trees (selected at random) is shown in Fig. 5 with branch lengths (ACCTRAN optimization) indicated above the branches and bootstrap percentages below. If ITS clones from a single plant were positioned in two different clades, we mark them as (copy type) I and II. Although PAUP calculated that there were 96 equally parsimonious shortest trees, the strict consensus tree is identical to the single tree illustrated.

Midpoint rooting of the ITS tree again divides subgenus *Ceterach* in two main groups: the Eurasian-Macaronesian species plus *A. dalhousiae* and the strictly African taxa. The former, maximally supported (BP 100) clade consists of the Eurasian and Macaronesian *Ceterach* species and bifurcates into two strongly supported subclades: the *aureum*-genome clade (BP 100) and the *ceterach*-genome clade (BP 90). The *aureum* clade consists of the ITS copy type I of both *A. aureum* (CV712) and (CV174). The *ceterach* clade splits into two highly supported groups: one (BP 89) with *A.* ×*mantoniae* and two diploid *A. javorkeanum* specimens from Italy and Slovenia and the other (BP 100) with tetraploid *A. ceterach* from Tenerife (CV187), copy type II of *A. punjabense* (TR7634), *A. aureum* (CV712), and *A. lolegnamense* (CV174).

The *A. dalhousiae* clade (BP 100) is most closely related to the Eurasian-Macaronesian clade (this is of course dependent on the topology created by midpoint rooting) and includes sequences from three taxa. Copy type I of the Pakistani allohexaploid *A. punjabense* (TR7675) has an identical sequence to that of the diploid *A. dalhousiae* (TR7634) from Pakistan, whereas the third sequence of the Mexican plant differs by two substitutions. Copy type II of the allohexaploid *A. punjabense* (TR7675) clusters with the *ceterach* clade and differs by only three substitutions from *A. ceterach* (autotetraploid) from Tenerife.

The African group is divided in two subgroups (each with BP 100). One subgroup corresponds to *A. haughtonii* in which two clusters are present, characterized by variation of up to 24 substitutions. The second subgroup bifurcates into two taxa: *A. cordatum* (BP 100) and *A. phillipsianum* (BP 81). The intermediate forms (*A.* sp.) turn up either in one of the sister taxa (*A. cordatum* or *A. phillipsianum*) or in both species (CV398b). The *cordatum* clade is homogenous; there are no cytological or geographical differences between the 15 specimens from different regions in eastern and southern Africa. The *phillipsianum* clade shows more variation, with a subcluster (BP 100) grouping the specimens from Ethiopia and Socotra.

DISCUSSION

Cytological results—Although new ploidies within several taxa are described elsewhere (e.g., Van den heede and Viane, 2002; Van den heede et al., 2002), we want to stress our cytological proof of the occurrence of tetraploid *A. ceterach* on Gran Canaria and Tenerife (C. J. Van den heede et al., unpublished data). Our study of micromorphology, cytology, and isozymes has shown that besides *A. aureum* and *A. octoploideum*, *A. lolegnamense* and *A. ceterach* occur in the Canarian Archipelago (Van den heede and Viane, 2002; C. J. Van den heede and R. L. L. Viane, unpublished data), and this has important implications for the assessment of the ancestry of tetraploid *A. aureum*.

Patterns for trnL-F/rbcL in Asplenium subgenus Ceterach—Intraspecific variation in *trnL-F* DNA was low and limited to single substitutions within some accessions in most species. Because double signals were never found in our *trnL-F* intergenic spacer and *rbcL* sequences, plastid inheritance must be at least uniparental (Soltis et al., 1992; Sewell et al., 1993). Maternal plastid inheritance has generally been recorded in ferns (Stein and Barrington, 1990; Gastony and Yatskievych, 1992; Vogol et al., 1998; Van den heede et al., 2002). Consequently, reticulate patterns of evolution cannot be detected. Reticulate evolution in *Asplenium* was documented in detail by e.g., Wagner (1954), Lovis (1977), and Reichstein (1981, 1984).

Internal transcribed spacer in Asplenium subgenus Ceterach—The ITS characteristics of *Asplenium* fall within the range of variation noted for other land plants, but the length of each of the spacers in these taxa was shorter than those reported by Gastony and Rollo (1998). The total length of the ITS regions (including the 5.8S gene) varies from 600 to 700 bp, which agrees with the range found across the angiosperms (Soltis and Soltis, 1998). Similarly, the GC content does not exceed the variation (45–75%) found within the angiosperms (Hershkovitz et al., 1999).

Although generally rare, intraspecific nrITS variability was reported by Sytsma and Schaal (1990), who detected differences of up to 100 bp among specimens of populations of *Lisianthus skinneri* (Gentianaceae). According to Smith (1976) large indels may be due to unequal crossing over (believed to be one of the driving forces of concerted evolution) of rDNA repeat units. Baldwin (1993) found intraspecific ITS sequence divergence in populations of *Calycadenia* (Asteraceae). Sim-

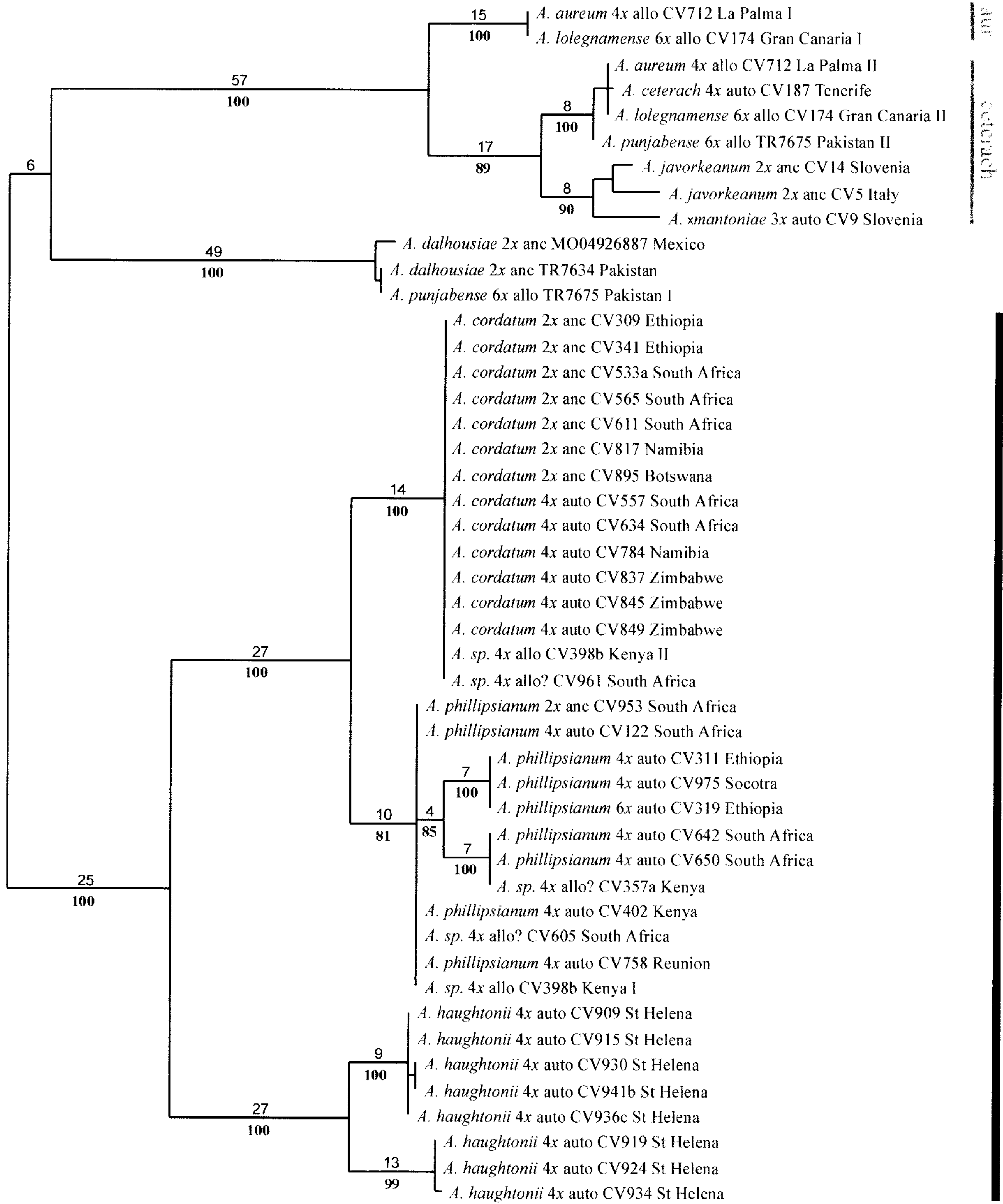

Fig. 5. One randomly selected of the 96 most parsimonious, midpoint rooted trees of *Asplenium* subg. *Ceterach* and *A. dalhousiae* based on analysis of nrITS sequence data; length = 335 steps, CI = 0.85, and RI = 0.98. Fitch branch lengths (ACCTRAN optimization) are shown above and bootstrap percentages greater than 50% (1000 replicates) below the branches. The tree shown is identical to the strict consensus tree. Next to ploidy (2*x*, 3*x*, etc.), putative ancestral (anc), autoploid (auto), or alloploid (allo) status is indicated. The aureum, ceterach, and African clades are indicated by a vertical bar.

ilarly, we found intraspecific variation (indels) in the two nuclear ribosomal spacers, ITS1 and ITS2, but not in the conserved 5.8S gene. Limited interspecific, substitutional variation in the 5.8S gene was observed among the three African species but not among the Eurasian-Macaronesian species.

Because gene conversion has not eliminated one allele of the biparentally inherited nuclear ribosomal ITS regions of the allopolyploids, their reticulate origins can be demonstrated. Autopolyploids show a single copy type, whereas allopolyploids are characterized by two, and according to A. Coleman (Brown University, personal communication) these are all functional ITS copies, which is also indicated by the uniform length and low numbers of substitutions observed in the 5.8S gene.

Gastony and Rollo (1998) did not report heterogeneity among ITS copies produced from a single accession. They do not give cytological data but most taxa used in their ITS analysis were diploids or autopolyploids, and gene conversion may have eliminated all but a single ITS allele in any allopolyploids. However, our results are concordant with those of Hoot and Taylor (2001), showing different copy types in allopolyploids and interspecific hybrids in *Isoetes*.

Polyphyly of Asplenium subgenus Ceterach—Only Moore (1857) excluded the strictly African species from *A.* subgenus *Ceterach* because of their free venation pattern, which contrasts with the anastomosing veins in the Eurasian-Macaronesian complex. Among "modern" pteridologists (e.g., Copeland, 1947; Pichi Sermolli, 1979), it is generally accepted that *A.* subg. *Ceterach* is natural (monophyletic), although this was never evaluated in a phylogenetic context. However, in our plastid analyses, all trees using *Dennstaedtia* as outgroup show that *A.* subg. *Ceterach* is polyphyletic. Moreover, in both our *trnL-F* and ITS matrices, species within each main group were easily alignable (i.e., required few gaps), but the Eurasian-Macaronesian group was more time-consuming to align with the African group and necessitated many more gaps, some of which were ambiguous in position.

Most comparative studies of *rbcL* and *trnL-F* data have produced highly similar but not always identical trees (e.g., Chase et al., 2000; Lledo et al., 2000; Richardson et al., 2000). If we accept the *rbcL* and *trnL-F* trees as an accurate reflection of phylogenetic patterns, then the following characters, present in most (99%) *Asplenium* species, are likely to be plesiomorphic: a pinnate lamina, subglabrous fronds, free venation, and an indusium. Because the two major groups of *Ceterach* taxa are not each other's closest relatives, two synapomorphies must have originated in parallel: (1) a pinnatisect lamina and (2) a dense scale cover. Adaptation to xeric habitats most likely resulted in the multiple origin of a dense scale cover. The presence of these extra scales made a protective indusium redundant; this consequently became reduced (in the European-Macaronesian species) or totally disappeared (in the African taxa). There is, however, no explanation why the typical pinnatisect lamina (Fig. 6) should have been evolved twice within the family.

The topology of the ITS tree is more in agreement with previous morphological interpretations than the plastid tree, and placement of *A. dalhousiae* as sole sister species to *A. aureum* in the plastid trees seems unreasonable to us on morphological grounds. It would imply the secondary loss in *A. dalhousiae* of four synapomorphies shared by *A. ceterach* and *A. aureum*: anastomosing veins, a reduced indusium, dense scale cover, and identical ceterachoid scales. It is more plausible that there was an ancient transfer of plastid DNA from an unknown diploid, implicated in the origin of *A. aureum*, to *A. dalhousiae*. This unknown progenitor of *A. aureum* is hereafter provisionally called "*A. semi-aureum*" (Fig. 6).

All molecular data collected to date reveal the polyphyly of *Asplenium* subgenus *Ceterach*, implicating homoplasy in the lamina shape and the dense scale cover, characters previously used to circumscribe this "taxon." The taxonomic consequences are that either *A.* subg. *Ceterach* should be limited to the Eurasian and Macaronesian species (excluding *A. dalhousiae*) and a new subgenus be created to accommodate the African taxa or that distinction at subgeneric level is abandoned altogether.

Comparison of trnL-F/rbcL and ITS phylogenetic trees—Because the ITS trees could not be rooted with an outgroup, they are not directly comparable to the rooted plastid trees. However, the plastid trees are congruent with the ITS tree in five fundamental ways: (1) recognition of six identical groups, i.e., *A. dalhousiae*, *A. ceterach*, "*A. semi-aureum*," *A. cordatum*, *A. phillipsianum*, and *A. haughtonii*; (2) division of the former subgenus *Ceterach* into two distinct groups, a Eurasian-Macaronesian and a strictly African alliance; (3) the position of *A. dalhousiae* in the ingroup; (4) a lack of molecular divergence in *A. cordatum* despite its morphological variability; and (5) clustering of autopolyploids with their diploid progenitor in the *A. ceterach* complex, in *A. cordatum*, and in *A. phillipsianum*.

The plastid and ITS trees differ in their placements of *A. haughtonii* and *A. dalhousiae*. These are "hard incongruences" (Seelanan et al., 1997, p. 286), and presumably such incongruence is due to different evolutionary histories of these regions, which evolved at their own rates (Page and Holmes, 1998; Wendel and Doyle, 1998). Various phenomena may lead to discordant patterns and include processes that operate at the organismal level as well as at the molecular level (reviewed in Wendel and Doyle, 1998).

The cause of the incongruence between taxa previously included in *A.* subgenus *Ceterach* remains unclear. However, it is possible that ancient hybridization including plastid DNA capture unaccompanied by nrDNA introgression might have produced this conflict (Rieseberg and Brunsfeld, 1992; Wendel and Doyle, 1998). This cytoplasmic "introgression" involves ancient hybridization among lineages that presently have separate ranges and/or are even incompatible. Evidence for the existence of this phenomenon is seen in *Gossypium gossypioides* (Wendel et al., 1995) and *Allium* section *Cepa* (van Raamsdonk et al., 1997). The occurrence of plastid capture is generally seen as a disadvantage for the use of plastid DNA to study phylogenetic relationships at lower taxonomic levels (Soltis and Soltis, 1998), but it also provides additional valuable evidence if hybridization is known to have occurred on the basis of other information.

Further results are discussed by clade or taxon.

Asplenium aureum clade—The *aureum* clade contains three Macaronesian species, *A. aureum*, *A. lolegnamense*, and *A. octoploideum*, sharing identical nuclear ("*semi-aureum*") and plastid sequences. Tetraploid *A. aureum*, endemic to the Canary Islands, was suggested previously (not fully confirmed) to be allotetraploid (Viane and Reichstein, 1992). From the ITS pattern, we know that one of its diploid pro-

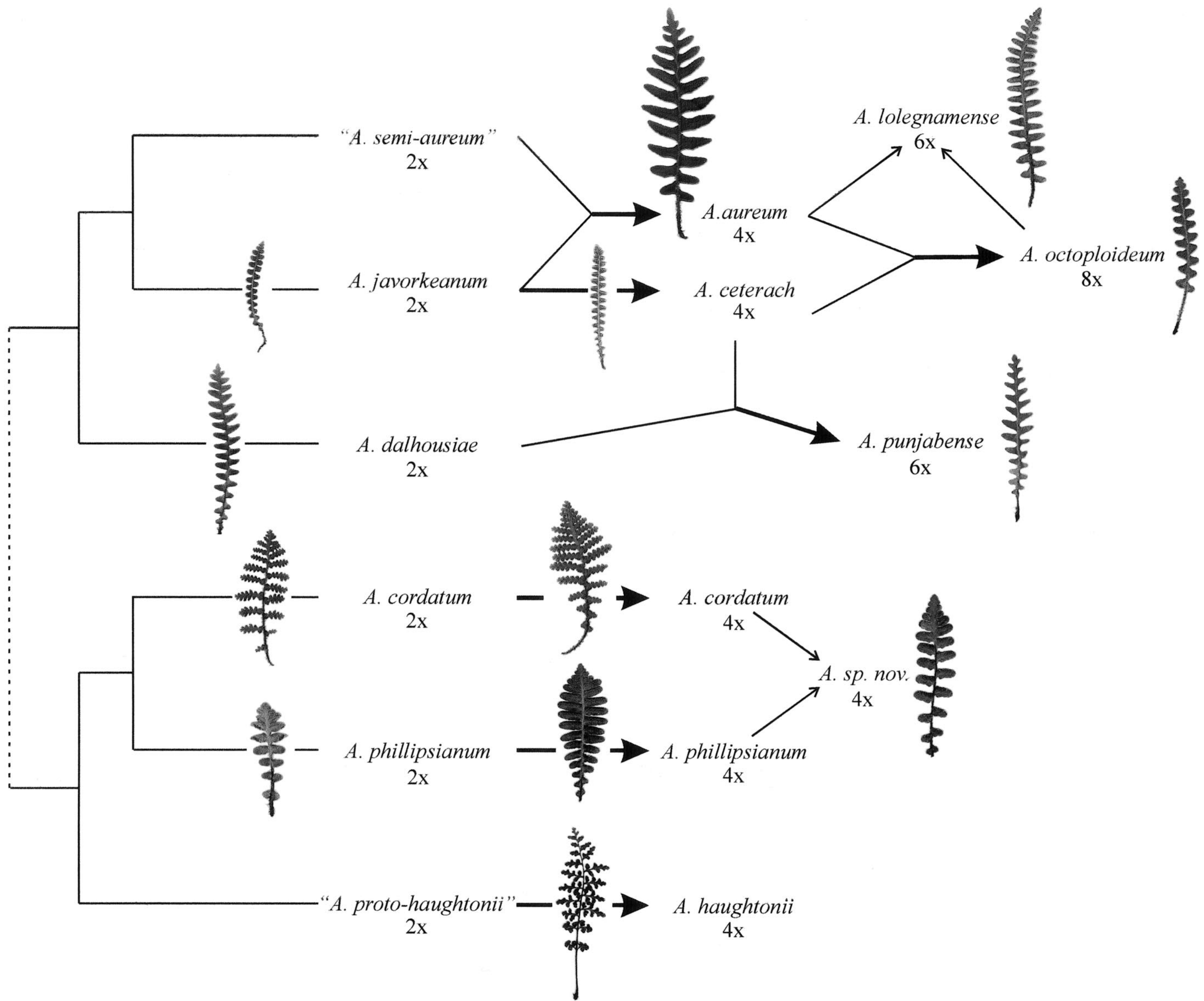

Fig. 6. Concept of relationships based on nuclear ITS data of the *Asplenium* taxa studied. Putative ancestral diploids, "*A. semi-aureum*" and "*A. proto-haughtonii*," may be extinct (they are at least undiscovered) and gave rise to the extant taxa via polyploidization and/or hybridization followed by chromosome doubling (sterile hybrids not shown). Thick lines and arrows indicate chromosome doubling.

genitors was *A. javorkeanum*: one of the ITS copies of *A. aureum* retrieved by cloning is nearly identical to the ITS copy of the autotetraploid *A. ceterach* from Tenerife. However, we still lack a diploid that carries the other ITS allele found in *A. aureum*. For *trnL-F/rbcL, A. aureum* has a sequence type that is similar to that of *A. dalhousiae* but not identical. We thus hypothesize the existence of a now extinct or at least unknown diploid ("*A. semi-aureum*"; Fig. 6) that donated its ITS allele and plastid genome to *A. aureum*. Our results from plastid *trnL-F/rbcL* also support the hypothesis that *A. aureum* is an ancestor of both allohexaploid *A. lolegnamense* (Gibby and Lovis, 1989) and allo-octoploid *A. octoploideum* (Manton et al., 1986). *Asplenium lolegnamense* produced two ITS alleles, one from "*A. semi-aureum*" and another nearly identical to *A. ceterach* from Tenerife.

These results not only document reticulate evolution within subgenus *Ceterach* sensu stricto, but also support the allopolyploid status of *A. aureum* and *A. lolegnamense* (Fig. 5). This confirms our interpretation of morphological, cytological, and isozyme data after the discovery of true *A. ceterach* on the Canary Islands (Van den heede and Viane, 2002).

Asplenium dalhousiae clade—In addition to the large population in southwestern Asia, diploid *A. dalhousiae* is also known from a few localities in Ethiopia, northwestern Mexico, and southwestern USA (Arizona). This disjunct distribution, which is similar to that of *Asplenium exiguum* Bedd. and once led Clute (1911) to hypothesize a multiple origin for this species, is now best explained by long-distance dispersal (Moran and Smith, 2001; Parris, 2001). According to our analyses of the *trnL-F/rbcL* data, the *A. dalhousiae* clade is sister to the *A. aureum* clade. This topology is unexpected because it was

generally accepted among pteridologists (e.g., Copeland, 1947; Pichi Sermolli, 1979) that the members of *A.* subg. *Ceterach* originated from an ancestor close to *A. dalhousiae*. The *trnL-F/rbcL* results show that *A. ceterach*, *A. dalhousiae*, and *A. aureum* form a clade with *A. dalhousiae* sister to "*A. semi-aureum*," the unknown diploid ancestor of *A. aureum*, *A. lolegnamense*, and *A. octoploideum*. Such a phylogeny would imply the secondary loss (reversal) of four apomorphic morphological ceterachoid characters in *A. dalhousiae*: (1) anastomosing venation, (2) indusium, (3) the dense scale cover, and (4) scale type. These synapomorphies are shared by *A. ceterach* and *A. aureum*. Given that the typical ceterach condition prevails in the allotetraploid *A. aureum*, we assume that the diploid "*A. semi-aureum*" that gave rise to it had the typical ceterachoid characters, but it most likely had much wider fronds than in either *A. ceterach* or *A. dalhousiae* (Fig. 6). In contrast, according to the ITS results, "*A. semi-aureum*" is sister to *A. ceterach*, which has at least two divergent alleles, and this group is then a distant sister to *A. dalhousiae*. This relationship makes more sense morphologically because it does not involve the four reversals mentioned above. The topological and divergence inconsistencies in the *trnL-F/rbcL* and ITS trees indicate that the evolutionary histories of the nuclear and plastid genomes in this clade are different: putative ancient hybridization and plastid capture, in which the unknown diploid progenitor of *A. aureum* hybridized with *A. dalhousiae*, may have left the latter with plastid sequences similar to those of "*A. semi-aureum*." Additional nuclear markers should be evaluated to determine if this incongruence relates to more of the nuclear genome than ITS; it could also be that the ITS copy obtained from *A. dalhousiae* is a paralogue to that retrieved from the *A. ceterach* group.

***Asplenium ceterach* clade**—*Asplenium ceterach* is an autotetraploid that originated via chromosome doubling in the morphologically identical diploid *A. javorkeanum* (see introduction). *Asplenium ceterach* occurs mainly in Europe, southwestern Asia to the Himalayas, and more rarely in northern Africa. The allohexaploid *A. punjabense* originated by chromosome doubling of a triploid hybrid between diploid *A. dalhousiae* and tetraploid *A. ceterach* and is endemic to the Punjab area in India and Pakistan (Bir et al., 1985). Such an origin is congruent with the two ITS alleles obtained from cloning (Fig. 5). The *trnL-F* sequence of *A. punjabense* (TR7675) is similar to that of a tetraploid *A. ceterach* specimen (CV494) from Italy. We were unable to investigate any *A. ceterach* from the Punjab where *A. punjabense* is endemic, so the match might be better with a specimen from there. For *trnL-F,* the "*A. ceterach* clade" includes all investigated taxa of the *A. ceterach* complex (2*x,* 3*x,* 4*x,* 6*x*), *A.* ×*chasmophilum*, and *A. punjabense*. For both the hexaploid hybrids (CV682a and CV 690a) between tetraploid *A. ceterach* and octoploid *A. octoploideum* from Tenerife (Van den heede and Viane, 2002) and *A. punjabense*, autotetraploid *A. ceterach* is probably the maternal parent. We found almost no intraspecific *trnL-F* variation in *A. ceterach*, whereas ITS variation was much higher.

Asplenium cordatum—Lovis (in Bir et al., 1985) found *A. cordatum* to be tetraploid but did not mention either auto- or allopolyploidy. The species is relatively common in rocky habitats in the drier parts of eastern and southern Africa. Both *trnL-F* and ITS data cluster all *A. cordatum* samples from all 18 localities in Africa and one intermediate plant (CV961) in which *A. cordatum* putatively served as maternal parent. Although this species shows considerable (micro)morphological (scale structure and leaf silhouette) variability, intraspecific *trnL-F* intergenic spacer variation is limited to 1–3 substitutions, whereas no intraspecific ITS divergence was detected, and neither cytological, nor geographical subdivisions are present. Most *trnL-F* variation (three substitutions; Fig. 3) is found among specimens from Ethiopia. The absence of length and sequence variation in the ITS region of the tetraploid specimens compared to that of their diploid progenitors supports the hypothesis of an autopolyploid origin.

Asplenium phillipsianum—Bir et al. (1985) reported diploid and tetraploid specimens within this taxon, although its exact polyploid status was not investigated. In East and South Africa, *A. phillipsianum* is less common than *A. cordatum*. However, it apparently is the only species occurring on the Indian Ocean islands of Socotra, Madagascar, and Réunion. The *trnL-F* clade includes 13 *A. phillipsianum* specimens and three plants intermediate in morphology between *A. cordatum* and *A. phillipsianum* (CV357a, CV398b, CV605), in which it is likely that *A. phillipsianum* was the female parent. In the ITS tree, *A. phillipsianum* shows some geographic variation with a subcluster grouping specimens from Ethiopia and Socotra. The Ethiopian specimens are the most divergent for both *trnL-F* and ITS data.

African intermediates—In the ITS tree, several morphologically intermediate specimens have ITS copies that turn up in *A. cordatum* (CV961), *A. phillipsianum* (CV357a, CV605), or in both species clusters (CV398b). Based on the ITS and chromosome results, the Kenyan tetraploid specimen (CV398b) must be allotetraploid and probably originated via hybridization between *A. phillipsianum* and *A. cordatum*. It thus can be considered to represent a new taxon on the African continent. This allotetraploid may have originated either by chromosome doubling in the sterile diploid hybrid between diploids of *A. phillipsianum* and *A. cordatum* (conventional alloploidy) or by direct hybridization between the autotetraploids (delayed alloploidy or allohomoploidy). Lovis (1977) introduced the term "delayed allotetraploidy" to denote the phenomenon whereby a fertile allotetraploid hybrid originates from a cross between two different autotetraploid species. At least three European taxa have originated this way (Reichstein, 1984). More recently, Haufler (1997) used the term "allohomoploidy" to define hybrid speciation that does not involve chromosome doubling.

Within the African *A. cordatum/phillipsianum* complex both conventional and delayed allotetraploidy could have taken place because the diploid as well as tetraploid parents are still extant. However, because diploid *A. phillipsianum* is rare in (South) Africa whereas the tetraploid cytotypes of both *A. cordatum* and *A. phillipsianum* are common in Africa, we consider hybridization by delayed allotetraploidy the most probable explanation (Fig. 6). The subtle gross morphological differences between *A. cordatum*, *A. phillipsianum*, and intermediate forms (Fig. 6) are supported by the molecular results. Additional micromorphological studies to distinguish these taxa are in progress.

Asplenium haughtonii—The Saint Helenian endemic, *A. haughtonii*, was reported to be tetraploid by Lovis in Bir et al. (1985). Several authors (Hooker, 1860, 1861; Sim, 1915;

Tardieu-Blot, 1958; Burrows, 1990) considered this plant conspecific with *A. cordatum*, although both species are easy to distinguish morphologically (Fig. 6). The 12 specimens studied have identical *trnL-F* sequences. According to the *trnL-F* and *rbcL* results, plastid DNA of *A. haughtonii* is more similar to that of *A. phillipsianum* than the latter is to that of *A. cordatum*. The ITS results show a tree with *A. haughtonii* sister to the pair *A. phillipsianum*/*A. cordatum*. This pattern is better supported by morphological and isozyme data (C. J. Van den heede, unpublished data), in which *A. haughtonii* comes out as a species distinct from its continental African relatives. In any event, the three African entities form distinct clusters, which should be recognized as distinct species.

General conclusions—Using DNA sequences, we were able to clarify interspecific relationships within *A.* subg. *Ceterach* (Fig. 6), evaluate earlier hypotheses about the phylogenetic patterns in this group, and document reticulate evolution in the production of some taxa. Morphological, cytological, and isozyme studies (C. J. Van den heede and R. L. L. Viane, unpublished data) provided hypotheses about the number of taxa and the ancestry of species within *A.* ''subg. *Ceterach*.'' DNA sequencing has corroborated these hypotheses independently, although we emphasize that cytological knowledge of individual plants was needed to accurately interpret the molecular results.

Our DNA results support the existence of several strictly African taxa. *Asplenium cordatum* and *A. phillipsianum* are distinct species or species complexes consisting of diploid and autopolyploid taxa. A third undescribed allotetraploid species has originated from *A. cordatum* and *A. phillipsianum*. These three taxa can also be recognized on morphological grounds. Furthermore, our results show that *A. haughtonii* from Saint Helena is a distinct endemic species apart from its African relatives, *A. phillipsianum* and *A. cordatum*.

Reticulate evolution could be demonstrated because gene conversion apparently did not eliminate the divergent nrITS alleles in the allopolyploids. Uniparentally inherited plastid DNA only discloses the maternal lineage, whereas in this case biparentally inherited nuclear ribosomal ITS sequences detected the allopolyploid status of *A. aureum*, *A. lolegnamense*, and *A. punjabense* and indicated that *A. ceterach* was one of their progenitors. The *trnL-L* and *rbcL* results support the hypotheses that *A. aureum* is an ancestor of both *A. lolegnamense* and *A. octoploideum*. Independently of our other studies, these DNA analyses demonstrate that *A. ceterach* occurs in the Canary Islands.

This study explored the usefulness of DNA sequence data for resolving interspecific relationships within Aspleniaceae. Because *A.* subg. *Ceterach* in its current circumscription is polyphyletic, increased taxon sampling is needed to reveal the position within the family of these two clades and identify the sister group of the strictly African taxa. Future work will include additional molecular data, both plastid and nuclear, of many more species, in combination with study of cytology, isozymes, and (micro)morphological characters. More effort needs to be focused on the apparent disagreement of the plastid and ITS patterns; in particular additional evidence from the nuclear genome should be sought. Nomenclatural changes indicated by our results will be published elsewhere.

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