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Polyploidy, phylogeography and Pleistocene refugia of the rockfern *Asplenium ceterach*: evidence from chloroplast DNA

S. A. TREWICK,*† M. MORGAN-RICHARDS,* S. J. RUSSELL,* S. HENDERSON,* F. J. RUMSEY,* I. PINTÉR,‡ J. A. BARRETT,§ M. GIBBY¶ and J. C. VOGEL*

**Cryptogamic Plants, Department of Botany, Natural History Museum, London, UK; ‡Eötvös Loránd University, Department of Genetics, Budapest, Hungary; §Department of Genetics, University of Cambridge, Cambridge, UK; ¶Royal Botanical Garden, Edinburgh, UK*

Abstract

Chloroplast DNA sequences were obtained from 331 *Asplenium ceterach* plants representing 143 populations from throughout the range of the complex in Europe, plus outlying sites in North Africa and the near East. We identified nine distinct haplotypes from a 900 bp fragment of *trnL-trnF* gene. Tetraploid populations were encountered throughout Europe and further afield, whereas diploid populations were scarcer and predominated in the Pannonian-Balkan region. Hexaploids were encountered only in southern Mediterranean populations. Four haplotypes were found among diploid populations of the Pannonian-Balkans indicating that this region formed a northern Pleistocene refugium. A separate polyploid complex centred on Greece, comprises diploid, tetraploid and hexaploid populations with two endemic haplotypes and suggests long-term persistence of populations in the southern Mediterranean. Three chloroplast DNA (cpDNA) haplotypes were common among tetraploids in Spain and Italy, with diversity reducing northwards suggesting expansion from the south after the Pleistocene. Our cpDNA and ploidy data indicate at least six independent origins of polyploids.

Keywords: chloroplast DNA, DNA sequence, multiple origins, Pleistocene, polyploidy, Pteridophyte

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Introduction

Phylogeographical methods have provided the opportunity to elucidate the effects of large-scale historical events (such as Pleistocene climate change) on the distribution and subdivision of biota and put into context the role of reproductive biology in the genetic structuring of species. A broad spectrum of organisms has provided grist to the phylogeographical mill including animal (e.g. grasshopper, Cooper *et al*. 1995; weta, Trewick *et al*. 2000; newt, Wallis & Arntzen 1989; bear, Taberlet & Bouvet 1994) and plant (e.g. moss, Shaw 2000; grass, Sahuquillo & Lumaret 1999; trees, Demesure *et al*. 1996; Dumolin-Lapègue *et al*. 1997) taxa, but as yet there are few published data relating to fern phylogeography (but see Rumsey *et al*. 1996).

Correspondence: S. A. Trewick. †Present address: Department of Plant and Microbial Sciences, University of Canterbury, Private Bag 4800, Christchuch, New Zealand. Fax +64 3364 2083; E-mail: s.trewick@botn.canterbury.ac.nz

Ferns produce abundant, very small, wind-dispersed haploid spores and are therefore capable of long-distance dispersal (van Zanten 1978), and in some instances can found populations from single spores via intragametophytic fertilization (Schneller & Holderegger 1996; Vogel *et al*. 1999a). Ferns may therefore be expected to have high levels of gene flow and thus be of little utility for phylogeographical studies (e.g. Hooper & Haufler 1997; Maki & Asada 1998; Schneller *et al*. 1998). However, several studies have revealed interpopulation diversity and evidence of restricted gene flow (Li & Haufler 1999; Ranker *et al*. 2000; Pryor *et al*. 2001), and it is evident that mating systems in ferns are highly variable (Soltis & Soltis 1987). In addition, the evolution of autopolyploids in many ferns (and other plants, Soltis & Soltis 1993), which is often associated with distinctive reproductive strategies, increases the likelihood of reproductive barriers and thus the potential for the development of spatial structure (Vogel *et al*. 1999b).

Asplenium ceterach is a relatively small fern with thick, simply divided fronds (up to 20 cm) that are heavily coated

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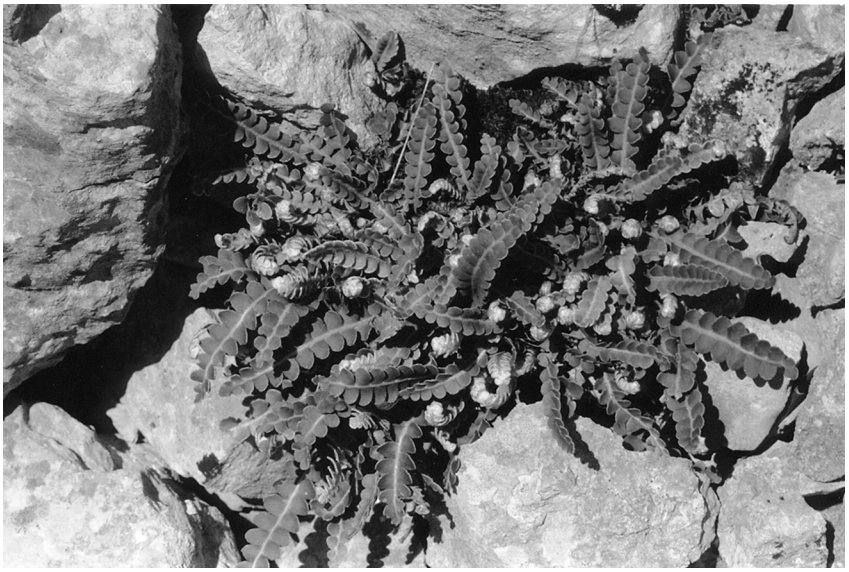

Fig. 1 Diploid *Asplenium ceterach* growing among rocks in coastal Croatia.

on the underside with pale reddish-brown scales (hence the common name, rustyback) (Fig. 1). *A. ceterach* inhabits base-rich rocks and mortared walls in Europe and north Africa, and the near East, expanding eastwards in scattered populations as far as western China. The taxon is a complex comprising diploid, tetraploid and hexaploid forms that are generally treated as subspecies (Lovis 1977). The tetraploid *A. ceterach* ssp. *ceterach*, is the most common and widespread, occurring throughout the range from the Canary Islands in the west to China in the east (Fig. 2). The diploid, *A. ceterach* supsp. *bivalens* (DE Meyer) Greuter & Burdet (1980), is known mainly from central eastern Europe (Croatia, Bulgaria, Hungary and Romania) with, as far as is known, peripheral populations in Italy, Greece and Turkey. Hexaploids have been reported from Sicily, Greece and Cyprus but there are few records (Vida 1963; Vianne *et al*. 1993; Pintér *et al*. 2002).

The distribution of diploid and tetraploid *A. ceterach* suggests that they have different colonizing abilities (Fig. 2). Much of the modern range of tetraploid *A. ceterach* is in regions that were glaciated or subject to periglacial conditions during the Pleistocene, implying range expansion in the Holocene. The modern occupation of walls is testimony to the colonizing ability of tetraploid *A. ceterach*, and contrasts with the localized distribution of diploid *A. ceterach*. This pattern fits well with the differing breeding systems of the two ploidy levels inferred from allozyme data: diploids are out-breeders and tetraploids are highly inbred (unpublished data).

Several Pleistocene glacial refugia have been proposed for the European biota (Taberlet *et al*. 1998; Hewitt 1999, 2000). The consensus from previous molecular studies is that taxa emerged from one or more of three southern areas (Iberia, Italy, Balkans) following the last glacial, and presumably also during interglacials. However, congruence

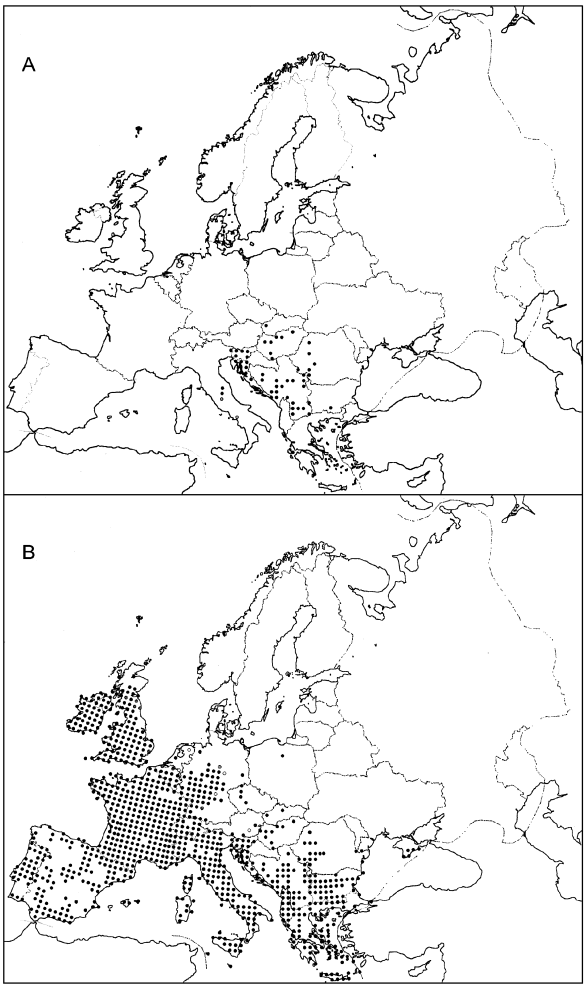

Fig. 2 European distribution of diploid (a) and tetraploid (b) *Asplenium ceterach*. (Images provided by Paul Williams, Natural History Museum- Worldmap/Atlas Florae Europeae, revised from Jalas & Suominen 1972).

in phylogeographical patterns is observed only on a broad scale and most taxa show distinctive patterns of genetic diversity throughout Europe. Molecular and fossil pollen evidence also indicates that some tree species such as common beech (Demesure *et al*. 1996), black alder (King & Ferris 1998) and Scots pine (Sinclair *et al*. 1999) survived the Pleistocene in relatively northern locations (i.e. close to the periglacial zone).

Vogel *et al*. (1999b) observed that high regional densities of diploid *Asplenium* species paralleled those of tree species inferred from fossil pollen data, and indicated the existence of several refugia in Europe. In contrast many polyploid *Asplenium* spp. are widespread in Europe and allozyme data from two species (*A. trichomanes* L., *A. ruta-muraria* L.) have shown them to have comparatively low genetic variability suggestive of recent expansion (Schneller 1996; Vogel *et al*. 1999a; Suter *et al*. 2000). A similar pattern is reported from the *Cardamine* polyploid complex (Brassicaceae) for which an historical biogeographical scheme has been proposed in which diploid taxa occupied southern European regions during and after the Pleistocene, and derived polyploids colonized northwards in the Holocene (Franzke & Hurka 2000). Likewise, in South America, populations of diploid *Turnera* are thought to be relictual, whereas the range of tetraploid populations increased at the end of the Pleistocene (Solís Neffa & Fernández 2001). It has been observed that the majority of plant polyploid complexes probably evolved during the Pliocene/Pleistocene, and that the widespread distributions of particular cytotypes may generally reflect an enhanced ability to colonize habitats revealed following climate amelioration after the Pleistocene (Stebbins 1950, 1971, 1985).

The current distribution of *A. ceterach* (Fig. 2) implies that the Balkans was a refugium for diploids and that the tetraploids colonized north and west following the last glaciation to occupy newly revealed habitat. If tetraploid *A. ceterach* that are widespread in Europe were derived from a single origin of polyploidy in the Balkans then we would expect little if any chloroplast diversity and thus little evidence of phylogeographical structure. Alternatively, if several distinct diploid lineages exist and have independently produced tetraploid taxa this could provide informative phylogeographical signal.

We used cpDNA sequences to explore patterns of diversity and distribution of the *A. ceterach* polyploid complex throughout Europe. We seek to identify chloroplast diversity within the known populations of diploid *A. ceterach* in the Pannonian-Balkans and Italy, locate unreported diploid populations that might be alternative progenitors of colonizing tetraploids, determine whether there have been multiple origins of polyploids, identify the origins of hexaploids, and map phylogeographical structure with a view to further resolution of Pleistocene refugia. We believe that the dataset presented here is one of the geographically broadest studies of plant phylogeography in Europe and to be among the first to report on the population level genetic structure of a fern on such a spatial scale.

Materials and methods

We obtained fronds of *Asplenium ceterach* from plants in populations throughout the extent of their range in Europe (see Fig. 2). Supplementary material from outlying populations in North Africa and Asia was sourced from herbaria at the Natural History Museum, London (BM), and Royal Botanical Garden, Edinburgh (E). Vouchers from each population are deposited at BM.

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 pH 8.0, 1.4 M NaCl, 20 mM EDTA), 50 μL sarkosyl (10% *N*-lauryl sarcosine, 100 mM Tris–HCl pH 8.0, 20 mM EDTA) and 5 μL β-mercaptoethanol, and were incubated at 60 °C for 1 h. An equal volume of sevac (chloroform/isoamyl alcohol 24:1) was added and the mixture shaken and centrifuged at 13 000 r.p.m. for 3 min. Supernatants were pipetted into fresh tubes and combined with a 2/3 volume of cold 100% isopropanol. DNA was pelleted after 15–60 min by spinning at 13 000 r.p.m. for 3 min and then washed with 500 μL 70% ethanol by spinning briefly. The 70% ethanol was discarded and the pellet dried and dissolved in 30 μL of water.

We targeted noncoding regions of cpDNA as described by Taberlet *et al*. (1991). A previous survey demonstrated that this fragment was likely to reveal an informative amount of diversity (Vogel *et al*. 1996) and it has been shown that the chloroplast is maternally (i.e. uniparentally) inherited in *Asplenium* ferns (Vogel *et al*. 1998), as in most other plants. Polymerase chain reaction (PCR) was used to amplify the *trnL-trnF* chloroplast gene fragment (Taberlet *et al*. 1991; Vogel *et al*. 1996). The primers Fern-1 (5′-GGCAGCCCCCARATTCAGGGRAACC-3′) and f (Taberlet *et al*. 1991) were used to amplify the *trnL* intron and *trnL/trnF* intergenic spacer. PCR 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 utilized Big Dye v2.0 chemistry (PE Biosystems) and the original PCR primers in quarter volume reactions but otherwise following the manufacturer's protocols. Cycle sequencing products were electrophoresed on an ABI automated sequencer (PE

Biosystems). Sequence alignments used SEQED Version 1.03 or SEQUENCHER Version 3.0.

Ploidy was determined for the majority of material using the evidence of allozyme banding patterns (Vogel *et al*. 1999a; Suter *et al*. 2000; unpublished data) and confirmed for a subsample of specimens using cytology (Manton 1950) and spore measurement (Vida 1963; Nyárády & Vicol 1967; Pintér 1995a).

Results

Sequence data

Primers Fern-1 and f amplified a fragment of ≈ 900 bp comprising the chloroplast *trnL* intron and the intergenic spacer between *trnL* and *trnF*. Nine distinct haplotypes were revealed among our data, and these are identified with colour codes (Table 1). Eight of the haplotypes differed by between one and three single-nucleotide substitutions or single-nucleotide indels. One (green) contained six single-nucleotide substitutions and a 17-bp tandem repeat. Sequence variation distinguishing the majority of haplotypes was within the *trnL* intron, but the 17 bp tandem repeat and three of the single-nucleotide substitutions specific to the green haplotype were in the intergenic spacer between *trnL* and *trnF* (alignments are available on request). Representative sequences are deposited at GenBank (Accession nos AF516256–AF516264).

We obtained cpDNA sequences from 331 individuals representing 143 populations (Table 2). Between 1 and 8 (mean = 2) individuals were sequenced per population. Most populations in our sample contained a single haplotype, although this must to a greater or lesser extent reflect sample sizes, 60% of population samples comprised a single individual. One population in Romania contained four haplotypes (three of them rare) among a sample of eight plants.

Table 1 Summary of chloroplast *trnL* sequence variation among nine haplotypes observed in *Asplenium ceterach*. Nucleotide positions in bold highlight the least common variant within the ingroup. An aligned reference sequence from the closely related species, *Asplenium dalhousiae*, is provided for comparison

	11222333334466788 8
	46138227782303056----------7
	98479793408359963 9
A. dalhousiae	GTTCGCACC-GCCCGT----------
GREEN	**A**CT**C**GT-CC-**A**C**A**C**CTTCACTAATTCTTAAGT**
BLUE	GCT**CA**T-CC-GCCCGT----------
YELLOW	GCT**C**GT-CC-GCC**T**GT----------
RED	GCTTGT-CC-GCCCGT----------
TURQUOISE	GC-TGT-CC-GCCCGT----------
ORANGE	GCTTGT-CC**G**GCCCGT----------
PINK	G**A**TTGT-CC-GCCCGT----------
BROWN	GCTTGT-**TT**-GCCCGT----------
PURPLE	GCTTG**CA**CC-G**A**CCGT----------

Phylogenetics

Using a reference sequence from the closely related diploid species *Asplenium dalhousiae* (Pintér *et al*. 2002) we constructed a minimum-spanning tree for the nine *A. ceterach* haplotypes scoring nucleotide substitutions, single-nucleotide indels and the 17 bp tandem repeat equally (Fig. 3a). This tree is robust and fully resolved with no conflicts. The single internal branch clusters two groups of haplotypes that to some extent also share geographical associations. The single step differences among all of the known Balkan region diploid lineages (red, turquoise, pink, orange) support other inferences that these lineages are native to this area. Similarly the Greek haplotypes (blue, yellow) share a common (missing) ancestor.

Multiple origins of polyploids

Seven haplotypes were observed in diploids. Three rare haplotypes (turquoise, orange, pink) were found in diploids only in the Pannonian-Balkan region, the area where the majority of diploid populations have been reported (Fig. 2). The most common diploid lineage (red) is present in the Balkans, Italy and Kephalonia (Greece). Three other diploid lineages (brown in Sicily, yellow and blue in Greece) were endemic to the southern Mediterranean. Only four of the haplotypes (red, turquoise, yellow, blue) found in diploids were also found in polyploid individuals (4× or 4× and 6×). The two most divergent haplotypes in our dataset (green, purple) were observed only in tetraploid and hexaploid populations, respectively.

From the haplotype tree (Fig. 3a) we derived a minimum-spanning hypothesis for the relationship of each of the observed haplotype–polyploid combinations with the assumptions that each haplotype evolved only once and that chromosome multiplication arose through autopolyploidy (Vida 1965) (Fig. 3b). Four separate chromosome duplication events are indicated by tetraploids with turquoise, red, yellow and blue haplotypes. The blue haplotype is also found in the scattered but widespread hexaploid taxon from the islands of Pantellaria, Sicily, Kephalonia, Cyprus and mainland Greece. A further two chromosome multiplication events are implied by the existence of the green tetraploid and purple hexaploid even though the diploid ancestors of these have not been located. Multiple polyploidization events may well have occurred within the lineages, but this cannot be elucidated from chloroplast data alone. The two polyploid haplotypes that lack diploid ancestors have the most divergent chloroplast sequences.

Table 2 Summary of *Asplenium ceterach* haplotype data by region including numbers of populations and individuals sampled

Region		*N* popln.	*N* plants	Haplotype/ploidy *N* 2×	4×	6×	?×
Canary Is.		2	3		3R		
Corsica		5	6		6G		
Crimea		1	1		1G		
Croatia		12	17	15R, 2O			
Cyprus		6	14		4R	5B	6P (4x/6x)
France		14	53		41G, 12T		
Germany		6	24		21G, 2T, 1R		
Georgia		2	5		1G, 4R		
Greece	Corfu	1	1		1B		
	Crete	3	14	10B	4Y		
	Kephalonia	8	20	3R	4T, 2B		10B, 1R
	Peloponnisos	14	28	14Y	3T	1B	1R, 3Y, 7B
Hungary		16	23	16R	5G, 2R		
Italy	mainland	11	29	4R	4G, 1T, 20R		
	Sicily	10	31	4R, 2Br	15T, 3R	7B	
	Pantellaria	1	3			3B	
Romania		2	9	2T, 5R, 1O, 1Pi			
Serbia		1	1	1R			
Spain	mainland	14	22		9G, 3T, 10R		
	Majorca	3	3		2T, 1R		
Switzerland		2	11	10G, 1T			
Turkey		2	2	1G, 1R			
UK		7	11	11T			
	Europe total	143	331				
	Morocco	2	4		4R		
	Pakistan	1	1		1G		
	India	1	1				1R
	Golan	1	1		1B		
	S. Arabia	2	2		2R		
	China	1	1		1R		

Phylogeography

We found that most populations of *A. ceterach* we examined from the Balkans were diploids but that diploid populations were less common in Italy and Greece, and absent from most of western Europe including Spain (Figs 3b and 5a). Of seven diploid cpDNA lineages, four occurred in the Balkans, one (red) being shared with Italy (mainland and Sicily) and Kephalonia (Greece), and three being endemic (orange, turquoise, pink). Greece has two endemic diploid lineages (yellow in Peloponnisos, blue in Crete) and polyploid derivatives of these which are restricted to Greece or the southern Mediterranean, and Sicily has an endemic diploid haplotype (brown) (Figs 3b and 5a). Excluding the Mediterranean lineages, the overwhelming majority of diploids were red (89%) and we found no diploid with the green haploytpe.

The majority of north Africa, western Europe including the Canary Islands, Morocco, Spain and France contained no diploid populations of *A. ceterach*, but tetraploids of three lineages (green, turquoise, red) were widespread (Figs 4 and 5b). Despite the absence of diploids to the west, haplotype diversity in Spain and mainland Italy are the same, and higher than tetraploid haplotype diversity in the Balkans. In the west the number of tetraploid haplotypes diminishes northwards, three in Spain, two in France, one in the UK (Fig. 4). We calculated regional diversity as the proportion of the total number of ploidy/haplotype permutations observed in the entire data set that were encountered in each region. We then adjusted this diversity score according to the overall rarity of each ploidy/haplotype by weighting presence scores. For example diploid/red was encountered in 4 of 8 regions and therefore has a rarity-adjusted score of 2 (Table 3). This highlights that overall diversity is highest in Greece and lowest in the northwest (UK). When diversity scores are weighted for rartity of ploidy/haplotype the distinctiveness of the southern Mediterranean is evident (Table 3).

Discussion

Multiple origins of polyploids

Our data provide further evidence that in ferns (see Werth *et al*. 1985a,b) just as in seed-forming plants (Doyle

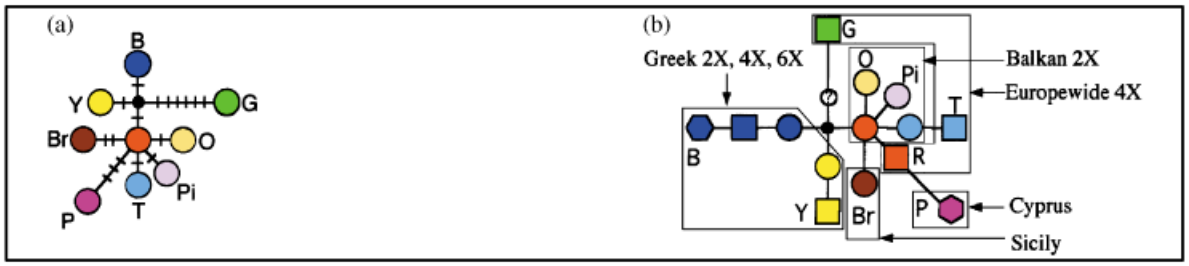

Fig. 3 Minimum spanning trees of nine *Asplenium ceterach* haplotypes (a), and observed haplotype/ploidy combinations (b). Geographic ranges of these are indicated.

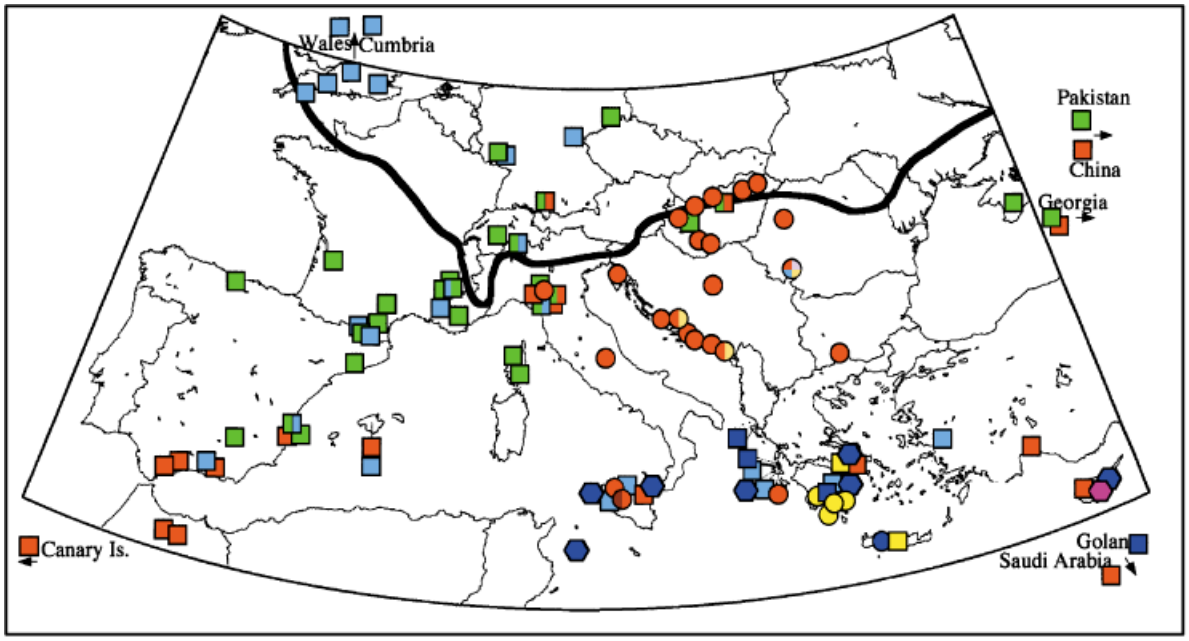

Fig. 4 Distribution of *trnL* haplotypes in populations of *Asplenium ceterach* in and around Europe. Symbol colour indicates haploytpe and symbol shape indicates ploidy (circle, 2×; square, 4×; hexagon, 6×). Polymorphic populations are indicated by combination of fill colour. The approximate extent of the Pleistocene periglacial (including montane glaciers in Italian Alps) is indicated by a black line (redrawn from Taberlet *et al*. 1998).

et al. 1999; Segraves *et al*. 1999; Sharbel & Mitchell-Olds 2001; Yamane & Ohnishi 2001), polyploidy can evolve many times. Current evidence indicates that gene flow between ploidal levels in *Asplenium ceterach* is predominantly unidirectional and occurs only during the generation of tetraploids from diploids; most spores produced by triploid hybrids are sterile (but see Pintér 1995b) or may result in hexaploid formation via unreduced gametes (see Reichstein 1981). Populations of mixed ploidy were not observed in *A. ceterach*, and few triploid hybrids have been encountered, apparently arising from rare migration events (unpublished data). Introgression into an established diploid or tetraploid population by spores from the alternative ploidal level is unlikely. However, we have evidence of some diploid and tetraploid populations with more than one haplotype, and although tetraploids are primarily inbreeders, gene flow between haplotypes has been detected with allozymes (unpublished data).

Several attributes of polyploids have been cited as potentially giving them competitive advantages over diploid relatives (e.g. increased heterozygosity, allelic diversity and enzyme multiplicity) (Soltis & Soltis 2000). However, for ferns the potential for intragametophytic selfing that can arise in polyploids offers an obvious and profound advantage in colonization and establishment (Schneller & Holderegger 1996), especially in the context of large-scale shifts in habitat availability such as would have resulted from glacial cycling in the Pleistocene. Long-distance, single-spore colonization is very probably the principal reason for the geographical success of tetraploid *A. ceterach* which has been able to take advantage of virgin habitat revealed by the retreat of Pleistocene glaciers and periglacial conditions. This advantage operates where new habitat is available because minority cytotype exclusion is expected to prevent invasion of existing diploid populations (Levin 1975; Vogel

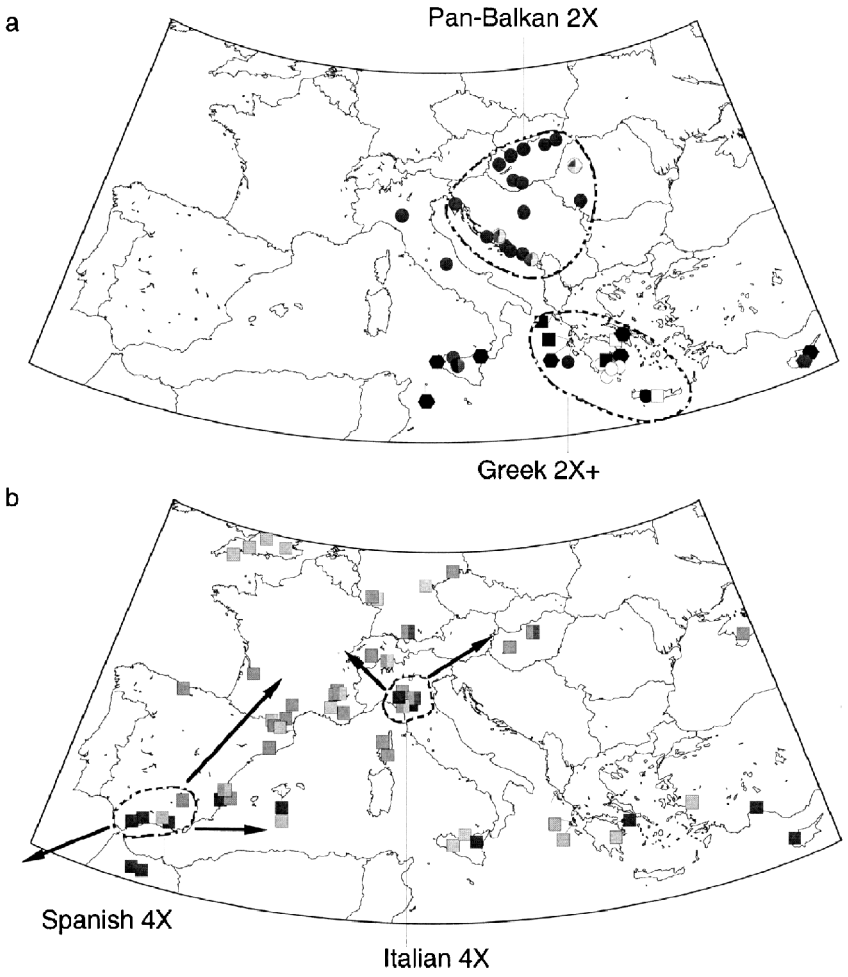

Fig. 5 Distribution of diploid and localized polyploids (a) and widespread tetraploids (b). Principal refugia and proposed expansion routes are indicated.

Table 3 Presence/absence of haplotype–ploidy combinations in *Asplenium ceterach* by region, with diversity scores for each region adjusted by rarity (i.e. the frequency of their occurrence among regions)

	2×T	2×R	2×B	2×Y	2×Br	2×O	2×Pi	4×G	4×T	4×R	4×B	4×Y	6×B	6×Pu	Diversity score
Presence by region															
UK	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0.07
Cyprus	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0.21
France+	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0.21
Spain	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0.21
Italy	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0.29
Sicily	0	1	0	0	1	0	0	0	1	1	0	0	1	0	0.36
Balkans	1	1	0	0	0	1	1	1	0	1	0	0	0	0	0.43
Greece	0	1	1	1	0	0	0	0	1	1	1	1	1	0	0.57
Rarity weighting	8	2	8	8	8	8	8	2	1.6	1.1	8	8	2.7	8	
															Rarity adjusted diversity
UK	0	0	0	0	0	0	0	0	1.6	0	0	0	0	0	0.11
Cyprus	0	0	0	0	0	0	0	0	0	1.1	0	0	2.7	8	0.84
France+	0	0	0	0	0	0	0	2	1.6	1.1	0	0	0	0	0.34
Spain	0	0	0	0	0	0	0	2	1.6	1.1	0	0	0	0	0.34
Italy	0	2	0	0	0	0	0	2	1.6	1.1	0	0	0	0	0.48
Sicily	0	2	0	0	8	0	0	0	1.6	1.1	0	0	2.7	0	1.10
Balkans	8	2	0	0	0	8	8	2	0	1.1	0	0	0	0	2.08
Greece	0	2	8	8	0	0	0	0	1.6	1.1	8	8	2.7	0	2.81

et al. 1999b; and references therein). The genetic advantage of having multiple genomic copies and the opportunity for recombination among polyploids is expected to generate sufficient fitness benefits to overcome competition only in restricted circumstances (Felber 1991; but see also Rodríguez 1996). In plants in general, polyploidy does not appear to confer a consistent advantage, such as greater tolerance of severe ecological or climatic conditions (Ehrendorfer 1980; Stebbins 1985). In *A. ceterach* there is little, if any, evidence that tetraploids have been able to out-compete diploids that are already established (Fig. 5). Inbreeding tetraploids will also be free from the negative effect of gene flow from which outccrossing diploids on the edge of a range might suffer (Peck *et al*. 1998).

Phylogeography of A. ceterach

Our data reveal two distinct phylogeographical patterns within the *A. ceterach* polyploid complex that relate to the diploid and polyploid taxa. Within this division a further distinction is evident in the geographical relationship of the diploid and polyploid taxa of central Europe, and of the southern Mediterranean. As obligately outcrossing diploid *A. ceterach* is expected to have limited likelihood of establishment at new sites, the existence of diploid populations comprising endemic and/or multiple chloroplast lineages is strong evidence of long-term occupation of an area. These characteristics are seen in two regions of Europe (Pannonian-Balkans and Greece) and indicate that these regions were refugia for diploid *A. ceterach*. The Pannonian-Balkans, Kephalonia (Greece), Sicily and Italy are linked by the co-occurrence of red diploid populations which might be evidence of a now fragmented older pattern, but in other respects these regions are quite distinct.

In the Pannonian-Balkans, tetraploid populations are comparatively rare and comprise lineages that are widespread across Europe. Although two of these lineages are represented in Balkan diploids (turquoise, red), the tetraploids are not necessarily recently derived from the diploids. In Hungary, allozyme analysis of all known populations of *A. ceterach* indicates that red tetraploids sampled alleles that are not found in the present red diploids (unpublished data). This implies that if, as it appears, the Pannonian-Balkans was the initial source of the widespread European red tetraploids, they probably did not arise very recently (i.e. not following the last glacial). Alternatively, tetraploids with the red haplotype may have evolved independently, in different regions. The common tetraploids may well have reinvaded Hungary in just the same manner that they colonized the rest of Europe, perhaps from Weichselian refugia in Spain and/or Italy. The rarity (and possible absence in one instance) of the turquoise and green haplotypes among diploids in the Pannonian-Balkan is consistent with the notion of an earlier origin of the tetraploids, as glacial cycling could have resulted in frequency changes or extinction via lineage sorting in the diploid refugium. Importantly, the dominance of diploids among Hungarian and Balkan *A. ceterach* populations surveyed, and the existence of four diploid cpDNA lineages in the Pannonian-Balkan region suggest a northern Pleistocene refugium (Willis *et al*. 2000; Stewart & Lister 2001).

In stark contrast, the endemic Greek diploids (blue, yellow) and their polyploid derivatives occur in adjacent populations, and nowhere else except on other southern Mediterranean islands. Northward colonization from Greece may have been impeded by the presence of the Balkan diploids, ubiquitous common tetraploids, or poor adaptation for the northern conditions (Fig. 4). A similar pattern of Greek or southern Mediterranean isolation of lineages is reported for black alder (King & Ferris 1998) and the meadow grasshopper (Hewitt 1999).

Superimposed on the Balkan diploid and Greek patterns is the distribution of three wide-ranging tetraploid lineages (Fig. 5). This group of tetraploids (green, turquoise, red) have spread widely throughout Europe and beyond, one or more have reached the Canary Islands, Sicily, Kephalonia and Cyprus, and pairs of lineages occur in polymorphic populations. Populations of tetraploids on oceanic islands probably arose from chance single-spore colonization events. The clinal distribution in northwestern Europe is typical of the gradient of diversity observed in many taxa (Hewitt 1999). All three lineages are found in two areas, southern Spain and northern Italy in the Alpe Apuani. Northwards of these areas, first the red then the green tetraploid becomes rarer, so that in the UK only turquoise is found. This suggests that one or both of these areas (southern Spain, northern Italy) were refugia for the tetraploid lineages and that the local mountain ranges provided habitat gradients through Pleistocene climate cycling (Hewitt 1996). The existence of red diploids in the Alpe Apuani is further evidence that this area served as a refugium.

Thus, *A. ceterach* demonstrates the trait now widely observed among European taxa, of reduction in genetic diversity during northward postglacial range expansion from southern Weichselian refugia. But, in addition *A. ceterach* provides compelling evidence for a northern Pleistocene refugium in the Pannonian-Balkans and an extensive southwestern refugium in the Mediterranean centred on Greece. It is expected that continuing work using nuclear markers will allow us to explore in more detail the evolution of polyploids, colonization routes and speciation processes in this complex.

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