Phylogenetic and biosystematic relationships in four highly disjunct polyploid complexes in the subgenera *Ceterach* and *Phyllitis* in *Asplenium* (Aspleniaceae)

Istvan Pinter[1], Freek Bakker[2], John Barrett[3], Cymon Cox[4], Mary Gibby[5], Sally Henderson[6],
Mary Morgan-Richards[6], Fred Rumsey[6], Stephen Russell[6], Steve Trewick[6], Harald Schneider[4,7], Johannes Vogel[6,*]

[1] Department of Genetics, Eötvös Loránd University, Budapest, Hungary
[2] Wageningen University Branch, National Herbarium Netherlands, The Netherlands
[3] Department of Genetics, University of Cambridge, UK
[4] Department of Biology, Duke University, Durham, NC, U.S.A.
[5] Royal Botanic Garden Edinburgh, Scotland
[6] Department of Botany, The Natural History Museum, London, UK
[7] Albrecht-von-Haller Institut für Pflanzenwissenschaften, Universität Göttingen, Germany

Abstract

Phylogenetic studies using DNA sequences of two chloroplast regions, *rbc*L and *trn*L-F, demonstrate that the proposed genus *Ceterach* is a small clade within the large genus *Asplenium*, and sister to the *Phyllitis* clade. The *Ceterach* clade is characterised by irregular anastomosing veins and often densely scaled leaf blades. Its taxonomic status as a group nested within *Asplenium* is confirmed, and it is accepted here as a subgenus with seven species. The *Ceterach* clade comprises four lineages that correspond to disjunct polyploid complexes: the *A. aureum* clade forming a polyploid complex (4×, 6×, 8×) in Macaronesia, the *A. ceterach* clade forming a polyploid complex (2×, 4×, 6×) in the Mediterranean Basin, the *A. paucivenosum* clade (4×, 6×) in central Asia, and the *A. dalhousiae* clade (2×) with a disjunct distribution in the Himalaya, Yemen and Eritrea, and southwestern North America. *Asplenium paucivenosum* is sister to all other members of the *Ceterach* clade, whereas *A. dalhousiae* is sister to the *A. aureum* clade that includes tetraploid *A. aureum*, hexaploid *A. lolegnamense*, and octoploid *A. parvifolium*. *Asplenium ceterach* and its variations – including the hexaploid *A. ceterach* subsp. *mediterraneum* subsp. nov. first described below – form a monophyletic unit, sister to a clade consisting of *A. aureum* and *A. dalhousiae*. *Asplenium cordatum* from Africa and *A. haugthonii* from the isolated atlantic island of St. Helena are not members of the *Ceterach* clade, which suggests that leaf blades with dense indumenta have evolved at least twice within asplenioid ferns. The allotetraploid species *A. hybridum* has the chloroplast DNA from *A. ceterach*, and therefore the latter species is the maternal ancestor of the former. The other parent of this hybrid species is *A. sagittatum* that is nested within the sister clade of *Ceterach*, the *Phyllitis* clade comprising *A. sagittatum* and *A. scolopendrium*. The findings suggest that the current distribution of *Ceterach* is either the result of long-distance dispersal or represents fragmented relics of a previously more widely distributed species.

Key words: biogeography, long-distance dispersal, oceanic islands, radiations, molecular phylogeny, plant taxonomy

Introduction

Asplenium L. is a cosmopolitan genus of some 700 taxa with fairly homogeneous morphology. Several groups have been separated as (sub-)genera within Aspleniaceae but these are for the most part rather small, comprising relatively few taxa. Such "satellite" genera have been regarded as having very close affinities with *Asplenium* based on morphology (Copeland 1947: 164), and for some genera (e.g. *Ceterach* Willd. and *Phyllitis* Hill), on the evidence of inter-generic hybrids with *Asplenium* species, the cases for and against merging them into *Asplenium* have been discussed (Lovis 1973, 1977: 224). In recent taxonomic treatments most authors have in-

cluded all of these "satellite" genera within *Asplenium* (Kramer & Viane 1990) or applied sub-generic rank – e.g. Viane et al. (1993): subgenus *Ceterach* (Willd.) Vida ex Bir, Fraser-Jenkins & Lovis; subgenus *Phyllitis* (Hill) Jermy & Viane – although their phylogenetic position within *Asplenium* has not been explored. Phylogenetic studies based on DNA sequence data have provided further evidence that most of these "genera" are nested within a highly a paraphyletic genus *Asplenium* (Murakami et al. 1999). *Ceterach* is distinguished from other putative monophyla of asplenioid ferns by the presence of dense scales on the underside of the lamina, the veins usually anastomosing towards the margins, and the indusium being obsolescent or obsolete. *Phyllitis* is distinguished from *Asplenium* by its "double sorus" comprising two closely placed sori that open towards each other on parallel veins and appear confluent at full maturity.

Subgenus *Ceterach*, as defined in Bir et al. (1985) and Viane et al. (1993), has undergone four radiations: 1) in the Mediterranean Basin, 2) in Macaronesia, 3) in the Himalaya and adjacent China, and 4) in southern Africa and St Helena (Fig. 1, Table 1). In all areas, the polyploid complexes have distinct morphological features, suggesting regional radiations.

Asplenium ceterach L. has diploid, tetraploid and hexaploid cytotypes that are treated as subspecies. They are very similar in gross morphology but can be distinguished by comparisons of exospore measurements and cytology. The tetraploid taxon, *A. ceterach* subsp. *ceterach*, is by far the most common, and is found on base-rich rocks and mortar in walls in southern and central Europe, north Africa, western Asia, Afghanistan, and north-west Himalaya (Jalas & Suominen 1972, Bir 1998, Trewick et al. in press). From biosystematic studies Vida (1963, 1965) inferred that the tetraploid *A. ceterach* subsp. *ceterach* was an autopolyploid taxon derived from the diploid subsp. *bivalens,* although Lovis (1977) regarded the evidence as inconclusive. The diploid *A. ceterach* subsp. *bivalens* (D.E. Meyer) Greuter & Burdet is known only from southern Europe close to the Mediterranean Sea in Italy, Croatia, Greece, Bulgaria, Hungary, Romania, and tentatively from Turkey and Algeria. Hexaploid plants of *A. ceterach* s.l. have been reported from Sicily, Cyprus and Greece (Vida 1963, Viane et al. 1996, Trewick et al. 2002), and are formally described as a new subspecies below.

The *Asplenium aureum* Cav. complex of Macaronesia comprises tetraploid, hexaploid and octoploid taxa, but in contrast to the other three regions a putatively ancestral diploid taxon is not known from the area. Tetraploid *A. aureum* and octoploid *A. parvifolium* Benl & Kunkel are restricted to the Canary Islands, whereas the hexaploid *A. lolegnamense* Gibby & Lovis is found exclusively in Madeira (Gibby & Lovis 1989, Ormonde
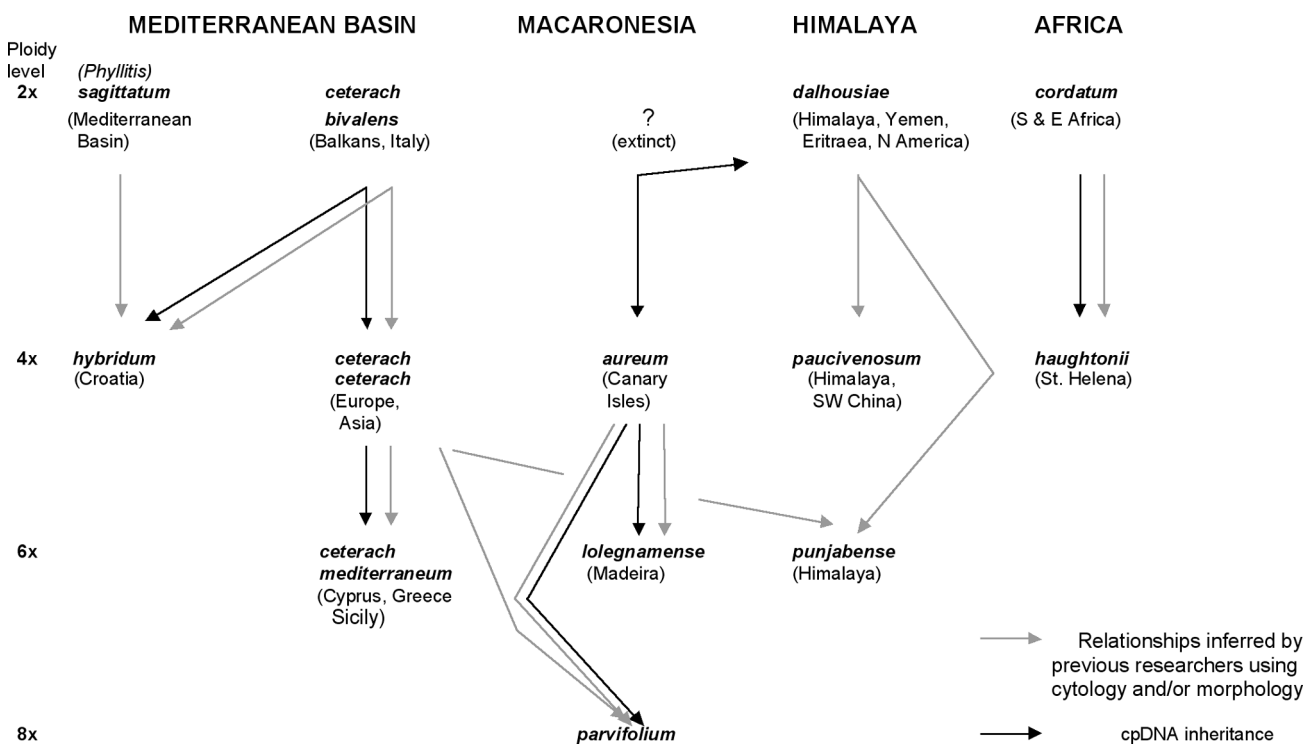

Fig. 1. Reticulation diagram of the four *Asplenium* polyploid complexes in the subgenus *Ceterach*.

1990). The three extant diploid taxa in subgenus *Ceterach*, *A. ceterach* from the Mediterranean Basin, *A. dalhousiae* from northeastern Africa and the southern Arabian Peninsula, southwestern North America and the Himalaya, and *A. cordatum* (Thunb.) Sw. from southern and eastern Africa, all have to be considered as possible ancestral taxa, since phylogenetic and biogeographic studies on angiosperms have identified close links of endemic Macaronesian taxa to all of the above regions (Sunding 1979, Francisco-Ortega et al. 2001).

The genus *Ceterachopsis* J. Smith ex Ching was applied to taxa that are classified as *Asplenium* and *Ceterach* (Copeland 1947), but are distinguished from the latter by the presence of an indusium and absence of scales on the lamina. These include *A. dalhousiae* Hook., a diploid taxon from the southwestern USA and Mexico, NW Himalaya, Eritrea and Yemen; *A. paucivenosum* Ching, a tetraploid from central and eastern Himalaya and Yunnan; and *A. punjabense* Bir, Fraser-Jenkins & Lovis, a hexaploid from central and eastern Himalaya (Bir 1998).

Two further species were assigned to *Ceterach* (Desvaux 1827, Cronk 2000): *A. cordatum* and *A. haugthonii*. *Asplenium cordatum* is a widespread diploid taxon ranging from northeastern to southern Africa, and reported to be related to the morphologically somewhat similar St. Helenan endemic *A. haugthonii* Hook. (Cronk 2000).

Asplenium sagittatum (DC.) A.J. Bange and *A. scolopendrium* L. are members of subgenus *Phyllitis*. While the former species is confined to coastal regions around the Mediterranean Basin, the latter is widespread in western Europe in moist calcareous woodland and on calcareous rocks in shade. Both taxa are diploid in Europe, but tetraploid taxa have been reported from North America and Japan (Britton 1953, Emmott 1964). Species of subgenera *Ceterach* and *Phyllitis* are known to hybridise with each other and with species of *Asplenium* (Emmott 1964; Lovis 1973, 1977), and give rise both to sterile hybrids and fertile polyploid taxa. *Ceterophyllitis* Pic. Serm. (syn. *Phyllitopsis* Reichstein) has been applied to a single taxon, *A. hybridum* (Milde) A.J. Bange (= *Ceterophyllitis hybrida* (Milde) Pic. Serm.) from Croatia, that combines features of *Ceterach* and *Phyllitis*. Similarly the hybrid genus X *Asplenophyllitis* Alston has been applied to sterile triploid plants that have arisen through hybridisation of *A. scolopendrium* with tetraploid taxa of *Asplenium*. Taxa that combine features of *Ceterach*, *Phyllitis* and *Asplenium* indicate their close affinity. Here, we are using DNA sequence data from chloroplast-encoded *trn*L (UAA) 5´ exon - *trn*F (GAA) exon regions and *rbc*L to develop a phylogenetic hypothesis for *Asplenium, Ceterach*, *Ceterachopsis*, *Ceterophyllitis and Phyllitis*, and to explore the phylogeographic and biosystematic relationships of species complexes within this group by determining the maternal parent in polyploid lineages.

Materials and methods

Extensive field work was carried out in Europe to clarify the distributions of diploid and polyploid taxa of *A. ceterach*. For the phylogenetic analysis we sampled representatives of all putative groups of *Ceterach*, the putative sister group *Phyllitis*, and 12 species of other groups of asplenioid ferns (see Table 2). *Asplenium unilaterale*, a member of the *Hymenoasplenium* clade, was assigned as an outgroup because this group was found to be the sister of all other asplenioid ferns in phylogenetic studies based on *rbc*L sequence data (Murakami et al. 1999).

Cytology and spore measurements

Cytological material was prepared following the method of Manton (1950) to examine meiosis in spore mother cells, and photographed on a Leica DMRB microscope. For exospore

Table 1. Geographic distribution of the different cytotypes/taxa in the four *Asplenium* polyploid complexes in subgenus *Ceterach*.

	A. ceterach, 2x	A. ceterach, 4x	A. ceterach, 6x	A. aureum, 4x	A. lolegnamense, 6x	A. parvifolium, 8x	A. dalhousiae, 2x	A. paucivenosum, 4x	A. punjabense, 6x	A. cordatum, 2x	A. haugthonii, 4x
Himalaya/China		x					x	x	x		
Arabian Peninsula		x					x				
Northeastern Africa		x					x			x	
Mediterranean basin/Europe	x	x	x								
Macaronesia		x		x	x	x					
Southern Africa										x	
St Helena											x
Southwestern USA, northern Mexico							x				

measurements, untreated fresh spores were mounted on slides in Euparal, and a minimum of 30 spores per sample were measured. Detailed numbers and origins of investigated plants are given in Trewick et al. (2002).

DNA extraction, amplification and sequencing

DNA extraction used a method derived from that of Rogers & Bendich (1994). Individual pinnae were removed from fresh, herbarium or silica-dried fronds and stripped of scales and sporangia. Samples were ground either using a pestle and mortar with acid-washed sand or, more frequently, crushed in 1.5 ml tubes with liquid nitrogen. Extractions used 500 μl CTAB buffer (2% CTAB in 100 mM Tris-HCl pH8.0, 1.4 M NaCl, 20 mM EDTA), 50 μl sarkosyl (10% N-Lauryl sarcosine, 100 mM Tris-HCl pH8.0, 20 mM EDTA) and 5 μl β-mercaptoethanol, and were incubated at 60 °C for 1 hour. An equal volume of sevac (chloroform: isoamyl alcohol 24:1) was added and the mixture shaken and centrifuged at 13,000 rpm for 3 min. Supernatants were pipetted into fresh tubes and combined with a 2/3 volume of cold 100% isopropanol for 15–60 minutes. DNA was pelleted by centrifugation at 13,000 rpm for 3 min and then briefly spun with 500 μl 70% ethanol. The ethanol was discarded and the pellet dried and dissolved in 30 μl of water.

Polymerase chain reaction (PCR) was used to amplify the *rbc*L and *trn*L-*trn*F chloroplast DNA fragments (Haufler & Ranker 1995, Taberlet et al. 1991, Vogel et al. 1996). PCR reactions were carried out in 25 μl volumes containing 2.5 mM MgCl$_2$, 200 μM dNTPs, 1 ng BSA, 1× PCR buffer, 0.625 U Red Hot *Taq* (ABgene), and 1.5 μl of diluted (1:20) DNA template. Thermal cycling conditions were: 2 min at 94 °C, 35 cycles of 15 s at 94 °C, 30 s at 48 °C and 90 s at 72 °C, followed by 3 min at 72 °C. PCR products were purified using Qiaquick spin columns (Qiagen). Cycle sequencing utilised Big Dye v2.0 chemistry (PE Biosystems) and the original PCR primers in quarter volume reactions but otherwise following the manufacturers protocols. Cycle sequencing products were electrophoresed on an ABI automated sequencer (PE Biosystems). Sequences were assembled using SequEd v3.01 or Sequencher v3.0 and aligned manually using MacClade 4.0 (Maddison and Maddison 2000). Newly obtained sequences have been submitted to GenBank, accession numbers are given in Table 2.

Alignment and phylogenetic analyses

We sampled two gene data sets: *rbc*L (1322 nucleotides) and a *trn*L (UAA)5' exon - *trn*F (GAA) exon region (about 400 nucleotides). Ambiguously aligned regions of the *trn*L-F spacer were excluded. Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses using PAUP 4.0b8 (Swofford 2000) were performed for the *rbc*L data set, the *trn*L-F data set, and a combined set that included data for both chloroplast regions. A homogeneity partition test as implemented in PAUP 4.0b8 (Swofford 2000) was performed to estimate incongruent length differences within the two-gene data set (Farris et al. 1995). Congruence between separately analyzed data sets was inferred by inspecting bootstrap scores above 70% resulting from separate MP analyses of the *rbc*L and *trn*L data sets (Mason-Gamer & Kellogg 1996).

Maximum Parsimony trees were obtained (PAUP 4.0b8, Swofford 2000) using the heuristic search mode with 1000 random-addition-sequence replicates, tree bisection-reconnection (TBR) branch swapping, and MULTrees option on. Character-state changes were treated as equally weighted, and if more than one tree was generated, a strict consensus tree was determined. Branch support was estimated by bootstrap analyses (Felsenstein 1985) from 1000 replicates using full heuristic searches and 10 random-addition-sequence replicates per bootstrap replicate. The options of TBR branch swapping and MULTrees options were selected; all trees generated were saved.

The nucleotide substitution model with the smallest number of parameters that best fits the data presented here was determined using Modeltest Ver. 3.04 (Posada & Crandall 1998). Maximum Likelihood analyses specifying the Transversion Model + Proportion of Invariant sites + Gamma distribution (TIM+I+G) model (Rodriguez et al. 1990) of sequence evolution were implemented (PAUP 4.0b8, Swofford 2000) as heuristic searches with 100 random-addition-sequence replicates. TBR branch swapping and MULTrees options were selected and all trees were saved. Clade credibility values were estimated calculating the posterior probability for each node using the Bayesian statistical procedure with a Markov-Chain Monte Carlo sampling method as implemented in MrBayes 2.1 (Huelsenbeck 2000, Huelsenbeck & Bollback 2001, Huelsenbeck & Ronquist in press). One out of every 100 trees was sampled for 1,000,000 generations, with kappa and DNA substitution parameters estimated during the search. The consensus tree was computed (PAUP 4.0b8, Swofford 2000) on the last 8500 sampled trees, excluding the 1500 trees found in the "burn-in period". Posterior probability values above p = 0.95 are considered to be statistically significant.

Character state changes were reconstructed using MacClade 4.0 (Maddison & Maddison 2000) using both ACCTRAN and DELTRAN optimisations.

Analysis of biogeographic patterns

To explore the biogeographical pattern, we used the program DIVA (Ronquist 1996, 1997) to assign the distribution of the internal nodes in the tree. This program optimises the distribution for each node by favouring vicariance events and minimising dispersal and extinction events. Six main areas were assigned based on the distribution of terminal taxa of the *Ceterach* clade: (1) Himalaya including Pakistan, (2) Mediterranean Basin, (3) Yemen and Eritrea, (4) North America, (5) Canary Islands, and (6) Madeira. The analyses were run with unlimited number of areas (= 6), and subsequent analyses with reduced maximal number of areas (5, 4, 3, 2, 1).

Results

Taxonomy

Our studies have discovered many new populations of hexaploid *A. ceterach* on central and eastern Mediterranean islands such as Pantelleria, Sicily, Kephalonia, Korfu and Cyprus, and on the eastern Peloponnes in

Table 2. Voucher specimens and accession numbers in GenBank.

Species	Subspecies	Synonym	GB-*rbcL*	*rbcL*	GB-*trnL*-F	*trnL*-F
A. aethiopicum (Burm.F.) Bech.			AF240654	BM, Hemp, A. 22, 2–3 1999, Marangu-Ogate, Kenya	AF525233	same as *rbcl*
A. aureum Cav.		*Ceterach aureum*	no *rbcl*	BM, JCV AUR-1, El Hierro, Canary Isles	AF525256	same as *rbcl*
		Ceterach aureum	AF240642	BM, JCV Cet-116, Tenerife, Canary Isles	AF525258	same as *rbcl*
A. auritum Sw.			AF240651	BM, Hughes 64, Belize	AF240667	same as *rbcl*
A. caudatum G.Forst.			AF525264	UC Berkeley 58.1094, R.C. ex Bot. Garden, Java	AF525234	same as *rbcl*
A. ceterach L.	*bivalens*	*Ceterach officinarum*	no *rbcl*	BM, JCV C21, Dubrovnik, Croatia	AF525251	same as *rbcl*
	bivalens	*Ceterach officinarum*	no *rbcl*	BM, JCV CET-121, Bulgaria	AF525252	same as *rbcl*
	bivalens	*Ceterach officinarum*	no *rbcl*	BM, JCV CET-110/1, Crete, Greece	AF525263	same as *rbcl*
	ceterach	*Ceterach officinarum*	no *rbcl*	BM, JCV CET-218, Corfu, Greece	AF525263	same as *rbcl*
	mediterraneum	*Ceterach officinarum*	no *rbcl*	BM, JCV CET-19/2, Poros, Greece	AF525235	same as *rbcl*
A. cordatum (Thunb.) Sw.		*Ceterach cordatum*	AF240650	BM, JCV, CET-119, South Africa	AF525241	same as *rbcl*
A. cuneifolium Viv.			AF525265	BM, JCV,CUN-D-6, Bavaria, Germany	AF525253	same as *rbcl*
A. dalhousiae Hook.		*Ceterach dalhousiae*	no *rbcl*	BM 1844214, Wood, JRI 2123, Yemen	AF525255	same as *rbcl*
		Ceterach dalhousiae	no *rbcl*	BM, JCV DAL-1, ex MO, New Mexico, USA	AF525254	same as *rbcl*
		Ceterach dalhousiae	AF240641	Gent, TR 7634, Pakistan		same as *rbcl*
A. emarginatum P.Beauv.			AF525266	BM, Nick Mundy, 123-124, Gabon, Africa	AF525243	same as *rbcl*
A. feei Kunze ex Fée			AF525267	BM, NYBG 393/94A	AF525244	same as *rbcl*
A. fontanum (L.) Bernh.			AF525268	BM, JCV F-3-92, ex. H & K. Rasbach, Germany	AF525239	same as *rbcl*
A. haugthonii Hook.		*Ceterach haugthonii*	no *rbcl*	BM, JCV Haug-1, St Helena	AF525236	same as *rbcl*
A. hemionitis L.			AF240648	BM, JCV, HEM-9, Azores	AF240663	same as *rbcl*
A. hybridum (Milde) Bange			AF249644	BM, JCV, HYBR-2, Croatia	AF525250	same as *rbcl*
A. juglandifolium Lam.			AF525269	BM, Boudrie M 3249, 1999 French Guiana	AF525245	same as *rbcl*
A. lolegnamense Gibby & Lovis		*Ceterach lolegnamense*	no *rbcl*	CV, LOLEG-5, Madeira	AF525257	same as *rbcl*
A. marinum L.			AF240647	BM, JCV, MAR-5, UK	AF240662	same as *rbcl*
A. nidus L.			AF525270	UC Berkeley 68.0392, R.C. ex Bot. Garden, Madagascar	AF525246	same as *rbcl*
A. parvifolium Benl & Kunkel		*Ceterach parvifolium*	AF240640	Gent, TR 5075, La Palma, Canary Isles	AF525259	same as *rbcl*
A. paucivenosum Ching		*Ceterach paucivenosum*	no *rbcl*	RBGE, Giersch + Lang 1051, May 1979, India	AF525260	same as *rbcl*
A. petrarchae (Guérin) Dc	*bivalens*		AF525271	BM, JCV, PET-4, Mallorca, Spain	AF525249	same as *rbcl*
A. platyneuron (L.) Britton, Sterns & Poggenb.			AF525272	BM, JCV - PLATY-1b, Virginia, USA	AF525240	same as *rbcl*
A. ruta-muraria L.	*ruta-muraria*		AF525273	BM, JCV, RUT-16, Austria	AF525242	same as *rbcl*
A. sagittatum (Dc.) Bange		*Phyllitis sagittatum*	AF240646	BM, JCV, SAG-1, Mallorca, Spain	AF525261	same as *rbcl*
A. sandersonii Hook.			AF525274	BM, Hemp A. 12, 2-3 1999, Weru-Weru, Kenya	AF525247	same as *rbcl*
A. scolopendrium L.		*Phyllitis scolopendrium*	AF240645	BM, JCV, SCOL-73, Pyrenees, France	AF525262	same as *rbcl*
A. septentrionale (L.) Hoffm.	*caucasicum*		AF525275	BM, JCV, SEPT-17, ex M.Rickard, Turkey	AF525248	same as *rbcl*
A. trichomanes L.	*trichomanes*		AF525276	BM, JCV, TT-25, Germany	AF525237	same as *rbcl*
A. unilaterale Lam.			AF240652	BM, Hemp A. 18, Kenya	AF525232	same as *rbcl*
A. viride Huds.			AF240649	BM, JCV 1334, Austria	AF525238	same as *rbcl*
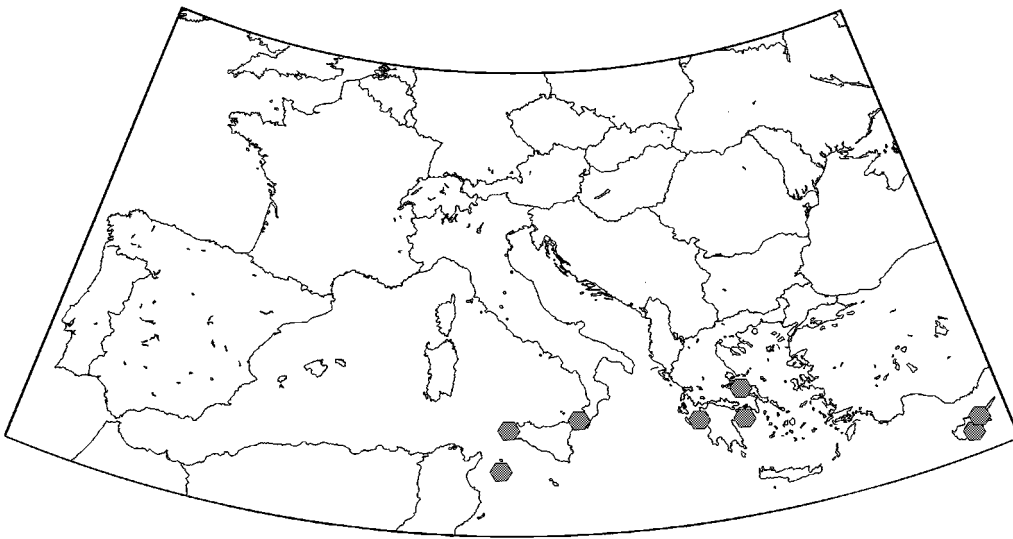
Fig. 2. Distribution of the hexaploid *Asplenium ceterach* subsp. *mediterraneum*.
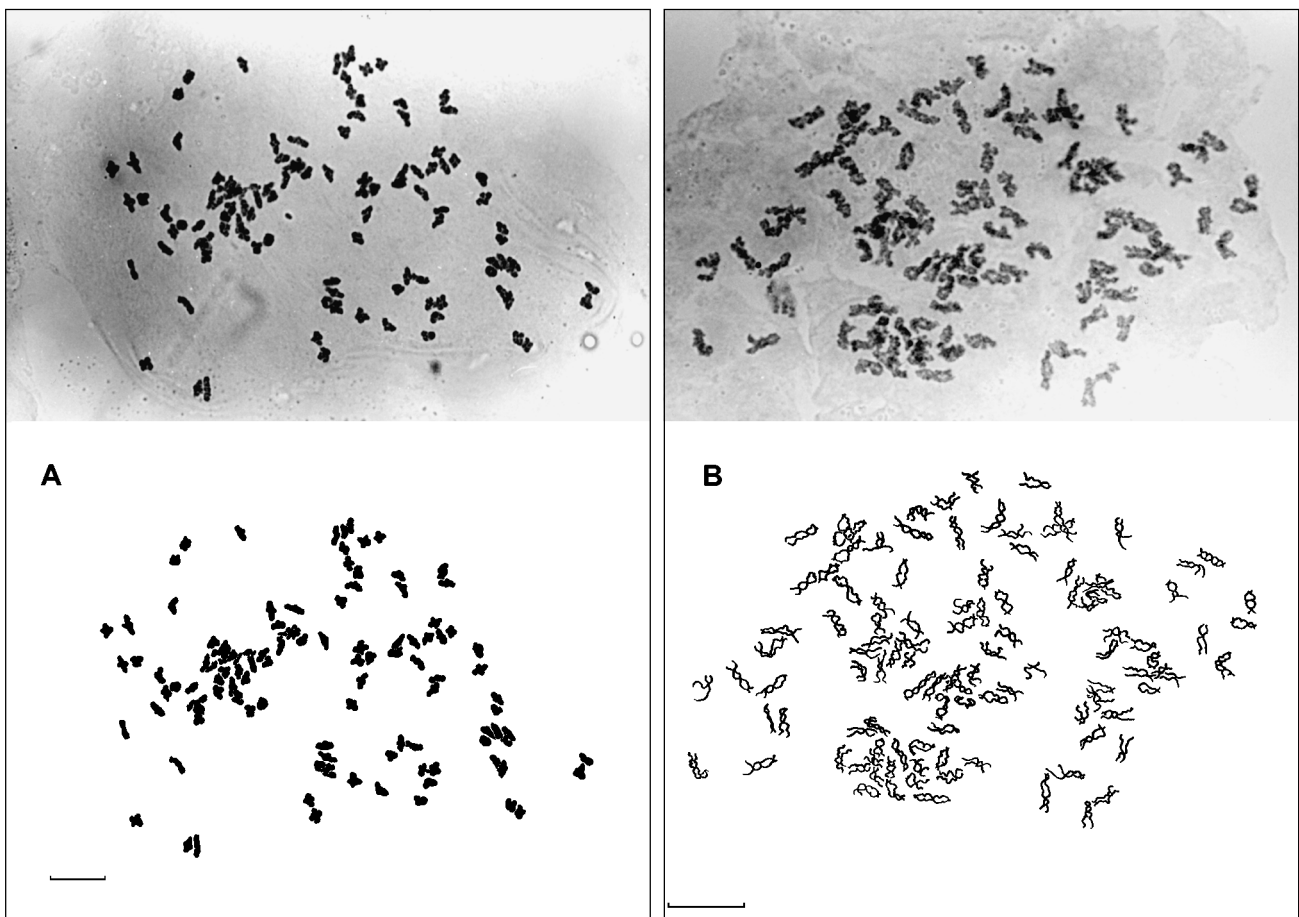
Fig. 3. Photomicrographs and explanatory diagrams for *Asplenium ceterach* subsp. *mediterraneum*.
A. Type specimen of the hexaploid plant from Poros, Greece (JCV CET-19B = PI 1071). Spore mother cells at meiosis metaphase I showing 108 bivalents. Scale = 10 μm. B. Hexaploid plant from Cyprus, grown from spores sent by T. Reichstein (TR 6842; originally collected by R. Viane 3577, north side of Mt Troodos at approx. 750 m). Spore mother cells at meiosis (diakinesis) showing 108 bivalents. Scale = 10 μm.

mainland Greece (Fig. 2). Plants from Cyprus and mainland Greece showed a regular meiosis with n = 108 (Fig. 3). These new records extend the distribution area of hexaploid *A. ceterach* which had been first reported from eastern Sicily (Vida 1963) and subsequently from western Sicily, Pantelleria and the eastern Peloponnes (Viane et al. 1996). This hexaploid taxon is here described as a new subspecies of *A. ceterach*:

Asplenium ceterach subsp. *mediterraneum* subsp. nov. I. Pinter

Habitu et textura *A. ceterach* subsp. *bivalens* et *A. ceterach* subsp. *ceterach* similis, sporae (40–) 43–47 (–51) longae et (30–) 34–38 (–46) latae (perispore exclusus) sunt. Numero chromosomatorum 2n = 216. Distributio geographica: plaga maris nostri.

Holotypus: I. Pinter PI 1071, April 1997, Greece, Peloponnes, Poros, top of hill above town, approx. 200 m a.s.l.; BP.

Etymology: the name "mediterraneum" is connected with the occurrence of this taxon in the Mediterranean Basin.

Diagnosis: the texture and size of fronds are similar to other *A. ceterach* subspecies, but the new taxon differs from the subspecies *bivalens* and subspecies *ceterach* by larger spores of (40–) 43–47 (–51) μm length and (30–) 34–38 (–46) μm width (excluding the perispore). The chromosome number is 2n = 216, hexaploid.

Phylogenetic analysis

In the phylogenetic analyses the two data sets contain a total of 1930 characters including 629 variable sites, of which 350 characters are parsimony informative. The *trn*L-F data set consists of 167 parsimony informative characters out of a total of 550 characters of which 278 are constant. The *rbc*L data set consists of 183 parsimony informative characters out of a total of 1380 characters of which 1023 are constant.

Maximum Parsimony and Maximum Likelihood analyses of the single-gene and the combined data sets recovered the *Phyllitis* group including *A. sagittatum* and *A. scolopendrium* as sister of the *Ceterach* group (Figs 4, 5). Maximum Parsimony analyses of the combined data set yielded 360 most parsimonious trees, whereas the Maximum Likelihood analysis of the same data set recovered two most likely trees of lg = –9126.1837.

Asplenium paucivenosum is recovered as the sister of all other *Ceterach* species. The Mediterranean *A. ceterach* forms a well-supported clade that is sister to a clade comprising *A. dalhousiae* and *A. aureum* and relatives. This clade is characterised by the occurrence of an insertion of 5 base pairs in the *trn*L-F spacer. *Asplenium dalhousiae* is recovered as a monophyletic lineage that is

sister to the *A. aureum* complex. *Asplenium dalhousiae* is characterised by the occurrence of two insertions of 10 + 9 base pairs in the *trn*L-F spacer. The American *A. dalhousiae* is sister to a clade including *A. dalhousiae* from Yemen and Pakistan. The *A. aureum* complex includes the tetraploid *A. aureum*, the hexaploid *A. lolegnamense*, and the octoploid *A. parvifolium*. The latter two taxa do not form a clade, and thus their chloroplast genomes are considered as inherited from different individuals of *A. aureum*.

Asplenium cordatum and *A. haugthonii* are not members of the *Ceterach* clade but are nested within a poorly resolved clade. Both taxa are recovered as sister clades despite significant differences between their chloroplast genomes. The selection of taxa used here is insufficient to identify the sister taxon of the *A. cordatum* clade.

Asplenium hybridum is nested within the *A. ceterach* clade.

Discussion

Phylogenetic position and description of the subgenera *Ceterach* and *Phyllitis*

The results presented here (Figs 4, 5) show that *Ceterach* is a monophyletic unit including *A. dalhousiae* (previously segregated as *Ceterachopsis*) but excluding *A. cordatum* and the St Helenan endemic *A. haugthonii*, and this is confirmed by a more extensive study of *Asplenium* s.l. (Schneider et al., manuscript submitted). The latter two species had been assigned as members of *Ceterach* based on similar leaf indumentum (Desvaux 1827, Fée 1852, Cronk 2000). However, their leaves are often pinnate to bipinnate and they have free veins, whereas the *Ceterach* lineage is characterised by pinnatifid leaves and mostly irregularly anastomosed veins. Thus, the results based on *rbc*L and *trn*L-F exon data are consistent with morphology, with the exception of leaf indumentum. Persistent leaf scales are found in several species of *Asplenium* such as *A. aethiopicum* and other members of the section *Sphenopteris* (Morton & Lellinger 1966) and dense indumenta may have evolved at least twice in reaction to similar ecological conditions. The absence of a dense leaf indumentum appears to be a reversal characterising *A. dalhousiae*.

The sister unit of *Ceterach* (Figs 4, 5) appears to be the monophyletic unit *Phyllitis* (including *A. sagittattum* and *A. scolopendrium*) with simple leaves, free veins and an unusual position of the sori. The important character of the unit is the presence of two parallel sori located opposite to and opening towards each other, the so-called scolopendrioid sori. Several unrelated monophyletic taxa within *Asplenium* have simple leaves, e.g. *A. hemionitis* and the *Thamnopteris* C.Presl group, but
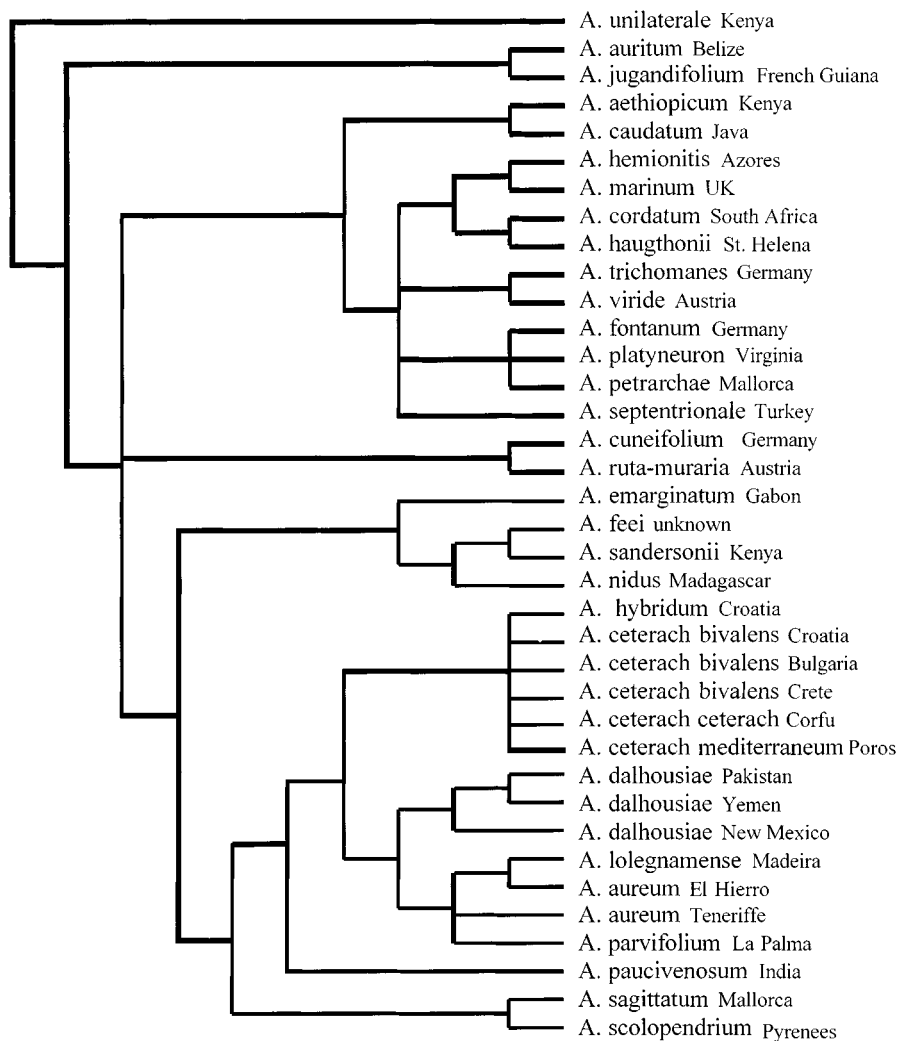
A. unilaterale Kenya
A. auritum Belize
A. jugandifolium French Guiana
A. aethiopicum Kenya
A. caudatum Java
A. hemionitis Azores
A. marinum UK
A. cordatum South Africa
A. haugthonii St. Helena
A. trichomanes Germany
A. viride Austria
A. fontanum Germany
A. platyneuron Virginia
A. petrarchae Mallorca
A. septentrionale Turkey
A. cuneifolium Germany
A. ruta-muraria Austria
A. emarginatum Gabon
A. feei unknown
A. sandersonii Kenya
A. nidus Madagascar
A. hybridum Croatia
A. ceterach bivalens Croatia
A. ceterach bivalens Bulgaria
A. ceterach bivalens Crete
A. ceterach ceterach Corfu
A. ceterach mediterraneum Poros
A. dalhousiae Pakistan
A. dalhousiae Yemen
A. dalhousiae New Mexico
A. lolegnamense Madeira
A. aureum El Hierro
A. aureum Teneriffe
A. parvifolium La Palma
A. paucivenosum India
A. sagittatum Mallorca
A. scolopendrium Pyrenees

Fig. 4. Strict consensus tree of the 360 most parsimonious trees using combined data set of the *rbc*L gene and *trn*L-F spacer (tree length 1214, CI = 0.5255, HI = 0.4815, RI = 0.6515, RC = 0.4218).

they differ from *Phyllitis* in the position of the sori (Murakami et al. 1999). Their sori are orientated in parallel lines, but the opening of all indusia is towards the leaf apex. Differences in venation and position of the sori indicate different development processes and independent evolution of simple leaves. Parallel sori with indusia opening towards each other are found in several species, and this pattern may have evolved more than once. Scolopendrioid sori are known from proposed taxonomic units such as *Antigramma* C.Presl, *Diplora* Baker, *Triphlebia* Baker, *Schaffneria* Fée. Many of these taxa share the root-type with *A. scolopendrium*, but smaller taxa such as *A. delavayi* (Franch.) Copel. and *A. nigripes* (Fée) Hook. have the same root-type as *A. ceterach* (Schneider 1997). Although *Asplenium* shows significant variation in root anatomy, similar roots to those in *Phyllitis* and *Ceterach* are found in other, possibly monophyletic units.

The *Phyllitis* and *Ceterach* clades share few morphological characters; the blade dissection is simple rather than pinnate, the scales on the stipe are persistent, and the relatively thick, coriaceous lamina tissue obscures the veins. Each of these characters is also found in other groups of *Asplenium*, but a future detailed phylogenetic study of morphology may reveal obscure autapomorphic characters.

Asplenium dalhousiae and the origin of Himalayan *Ceterach*

Asplenium dalhousiae (*Ceterachopsis*) is nested within *Ceterach*, and this supports the view that the segregate *Ceterachopsis* is a member of *Ceterach* despite the absence of a dense indumentum and irregular anastomosed veins. Recognition of *Ceterachopsis* would make *Ceterach* paraphyletic, thus we reject this proposal. *Asplenium dalhousiae (= Ceterachopsis)* is recovered as a monophyletic unit, although the species shows a disjunct distribution. In our study, we included one specimen from each of the three areas of occurrence: (1)
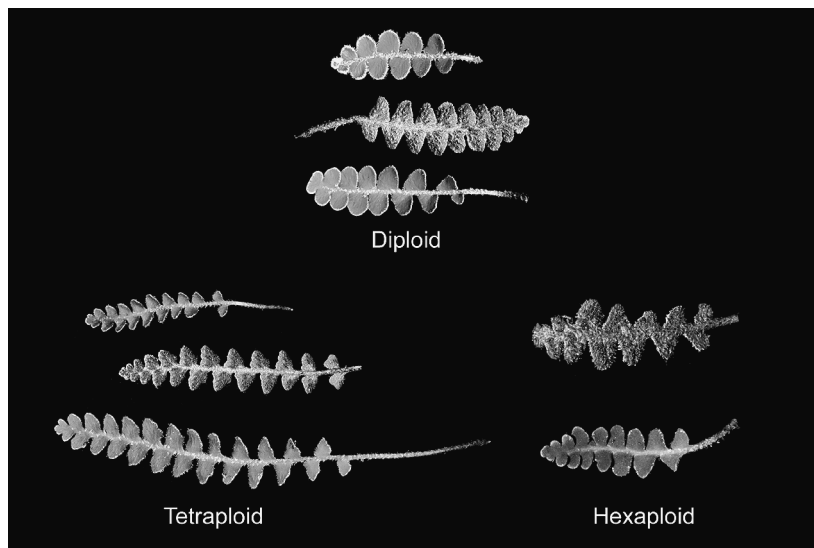
Fig. 5. Topology recovered by Bayesian inference of phylogeny using combined data set of the *rbc*L gene and *trn*L-F spacer. Values above branches indicate p-values above 0.50. Ploidy levels: 2×, 4×, 6×, 8×. Geographical distribution: Hi = Himalaya, Ye = Yemen, Me = Mediterranean Basin, Ca = Canary Islands, Ma = Madeira, NA = North America, Co = complete area excluding Yemen. Current distribution of terminal taxa indicated at right. Regions shown at nodes indicate probable centre of origin, inferred from DIVA.
Introns: A = 5 base pair intron of the *A. aureum* & *A. dalhousiae* clade. B = 10+9 base pair introns of the *A. dalhousiae* clade. C = 10 base pair intron of the *A. paucifolium* clade. D = 9+5 base pair introns of the *A. cordatum* clade. E = 7 base pair intron of the *A. haughtonii* clade.

southwestern North America, (2) Yemen, and (3) Pakistan (Table 2). The three specimens share two insertions of 10 + 9 base pairs in the non-coding *trn*L region. The North American specimen is sister to the specimens from Pakistan and Yemen, as expected from the geographical proximity of the latter two. The present day distribution of *A. dalhousiae* can best be explained either by long-range dispersal or as the result of extinctions. The second hypothesis assumes a widespread distribution of this taxon in North Africa, Europe and Asia, at least, most recently, before the last glacial periods in the Pleistocene. Such continuous widespread distributions are found in temperate species of the genus *Asplenium*, e.g. *A. trichomanes*. Note that *A. scolopendrium*, a member of the sister clade of *Ceterach*, has a widespread distribution in temperate Asia, Europe and some isolated populations in eastern North America. Various events

may have formed the current distribution of fern taxa, and denser sampling and more variable nuclear markers, such as allozymes or ITS sequences, combined with critical analyses using phylogenetic methods would be desirable to explore the historical biogeography of these ferns (Wolf et al. 2001).

Surprisingly, tetraploid *A. paucivenosum* turned out not to be part of a Himalayan polyploid radiation based on *A. dalhousiae* as suggested by Bir (1998) but instead is recovered as ancestral to all other *Ceterach* taxa. We therefore believe *A. paucivenosum* to be a palaeopolyploid. This phylogenetic-biogeographical pattern indicates the Himalayan region as a putative area of origin, but this area is geologically very young (Molnar 1986). So far, we have not found evidence for a radiation of *Ceterach* in the Himalayan region, but one critical taxon, the hexaploid *A. punjabense* (*Ceterachopsis*), that

Fig. 6. The three macromorphologically very similar and indistinguishable taxa of *Asplenium ceterach*. All three taxa were collected on the Greek island of Kefalonia by Drs Mary Morgans-Richards and Steve Trewick in May 2001. Diploid *A. ceterach* subsp. *bivalens*, population CET 342, Mt Enos (Mt Enos is the type locality for this taxon). Tetraploid *A. ceterach* subsp. *ceterach*, population CET 336-B, south of Mt Thorinthos. Hexaploid *A. ceterach* subsp. *mediterraneum*, population CET 340-B, Langaditica.

is believed to be derived from the cross of the diploid *A. dalhousiae* and a tetraploid member of the *Ceterach* clade (Bir 1998), is missing from our analysis.

The European *Asplenium ceterach* polyploid complex

Three morphologically very similar cytotypes of *A. ceterach* s.l. are known from Europe (Fig. 6), the Near East and Asia. Morphological and biogeographic evidence suggests regional origins for the polyploid taxa that may have evolved in the Pleistocene from relictual diploid populations such as those present in mainland Italy, Sicily or the Balkans (Vogel et al. 1999). A recent study, including 331 plants from 142 populations, of all three ploidy levels of *A. ceterach* s.l. throughout its range (Europe to Asia) has revealed sequence variation in an approx. 900bp fragment of non-coding cpDNA, the *trn*L-*trn*F intron and exon, that is phylogeographically and biosystematically informative (Trewick et al. 2002). Nine cpDNA haplotypes were detected in Europe and around the Mediterranean Basin, seven in the diploids, five in the tetraploids, and two in the hexaploids. While the data clearly support the monophyly of *A. ceterach* they also provide evidence for a dynamic evolution and polyploid radiation based on diploid lineages in the Balkans and the eastern Mediterranean Basin. Based on the cpDNA evidence we found multiple origins of polyploids, at least four in the tetraploids, and two in the hexaploids. Tetraploids from as far away as China and Saudi Arabia have cpDNA haplotypes that are present in European diploids. The data point to at least three distinct and extant diploid lineages in putative Pleistocene refugia in Europe; all three lineages are involved in the formation

of tetraploid taxa, and in one case in the formation of a hexaploid taxon.

Investigations of natural hybrids and the analysis of synthesised hybrids have demonstrated that chloroplast genes are maternally inherited in *Asplenium* (Vogel et al. 1998a, b). *Asplenium hybridum* (= *Ceterophyllitis hybrida*), a taxon endemic to islands off the coast of northern Croatia, was proposed on morphological evidence to be a hybrid between *Ceterach* and *Phyllitis*; the position of *A. hybridum* in the phylogeny (Fig. 4), as sister to *A. ceterach,* provides evidence that *A. ceterach* is the female parent of this allotetraploid taxon. This phylogenetic study includes a diploid *A. ceterach* from near Dubrovnik, a town on the southern Croatian coast, and one from the Rhodope mountains in Bulgaria (Table 2). Both accessions belong to the widespread and predominant Balkan lineage in *A. ceterach* (Trewick et al. 2002). These two sequences are similar with that of *A. hybridum*, thus supporting the hypothesis that local and extant diploid *A. ceterach* was involved in the formation of *A. hybridum*. On morphological evidence, particularly the scolopendroid sori, the male parent may be inferred as *A. sagittatum*. This is supported by evidence from cytology (Vida 1963) and allozyme electrophoresis (Fig. 1; Vogel et al., unpublished data). A wider study of this taxon provided no evidence for multiple origins in *A. hybridum* (Vogel et al. unpublished).

We recovered all the cpDNA diversity necessary to explain the variation in the *A. ceterach* polyploid complex from Europe and Asia and in the local endemic allotetraploid *A. hybridum*. Phylogenetic and phylogeographic evidence clearly support the hypothesis of a European origin and regional radiation of the *A. ceterach* (– *A. sagittatum*) polyploid complex in the Mediterranean Basin during the Pleistocene.

The Macaronesian *Asplenium aureum* polyploid complex

Data presented in this study clearly demonstrates a common ancestor of the Macaronesian *Ceterach* (*A. aureum* s.l.) clade, but a more detailed study using nuclear and population genetic markers is desirable to unravel this complex in more detail. Sequence variation in tetraploid *A. aureum* was resolved between the accessions from the islands of El Hierro and Tenerife. The cpDNA sequence of *A. aureum* from Tenerife is identical with the cpDNA of the octoploid *A. parvifolium* from Tenerife, whereas the cpDNA sequence of *A. aureum* from El Hierro is identical with the sequence of *A. lolegnamense* from Madeira. Morphological evidence suggested the octoploid *A. parvifolium* to be an allotetraploid between the endemic *A. aureum* and tetraploid *A. ceterach* (see Fig. 1; Benl & Kunkel 1967; Ormonde 1990, 1991). However, the latter taxon had not yet been reported from Macaronesia when Benl & Kunkel and Ormonde speculated about the origin of *A. parvifolium*. The cpDNA evidence clearly demonstrates *A. aureum* from Tenerife as the maternal parent of *A. parvifolium* from Tenerife. The discovery of tetraploid *A. ceterach* on Tenerife by I. Pinter in 1998 (Pinter in Trewick et al. 2002) would support the hypothesis of a recent and local evolution of *A. parvifolium* on Tenerife.

Ormonde (1990, 1991) suggested diploid *A. ceterach* subsp. *bivalens* and tetraploid *A. aureum* as the taxa involved in the formation of the hexaploid Madeiran endemic *A. lolegnamense*. However, three further hypotheses about the origin of *A. lolegnamense* can be proposed: 1) It is derived from *A. aureum* through backcrossing with the missing diploid ancestor to the Macaronesian polyploid complex; 2) It is derived from *A. aureum* and African *A. cordatum*; 3) It is a species of hybrid origin between tetraploid *A. aureum* and octoploid *A. parvifolium*, that has developed regular meiosis through "delayed polyploidy" as described by Lovis (1977) for other fertile hybrids between polyploid *Asplenium* species. To exclude any hypothesis without further evidence from nuclear or population genetic markers is difficult, but morphological evidence would rule out the involvement of any taxa from outside the well-supported *Ceterach* clade, such as *A. cordatum*.

The highly disjunct *A. dalhousiae* is the sister taxon to all observed specimens of the polyploid endemic *Ceterach* (*A. aureum* s.l.) from Macaronesia (Canary Isles, Madeira). The two clades, *A. aureum* s.l. and *A. dalhousiae*, differ in 10 unambiguous character changes from each other. However, the two lineages share 23 unambiguous character state changes that separate them from other members of the *Ceterach* lineage. Similarities in the pinna structure between *A. dalhousiae* and the *A. au-*

reum polyploid complex are congruent with this result. From analysis of meiosis in a synthesised triploid hybrid between *A. aureum* and the Mediterranean *A. ceterach* subsp. *bivalens*, Pintér & Vida (1993) demonstrated that the two taxa do not share a common genome, and suggested that *A. aureum* is probably an allotetraploid. All these results would thus exclude *A. ceterach* from being ancestral to the endemic Macaronesian polyploid complex, and support the results of the DIVA analysis which suggests that the European *Ceterach* is not ancestral to Macaronesian *Ceterach*. The diploid progenitor(s) of *A. aureum* remain unknown, and likely extinct.

Dispersal vicariance analyses were performed using DIVA (Ronquist 1996, 1997). They indicate a likely origin of *Ceterach* in the Himalaya, whereas the shared ancestor of the *Ceterach* and *Phyllitis* clades occurred throughout the whole range of the *Ceterach* clade (Fig. 5). The distribution of extant species must have resulted from subsequent dispersal, extinction and vicariance events. Models favouring vicariance events suggest a distribution of the ancestor of *A. aureum* from the Himalaya via Eritrea to the Canary Islands. This area may have expanded towards North America via long-distance dispersal. The extant populations of *A. dalhousiae* are likely to reflect the distribution of this ancestral taxon. The DIVA analyses also reject the hypothesis of a colonisation of the Canary Islands by *Ceterach* of Mediterranean origin.

Asplenium cordatum and *A. haughtonii*

Asplenium cordatum and *A. haugthonii* do not resolve within the *Ceterach* clade. Despite significant differences between their chloroplast genomes they are recovered as sister clades within a poorly resolved clade. Similarities in some morphological features with taxa of the *Ceterach* clade, especially the dense indumentum, are assumed to have arisen more than once within *Asplenium*. The selection of taxa used here is insufficient to identify the sister taxa of the *A. cordatum* clade, but their position within the phylogeny of *Asplenium* has been explored in more detail by Schneider et al. (manuscript submitted).

Conclusions

The interpretation of the data is limited by the absence of a phylogenetic study of the genus *Asplenium* including representatives of all possible monophyletic groups. Furthermore, the absence of a detailed and critical study of the morphological evidence for the phylogeny of *Asplenium* restricts the possibility of assignment of morphological characters to clades. Taxonomic conclusions are also restricted for these two reasons.

Within the *Ceterach* clade, polyploids evolved independently in at least two lineages. The first one characterises the Mediterranean radiation of *A. ceterach*, the second the Macaronesian *A. aureum* complex. A putative third lineage includes the Himalayan tetraploid, *A. paucivenosum*, the diploid relative of which is unknown, perhaps extinct.

The polyploid members of the *Ceterach* clades are the most widespread taxa in their subclades, and in several groups the diploid ancestors are likely extinct. So far, the diploid ancestors of *A. aureum* and *A. paucivenosum* are unknown. In addition, the diploid *A. dalhousiae*, which is not involved in any polyploid complex, shows remarkable disjunctions in its distribution. This observation suggests extinction of the diploid taxa and subsequent dispersal of the polyploid members of the subclades. This hypothesis will be explored by the authors by means of denser sampling of taxa, more variable genetic markers, estimation of divergence (Sanderson 2002), rigorous analyses of dispersal versus vicariance events (Ronquist 1997), and comparison with geological history of the area of distribution (Vinnersten & Bremer 2001).

Acknowledgements

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