Systematic Botany (2005), 30(1): pp. 52–59
© Copyright 2005 by the American Society of Plant Taxonomists

Isozymic Contribution to the Systematics of the *Asplenium seelosii* Group

SANTIAGO PAJARÓN,[1,3] LUIS G. QUINTANILLA,[1,2] and EMILIA PANGUA[1]

[1]Departamento de Biología Vegetal I, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain;
[2]Present address: Área de Biodiversidad y Conservación, Escuela Superior de Ciencias Experimentales y Tecnología, Universidad Rey Juan Carlos, 28933 Móstoles, Madrid. Spain.
[3]Author for correspondence: phone +34 913 945074; fax +34 913 945034; e-mail (spajbot@bio.ucm.es)

Communicating Editor: Paul S. Manos

ABSTRACT. The systematics of the *Asplenium seelosii* complex have been debated for a long time. This complex includes strictly rupicolous plants that live on limestone cliffs mainly in mountains of southwest Europe: the Alps, the Pyrenees, and several mountain ranges of the Eastern Iberian Peninsula. The disjunct distribution of the populations and several morphological characters, i.e., leaf indumentum and the structure of the perispore, have been used to distinguish species and subspecies. The goal of this study was to evaluate the different systematic treatments of this complex by means of isozyme electrophoresis. Seventeen populations throughout the range of the complex were studied, and 15 enzymatic systems were assayed. There was no within population genetic variation and genetic identity between populations varied widely. Analysis of isozymic data clearly differentiated two groups corresponding to the species proposed, *Asplenium seelosii* and *A. celtibericum*, but these data do not support the recognition of subspecies in this complex.

Plants of *Asplenium seelosii* s.l. are diminutive, strictly rupicolous and emerge from small crevices in more or less vertical limestone cliffs and rock overhangings (Figs. 1 and 2). This complex has a disjunct distribution in Europe. Some populations are located in the Alps, especially in northern Italy, around the Dolomites, and extending from there to Austria, Germany and Slovenia. The remaining populations may be found on scattered limestone outcrops in the eastern half of the Iberian Peninsula, from the Pyrenees in the north, including some localities in France, to the Betic Mountains in southern Spain. Two additional localities have been found in northern Morocco (Fig. 3).

The leaves consist of a relatively long green petiole and a small lamina which may vary from entire to divided into three separate segments (Figs. 1 and 2). Individuals from the Alps and surrounding regions mostly have a divided lamina, and the lamina and petiole are covered with glandular hairs. Plants from the Iberian Peninsula have an entire or almost entire lamina and are glabrous. Based on the presence or absence of glandular hairs, some authors recognize two subspecies: subsp. *glabrum* (Litard. & Maire) Rothm. and subsp. *seelosii* (Rothmaler, 1937; Nogueira and Ormonde 1986; Viane et al. 1993). Starting with this indumentum distinction, Rivas-Martínez (1967) used leaf morphology and ecological differences to separate the Iberian plants as a new species, *A. celtibericum* Rivas-Martínez. Lovis (1987) conducted cytological research on experimental hybrids between plants from the Alps and plants from the northeastern edge of the Iberian Peninsula (East Pyrenees) and demonstrated a close relationship between these entities. Moreover, plants from the Iberian Peninsula contain some morphological variation. Those living in the Pyrenees Mountains, in northeast Spain and southeast France, are similar to plants from the Alps, because both have mainly divided laminae. The remaining Spanish plants have mostly entire laminae, and show differences in the perispore (Pangua 1989). Because of this populational variation, Cubas et al. (1993) expanded the analysis of spore features by adding a detailed study of perispore morphology and spore wall structure. As a consequence they proposed a new subdivision of the complex into two species with two subspecies each. Thus, the proposed systematics of the *Asplenium seelosii* complex is as follows:

Asplenium seelosii Leybold

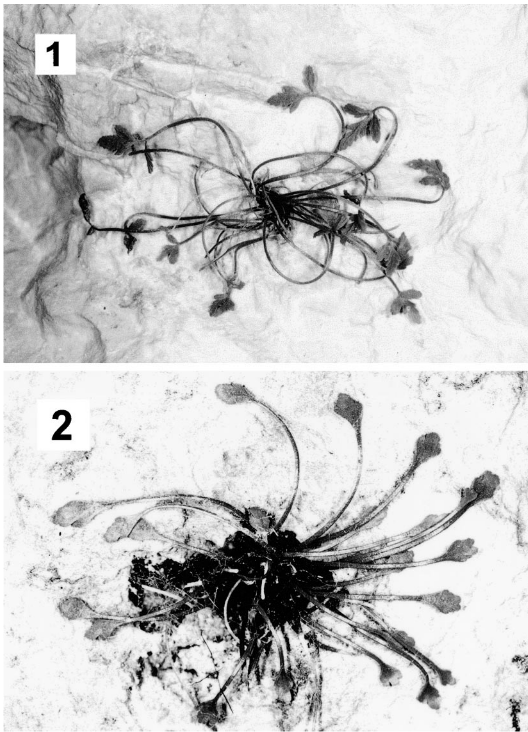
subsp. *seelosii*, from the Alps and neighboring areas.
subsp. *catalaunicum* (Bolòs & Vigo) Montserrat, from the eastern Pyrenees (NE Spain and SE France).

Asplenium celtibericum Rivas-Martínez

subsp. *celtibericum*, from the eastern half of the Iberian Peninsula and northern Morocco.
subsp. *molinae* Cubas, Pardo & Rivas-Martínez, from the central Pyrenees (NE Spain).

A complete historical revision of the taxonomic background of the group is found in Lovis (1987) and in Cubas et al. (1993). However, no general agreement has yet been reached about the classification of this complex (Prelli 2001).

Asplenium L. is one of the largest genera of pteridophytes in Europe with ca. 40 species and subspecies (Viane et al. 1993). Cytological studies have shown that most of these taxa are members of auto- or allopolyploid complexes (Lovis 1977; Reichstein 1981; Sleep 1983). The use of isozyme electrophoresis, in some cases, has confirmed proposed relationships, as for example in the *A. foreziense* Legrand *ex* Giraudias complex (Pajarón et al. 1996), or contributed to a better



FIGS. 1–2. Photographs of plants of *Asplenium seelosii* in their natural habitat. 1. Plant from population BAG with divided lamina, the typical habit of *Asplenium seelosii*. 2. Plant from population SOM with entire lamina, the typical habit of *Asplenium celtibericum*.

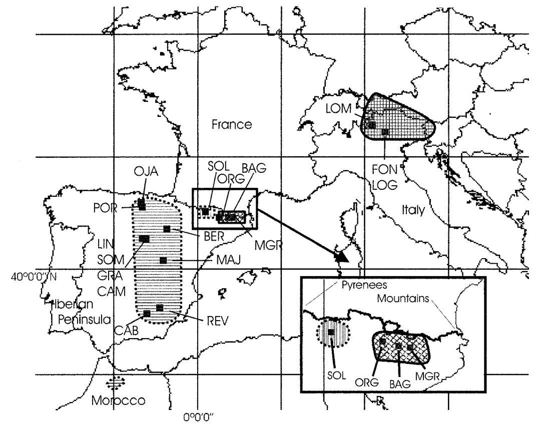


FIG. 3. Map showing the distribution of the four taxa accepted by Cubas et al. (1993), and the location of the studied populations according to the acronyms in Table 1. Solid line: *Asplenium seelosii*; Vertical squared area: *A. seelosii* subsp. *seelosii*; Oblique squared area: *A. seelosii* subsp. *catalaunicum*. Dotted line: *A. celtibericum*; Vertical lined area: *A. celtibericum* subsp. *molinae*; Horizontal lined area: *A. celtibericum* subsp. *celtibericum*.

understanding of the relationships among the taxa, as in the *A. billotii* F.W. Schultz complex (Herrero et al. 2001). Recently, the use of DNA sequencing techniques has contributed to the clarification of complicated and poorly understood groups, e.g., the *A. ceterach* L. group (Van den heede et al. 2003).

Asplenium seelosii s.l. is distinctive within this genus because it is not known to have contributed to any polyploid complexes. All plants examined of *A. seelosii* are diploid (Meyer 1957; 1967; Lovis 1987; Cubas et al. 1993) and no auto- or allopolyploids derived from them are known. Moreover, only one hybrid with another *Asplenium* species is known in the wild (Reichstein 1981; Prelli 2001). This is remarkable given how frequently most European *Asplenium* species hybridize in nature, and the fact that *Asplenium seelosii* often grows in close proximity (i.e., on the same rock faces) to *A. ruta-muraria* L., *A. fontanum* (L.) Bernh., and members of the *A. trichomanes* complex.

In the study of ferns isozyme electrophoretic data have been applied mainly to investigate the origin and evolution of polyploids (Werth et al. 1985; Bryan and Soltis 1987; Ranker et al. 1989; Barrington 1990; Gastony 1990; Pryer and Haufler 1993; Haufler et al. 1995), but isozymic data can also be used to study genetic divergence within and among species (Haufler 1985a; Levin 2001). When used in conjuction with other lines of evidence, allozyme divergence can be used to tip the scales in support of species recognition, especially in cases such as that presented here of two doubtfully distinct species that prove to be divergent at allozyme loci (Crawford 2000).

This study applies isozyme starch gel electrophoretic surveys of natural populations of *Asplenium seelosii* s.l. to investigate the presence of genetic differences among them. Such differences may be used to characterize populations from different parts of the distribution and validate the classification proposed for the complex.

MATERIALS AND METHODS

Plant material was collected from populations of each of the proposed subspecies as listed in Table 1, and represented in Fig. 3. In most populations leaves were gathered from about 30 plants. In some small populations fewer than 20 plants were sampled but the sample size always represented at least 75% of the total number of plants in those locations. It must be noted that only one population from *A. celtibericum* subsp. *molinae* was studied. This subspecies is known only from two sites, the area from which we collected population SOL, and another limestone mountain range, Sierra de Guara, in Rodellar not far from the former. We were not able to find it in the second location, from which it was last collected in 1967. Only herbarium material was used in the morphological study by Cubas et al. (1993).

Leaves were kept cold inside plastic bags until the extraction was performed. PVP-phosphate grinding buffer was used (Soltis et al. 1983), and extracts were absorbed on 6×4 mm paper wicks and then frozen and kept at−86°C.

Starch gel electrophoresis was conducted following Soltis et al. (1983) and Haufler (1985b). The enzymes analyzed with the names and acronyms used by Acquaah (1992) were: leucine aminopeptidase (LAP), phosphoglucoisomerase (PGI), isocitrate dehydro-

TABLE 1. Collection localities of the populations of *Asplenium seelosii* s.l. studied. Acronyms used in the text and number of plants used for electrophoresis are included. All vouchers are in MACB.

Population Acronym	Locality	Number of plants
CAM	Guadalajara, Campisábalos, 22-IX-1999, 1370 m, *S. Pajarón* & *L. G. Quintanilla*	24
GRA	Segovia, Grado del Pico, 22-IX-1999, 1325 m, *S. Pajarón* & *L. G. Quintanilla*	23
SOM	Guadalajara, Somolinos, 1340 m, 3-VII-1999, *J. M. Iriondo, M. J. Albert, S. Pajarón* & *E. Pangua*	50
LOM	Italy: Lombardía, prov. Varese, Valganna, 415 m, 24-VIII-1998, *S. Pajarón, E. Pangua, A., G.,* and *C. Peroni.*	12
MGR	Gerona, Montgrony, 1330 m, 6-X-1999, *S. Pajarón, E. Pangua,* & *L. G. Quintanilla*	27
BAG	Barcelona, between Bagá y Greixá, 1000 m, 6-X-1999, *S. Pajarón, E. Pangua,* & *L. G. Quintanilla*	27
BER	Soria, Plana de Beratón, 1450 m, 30-IX-1999, *S. Pajarón* & *E. Pangua*	20
LIN	Guadalajara, Somolinos, 1400 m, 29-VI-2000, *S. Pajarón* & *E. Pangua*	12
ORG	Lérida, Organya, Cañón del río Segre, 5-X-1999, *S. Pajarón, E. Pangua,* & *L. G. Quintanilla*	6
SOL	Huesca, Peña Solana, 7-X-1999, 1660 m, *S. Pajarón, E. Pangua,* & *L. G. Quintanilla*	15
OJA	Burgos, Quintanilla de Ojada, 650 m, 26-X-2000, *S. Pajarón* & *E. Pangua*	19
POR	Burgos, between Cascajares de Bureba y Aldea del Portillo del Busto, 1000 m, *S. Pajarón* & *E. Pangua*	30
MAJ	Cuenca, Las Majadas, Los Callejones, 1420 m, 2-X-2000, *S. Pajarón* & *E. Pangua*	35
REV	Murcia, Sierra de las Cabras, Revolcadores, 1800 m, 12-VI-2001, *S. Pajarón, E. Pangua* & *L. G. Quintanilla*	7
CAB	Jaén, Sierra del Pozo, Cabañas, 2000 m, 13-VI-2001, *S. Pajarón, E. Pangua* & *L. G. Quintanilla*	17
FON	Italy: Fortogna-Belluno, 445 m, 4-IX-1998, *C. Argenti*	9
LOG	Italy: Longarone-Belluno, 690 m, 9-IX-1998, *C. Argenti*	7

genase (IDH), shikimate dehydrogenase (SKDH), diaphorase (DIA), hexokinase (HEX), malate dehydrogenase (MDH), phosphoglucomutase (PGM), triosephosphate isomerase (TPI), glutamate dehydrogenase (GDH), aspartate aminotransferase (AAT), aldolase (ALD), aconitase (ACO), 6-phosphogluconate dehydrogenase (6-PGD), and malic enzyme (ME). When more than one activity zone was observed for an enzyme, these were identified as different loci. The loci were numbered sequentially with the most anodally migrating locus designated 1. Presumed allelic variants within loci, allozymes, were identified alphabetically, the most anodal one being ''a''. Samples were placed side-by-side on the same gel to determine band homologies.

Frequencies were calculated for all alleles and populations. Genetic similarities among the populations were obtained using GDA (Lewis and Zaykin 2001). An UPGMA tree was obtained based on Nei's (1978) genetic identities.

Using the characters discussed by Cubas et al. (1993) we calculated a similarity matrix of the populations to construct a phenetic tree only for comparison with the results of the genetic analysis. NTSYS-pc (Rohlf 1994) was used for these analyses and the dendrogram was obtained by the UPGMA method.

Geographic distances between populations were obtained with a Global Positioning System (GPS) device and then used to construct a geographic distance matrix. Mantel tests (Mantel 1967) were conducted to test for correlation between genetic and geographic distances in the whole set of populations and in the Iberian Peninsula populations alone. NTSYS-pc (Rohlf 1994) was also used for these analyses.

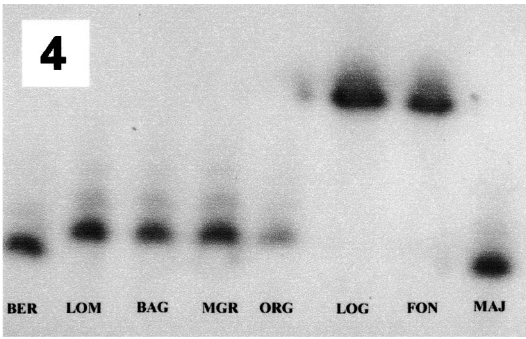
RESULTS

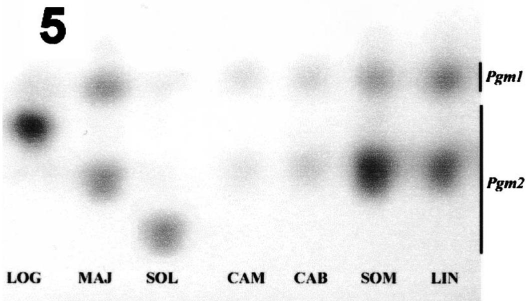
Not all of the 15 enzyme systems assayed, putatively encoded by 29 loci, were informative and useful. The behavior of these systems can be summarized as follows. Two activity zones were observed in PGI, IDH, DIA, PGM, TPI, AAT, ALD, and ACO. In AAT, ALD, and DIA, only one of these zones was consistent and interpretable: *Aat-1*, *Ald-1*, and *Dia-1*. *Aat-1* and *Ald-1* were monomorphic across all populations. Both loci detected in ACO were also monomorphic in all populations. Of the other four systems with two loci, *Pgi-1* was monomorphic, while *Pgi-2* (Fig. 4) showed four different alleles; *Tpi-1* showed two alleles, and *Tpi-2* was monomorphic; *Pgm-2* (Fig. 5) and *Idh-2* both showed four alleles, however, the latter was resolved only in half of the populations; *Pgm-1* and *Idh-1* were also variable, showing three different alleles each.

In each of LAP, HEX, SKDH, GDH, and 6PGD, only one locus was observed. The last one, *6Pgd–1*, was monomorphic but the other four showed variation. Four alleles were detected in *Lap-1* (Fig. 6) and *Gdh-1*, whereas only two were detected in *Hex-1*, but as in *Idh-2*, was well resolved in only half of the populations. *Skdh-1* (Fig. 7) yielded five different alleles.

Three activity zones were detected in ME; *Me-1* was poorly resolved, *Me-2* yielded a group of bands which we were not able to interpret and assign different alleles, and *Me-3* presented two alleles. In MDH four activity zones were observed, *Mdh-1* and *Mdh-4* were monomorphic, whereas two different alleles were found in both *Mdh-2* and *Mdh-3.*

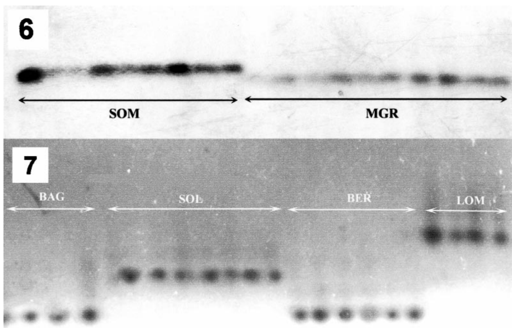
No variation was observed among individuals within populations. Thus, neither polymorphic alleles nor heterozygotes were found in any population. Allelic frequencies for each allele in each population were calculated and the data matrix is avalaible from the au-





FIGS. 4–5. Photographs of gels ran to depict several interpopulation comparisons. Anode in the upper edge. Each lane corresponds to a different population. 4. *Pgi-2* is represented showing four different alleles. 5. One allele of *Pgm-1* and three of *Pgm-2* are represented.

thors. Genetic identities (Nei, 1978) ranged from 0.43 to 1.00 (Table 2), with a mean value of 0.72. The dendrogram based on these relationships (Fig. 8) clearly shows five different clusters separated into two main groups. In group 1 cluster "a" contains populations CAM, SOM, MAJ and REV (I = 1.00) and is closely related (I = 0.95) to cluster "b" with populations GRA, BER, LIN, OJA, CAB, and POR (I = 1.00 among them).



FIGS. 6–7. Photographs of gels ran to depict interpopulation and intrapopulation comparisons. Anode in the upper edge. 6. Individuals of populations SOM and MGR showing two different alleles for *Lap-1,* but monomorphic within each population. 7. Individuals of populations BAG, SOL, BER, and LOM showing three different alleles for *Skdh-1,* but also monomorphic within each population.

TABLE 2. Matrix of Nei's (1978) unbiased genetic identity values between pairs of populations studied.

Population																	
CAM	0.000																
GRA	0.955	0.000															
SOM	1.000	1.000	0.000														
LOM	0.591	0.545	0.545	0.000													
MGR	0.636	0.591	0.636	0.870	0.000												
BAG	0.591	0.545	0.591	0.826	0.917	0.000											
BER	0.955	1.000	0.955	0.522	0.542	0.542	0.000										
LIN	0.955	1.000	1.000	0.545	0.591	0.545	1.000	0.000									
ORG	0.636	0.591	0.591	0.783	0.833	0.917	0.583	0.591	0.000								
SOL	0.714	0.714	0.682	0.500	0.500	0.545	0.727	0.714	0.591	0.000							
OJA	0.955	1.000	1.000	0.545	0.565	0.565	1.000	1.000	0.609	0.714	0.000						
POR	0.955	1.000	1.000	0.545	0.565	0.565	1.000	1.000	0.609	0.714	1.000	0.000					
MAJ	1.000	0.955	1.000	0.591	0.609	0.609	0.957	0.955	0.652	0.714	0.957	0.957	0.000				
REV	1.000	1.000	1.000	0.571	0.591	0.591	1.000	1.000	0.636	0.714	1.000	1.000	1.000	0.000			
CAB	0.955	1.000	1.000	0.545	0.565	0.565	1.000	1.000	0.609	0.714	1.000	1.000	0.957	1.000	0.000		
FON	0.429	0.429	0.429	0.619	0.571	0.619	0.429	0.429	0.571	0.476	0.429	0.429	0.429	0.429	0.429	0.000	
LOG	0.429	0.429	0.429	0.619	0.571	0.619	0.429	0.429	0.571	0.476	0.429	0.429	0.429	0.429	0.429	0.857	0.000

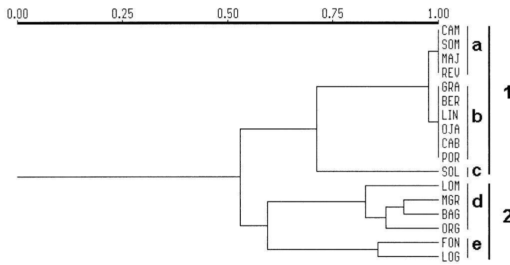


FIG. 8. Dendrogram based on Nei's (1978) genetic identities among populations. Numbers 1 and 2 refers to the main groups formed, and letters a-e identify the clusters of populations. Group 1 includes three clusters with populations from the Iberian Peninsula, a and b, plus c from the Pyrenees. Group 2 includes two clusters, d with populations from Italy and from the eastern Pyrenees (Spain), and e only with Italian populations.

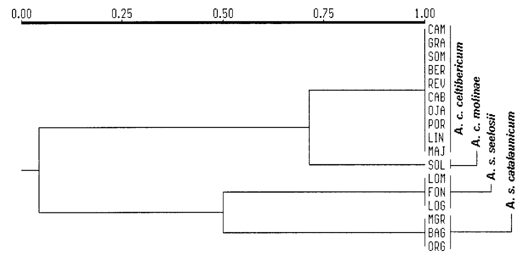


FIG. 9. Dendrogram representing the phenetic tree based on the morphological characters used by Cubas et al. (1993) in their taxonomic proposal. Populations are separated in two main branches. The upper one includes the populations with morphological characters corresponding to *Asplenium celtibericum*, population SOL is separated showing the characters that define subsp. *molinae*. The lower branch includes the populations with morphological characters corresponding to *Asplenium seelosii*, separated in two clusters, the Italian populations, subsp. *seelosii*, are grouped in the upper one, and the eastern Pyrenees populations, subsp. *catalaunicum*, in the other.

This group also includes cluster ''c'', comprising only population SOL, and with I = 0.68–0.73. Two more variable clusters comprised the other group. Cluster ''d'' contains populations LOM, MGR, BAG, and ORG (I = 0.78–0.87) separated from cluster ''e'' formed by populations FON and LOG (I = 0.86 between them). Clusters ''d'' and ''e'' are quite divergent (I = 0.57–0.62).

In the phenetic tree (Fig.9) populations are clustered in four groups, along two main branches. Populations CAM, GRA, SOM, BER, REV, CAB, OJA, POR, LIN, and MAJ, are the populations with characters that differentiate *A. celtibericum* subsp. *celtibericum*; and population SOL corresponded to subspecies *molinae*. On the other branch a group with populations LOM, FON and LOG corresponded to *A. seelosii* subsp. *seelosii*, while the other group with populations MGR, BAG and ORG corresponded to subspecies *catalaunicum*.

The Mantel test for all populations showed a significant correlation (r = 0.759; p = 0.001, after 999 permutations) between genetic and geographic distances, whereas no significant correlation was found (r = 0.297; p = 0.236, after 999 permutations) when applied to the populations of the Iberian Peninsula alone.

DISCUSSION

The main goal of this research was to evaluate the taxonomic scheme proposed for the *A. seelosii* complex, but before discussing this we would like to briefly comment on the striking lack of intrapopulational genetic variation revealed by our study. From its present habitat and distribution, and knowledge of glaciation patterns in Europe, *A. seelosii* s.l. may be interpreted as a species that is associated with glacial refugia (Pichi Sermolli 1979), and, therefore, might be expected to have high intrapopulational genetic variation (Vogel et al. 1999a). However, the 17 populations we studied throughout the range of this complex showed no intrapopulational genetic variation. Similar genetically depauperate populations have been found in several other rupicolous fern species including, for example, the diploid *Asplenium trichomanes* L. subsp. *trichomanes* (Vogel et al. 1999a), and the tetraploids, *A. septentrionale* (L.) Hoffm. (Holderegger and Schneller 1994), and *A. csikii* Kümmerle & Andras (Vogel et al. 1999b), although these species were not considered to be linked to glacial refugia. The lack of genetic variation in the populations of *A. seelosii* does not agree with the expected high variation proposed by Vogel et al. (1999a) for fern species linked to glacial refugia. On the other hand, in young populations of *A. ruta muraria* (autotetraploid and also rupicolous), genetic variation was low or lacking and increased as populations became older (Schneller and Holderegger 1996). There is no reason to think that populations of *A. seelosii* are young or recently established, and that the absence of genetic variation is due to this circumstance. Studies of the reproductive biology of these populations are now being carried out to investigate this further.

The taxonomic scheme proposed by Cubas et al. (1993) comprises two different levels, an infraspecific level and a specific one. First we will discuss the infraspecific level, and later, we will discuss whether, in our opinion, the proposed species deserve recognition.

The populations studied here are clustered in two main groups, numbered 1 and 2 (Fig. 8), and in each of these a second level of clusters appear. Populations belonging to group 1, all from the Iberian Peninsula, are arranged in three different clusters, ''a'', ''b'' and ''c''. Genetic identities among the 10 populations of clusters ''a'' and ''b'' are high and range from 0.95 to 1.00. High genetic identity values are commonly found among populations of the same species in ferns. For example, Haufler et al. (1995) reported values from 0.913 to 1.000 (mean 0.968) among populations within

species in the *Polypodium vulgare* complex, and Korpelainen (1995) reported similarly high values among populations of *Pteridium aquilinum* (L.) Kuhn. In other *Asplenium* species, such as *A. trichomanes* subsp. *quadrivalens* D. E. Meyer, genetic identities among populations in central Europe ranged from 0.921 to 0.999, as calculated from the data of Suter et al. (2000).

A comparison of both trees (Figs. 8 and 9) reveals that the 10 populations in clusters ''a'' and ''b'' (Fig. 8) are included in the same taxonomic unit, *A. celtibericum* subsp. *celtibericum* (Fig. 9). This indicates that our results are consistent with the classification proposed by Cubas et al. (1993). No isozyme samples were obtained from Moroccan plants. However, based on herbarium observations, there are no morphological differences between plants from these southernmost populations and plants from populations of clusters ''a'' and ''b'' (Pangua 1989). Only one population, SOL, from central Pyrenees, belongs to the other cluster of group 1, cluster ''c'' (Fig. 8). The mean value of the genetic identity between cluster ''c'' and clusters ''a'' and ''b'' is lower (0.71) than between ''a'' and ''b'' alone (as mentioned above). However, this does not mean that the populations comprising clusters ''a'', ''b'' and ''c'', cannot be considered conspecific. This is because there are other fern species whose genetic identity values among conspecific populations are in a similar range. For example, Haufler (1985b) reported genetic identity values ranging from 0.747 to 0.989 (mean 0.870) among populations of *Bommeria hispida* (Kuhn) Underw., and Ranker (1992), reported values from 0.71 to 1.00 (mean 0.87) among populations of *Hemionitis palmata* L. (Ranker 1992).

Plants from population SOL have some morphological similarities with the plants of populations from the eastern Pyrenees, MGR, BAG, and ORG (i.e., the presence of glandular hairs on sporelings and on the first adult leaves; pers. obs.), but they also share some morphological characters (e.g., perispore type) with plants from the rest of the populations from the Iberian Peninsula (Cubas et al. 1993). In view of this and considering their geographic position (Fig. 3), and the genetic differentiation revealed in this study, the plants in population SOL can be interpreted as an intermediate between the plants from the Alps and eastern Pyrenees and those from the eastern half of the Iberian Peninsula. On the other hand, the separation of populations into clusters ''a'' and ''b'' based on genetic differentiation is not correlated with their geographic distribution in the Iberian Peninsula, nor with any morphological differentiation. Overall, the relationships depicted within group 1 of the dendrogram (Fig. 8) support the systematics of this part of the complex with two entities, and little differentiation between them, clusters ''a'' and ''b'' on one hand, and cluster ''c'' on the other hand (Fig. 8). These correspond to *A. celtibericum* subsp. *celtibericum* and *A. celtibericum* subsp. *molinae* (Fig. 9), but, in our opinion, the differentiation between these taxa is too weak to warrant the recognition of subspecies.

More variation was found among populations clustered in group 2 (Fig. 8), which included populations from the eastern Pyrenees and from Italy. In fact, in stark contrast to the situation in group 1, there were no identical populations in group 2 (Fig. 8). Two clusters, ''d'' and ''e'', can be distinguished in group 2, and the genetic identity between them was lower than between clusters in group 1 (Fig. 8). The mean values of genetic identity within and between the clusters ''d'' and ''e'' match the values of genetic identity within conspecific populations, as discussed above in relation to the clusters in group 1. A comparison of the distribution of the populations in the clusters with their morphological differences may help to understand the relationships in this group. Plants from the Italian populations LOM, FON, and LOG, share morphological characters such as the presence of glandular hairs mainly on the lamina of adult plants, and have spores with a thin perispore (Cubas et al. 1993). Plants from populations MGR, BAG, and ORG, from eastern Pyrenees, lack glandular hairs on the lamina of adult plants and have spores with the perispore as in the mentioned Italian populations, but with larger areolae and unevenly, instead of evenly, distributed echinulae over the spore surface. Nevertheless, glandular hairs are present in sporelings and young plants, at least until the stage when two or three leaves are present (pers. obs.). As can be seen, morphological differences in the populations clustered in this group are subtle, and the clustering based on genetic identities (LOM, ORG, BAG, and MGR together in cluster ''d'', FON and LOG in cluster ''e'') does not agree with the clusters in the phenetic tree (Fig. 9), LOM, FON, and LOG as *A. seelosii* subsp. *seelosii*, and ORG, BAG, and MGR as *A. seelosii* subsp. *catalaunicum*. Thus, our results do not support the recognition of these subspecies. Moreover, the artificial hybridization experiments carried out by Lovis (1987) between plants from populations of group 2 (Fig. 8) fail to support subspecies designation. He crossed plants from Italy (same location as population LOM), and plants from Spain (same location as population ORG from the eastern Pyrenees), and meiosis in the resulting F1 individuals was normal, demonstrating that the genomes of these plants were very similar. The genetic differences among the populations in group 2 may be interpreted as being a consequence of isolation and distance. The correlation found between genetic and geographic distances supports this idea.

A comparison of both trees (Figs. 8 and 9) reveals that, at the specific level, groups 1 and 2 correspond respectively to *A. celtibericum* and *A. seelosii* sensu Cu-

bas et al. (1993). However, this fact alone is not enough to determine whether two or only one species may be differentiated. This becomes clear when we consider that the mean genetic identity between groups 1 and 2 is only 0.53, and that, in other studies of temperate ferns, values similar to this have been reported both among populations of the same species and among species of the same genus! In fact, in homosporous ferns genetic identities between congeneric species in temperate climates are usually low, although differences have been reported (Soltis and Soltis 1989; Soltis and Soltis 1990; Haufler et al. 2000) and the values are included in a broad range. For example, the lowest value of genetic identity between species in the genus *Bommeria* E. Fourn. (Haufler, 1985b) is only 0.099, between *B. hispida* and *B. ehrenbergiana* (Klotzsch) Underw., and the highest is 0.339, between *B. subpaleacea* Maxon and *B. ehrenbergiana*. In other genera the range of genetic identities is wider. For example, in a group of species of *Polystichum* Roth, Soltis et al. (1990) found a value of 0.187 between *P. lonchitis* (L.) Roth and *P. acrostichoides* (Michx.) Schott, but 0.812 between *P. munitum* (Kaulf.) C. Presl and *P. imbricans* (D. C. Eaton) D. Wagner. In this last case the authors considered that although allozymically very similar, those plants are genetically distinct and deserve recognition at the species level. Intermediate values were found by Haufler et al. (1995) in the *Polypodium vulgare* complex, including six species, whose genetic identities range from 0.117 to 0.608. In the genus *Asplenium* genetic identity values between species are also variable. Werth et al. (1985), using Rogers's genetic similarity instead of Nei's genetic identity, found values from 0.296 between *A. platyneuron* (L.) Britton, Sterns & Poggenb. and *A. montanum* Willd., to 0.498 between *A. montanum* and *A. rhizophyllum* L. Also in *Asplenium*, Herrero et al. (2001) found identities that ranged from 0.504 to 0.575 between two subspecies, *A. obovatum* Viv. subsp. *obovatum* and *A. obovatum* subsp. *protobillotii* Herrero, Pajarón & Prada. In view of the broad range of genetic identities in these examples it is not easy to decide whether the two main groups (1 and 2 in Fig. 8) found in the present analysis (and differentiated by a genetic identity of 0.53) indicate the existence of two different species. However, in view of the values of genetic identities between the species mentioned above, and considering the morphological characters, our opinion is that two species may be distinguished.

The analysis of meiosis in an artificial cross between plants of these main groups would help to clarify this situation. Note that although Lovis (1987) extended the interpretation of the results of his hybridization experiments (mentioned earlier in the Discussion) to the whole Iberian area, he, in fact, did not attempt any crosses between groups 1 and 2 as defined here. All his hybrids were produced from crosses involving only plants in group 2 (Fig. 8) (i.e., *A. seelosii*). Our analysis of isoenzymes suggests that these two groups should be maintained as distinct species, *Asplenium seelosii* and *A. celtibericum*, but further division of these into subspecies seems to be unwarranted.

There is no doubt that both taxa are related, but we can not determine from our data whether the differentiation took place from North to South or vice-versa. In the opinion of Pichi Sermolli (1979), *A. celtibericum* could have originated from the populations of the Pyrenees and then extended southwards to the Iberian Peninsula and Morocco. Molecular data may be useful for addressing this question.

Acknowledgements. We are grateful to Carmen Prada for criticism, comments and suggestions on an earlier draft of the manuscript. We appreciate very much the generous work of Chris Haufler and an anonymous reviewer on an earlier version of the manuscript, and of Associate Editor Paul S. Manos. We also appreciate the help with English and valuable comments of Tom Ranker, and especially of Stuart Lindsay. We thank José Luis Sánchez Morales, Director of the Parque Natural de Cazorla, Segura y Las Villas, for a permit to collect. Thanks also to Carlo Argenti for collecting plants for us in Italy, and to Adalberto, Cleo and Gabriele Peroni for leading us to collect more Italian plants. This study was funded by the Dirección General de Ciencia y Tecnología, project PB97–0307.

Literature Cited

Acquaah, G. 1992. *Practical protein electrophoresis for genetic research.* Portland, OR: Dioscorides Press.

Barrington, D. S. 1990. Hybridization and allopolyploidy in Central American *Polystichum*: cytological and isozyme documentation. *Annals of the Missouri Botanical Garden* 77: 297–305.

Bryan, F. A. and D. E. Soltis. 1987. Electrophoretic evidence for allopolyploidy in the fern *Polypodium virginianum*. *Systematic Botany* 12: 553–561.

Crawford, D. J. 2000. Plant macromolecular systematics in the past 50 years: one view. *Taxon* 49: 479–501.

Cubas, P., C. Pardo, and S. Rivas-Martínez. 1993. The *Asplenium seelosii* aggr. (Aspleniaceae: Pteridophyta): morphology, cytology, ecology and taxonomy. *Rivasgodaya* 7: 99–117.

Gastony, G. J. 1990. Electrophoretic evidence for allotetraploidy with segregating heterozygosity in South African *Pellaea rufa* A.F. Tryon (Adiantaceae). *Annals of the Missouri Botanical Garden* 77: 306–313.

Haufler, C. H. 1985a. Pteridophyte evolutionary biology: the electrophoretic approach. *Proceedings of the Royal Society of Edinburgh* 86B: 315–323.

——— 1985b. Enzyme variability and modes of evolution in *Bommeria* (Pteridaceae). *Systematic Botany* 10: 92–104.

———, M. D. Windham, and E. W. Rabe. 1995. Reticulate evolution in the *Polypodium vulgare* complex. *Systematic Botany* 20: 89–109.

———, E. A. Hooper, and J. P. Therrien. 2000. Modes and mechanisms of speciation in pteridophytes: Implications of constrasting patterns in ferns representing temperate and tropical habitats. *Plant Species Biology* 15: 223–236.

Herrero, A., S. Pajarón, and C. Prada. 2001. Isozyme variation and genetic relationships among taxa in the *Asplenium obovatum* group (Aspleniaceae, Pteridophyta). *American Journal of Botany* 88: 2040–2050.

Holderegger, R. and J. J. Schneller. 1994. Are small populations of *Asplenium septentrionale* variable? *Biological Journal of the Linnean Society* 51: 377–385.

KORPELAINEN, H. 1995. Mating system and distribution of enzyme genetic variation in bracken (*Pteridium aquilinum*). *Canadian Journal of Botany* 73: 1611–1617.

LEVIN, D. A. 2001. 50 years of plant speciation. *Taxon* 50: 69–91.

LEWIS, P. O., and ZAYKIN, D. 2001. Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c). Free program distributed by the authors from http://lewis.eeb.uconn.edu/lewishome/software.html

LOVIS, J. D. 1977. Evolutionary patterns and processes in ferns. Pp. 229–415 in *Advances in Botanical Research. Vol. 4,* eds. R. D. Preston and H. W. Woolhouse. London: Academic Press.

———. 1987. Hybridisation experiments between *Asplenium seelosii* and *A.celtibericum* (= *A. seelosii* subsp. *glabrum*) (Aspleniaceae: Pteridophyta). *Fern Gazette* 13: 151–156.

MANTEL, N. A. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* 27: 209–220.

MEYER, D. E. 1957. Zur Zytologie der Asplenien Mitteleuropas (I-XV). *Berichte der Deutschen Botanischen Gesellschaft* 70: 57–66.

———. 1967. Uber neue und seltene Asplenien Mitteleuropas, 4. Mitteilung. *Berichte der Deutschen Botanischen Gesellschaft* 80: 28–39.

NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583–590.

NOGUEIRA, I. and J. ORMONDE. 1986. *Asplenium* L. Pp. 90–104 in *Flora Iberica. Vol. I. Lycopodiaceae-Papaveraceae,* eds. S. Castroviejo, M. Laínz, G. López González, P. Montserrat, F. Muñoz-Garmendia, J. Paiva, and L. Villar. Madrid: Real Jardín Botánico, CSIC.

PAJARÓN, S., C. PRADA, A. HERRERO, and E. PANGUA. 1996. Isozymical study of genetic variability in *Asplenium foreziense* and related taxa. Preliminary results. Pp. 307–311 in *Pteridology in Persppective,* eds. J. M. Camus, M. Gibby, and R. J. Johns. Kew: Royal Botanic Gardens.

PANGUA, E. 1989. *Estudios palinológicos en pteridófitos ibéricos: familia Aspleniaceae.* Ph.D. Thesis. Universidad Complutense de Madrid, Madrid. 377 pp.

PICHI SERMOLLI, R. E. G. 1979. A survey of the pteridological flora of the Mediterranean Region. *Webbia* 34: 175–242.

PRELLI, R. 2001. *Les Fougères et plantes alliées de France et d'Europe occidentale.* Paris: Éditions Belin.

PRYER, K. M. and C. H. HAUFLER. 1993. Isozymic and chromosomal evidence for the allotetraploid origin of *Gymnocarpium dryopteris* (Dryopteridaceae). *Systematic Botany* 18: 150–172.

RANKER, T. A. 1992. Genetic diversity, mating systems, and interpopulation gene flow in neotropical *Hemionitis palmata* L. (Adiantaceae). *Heredity* 69: 175–183.

———, C. H. HAUFLER, P. S. SOLTIS, and D. E. SOLTIS. 1989. Genetic evidence for allopolyploidy in the neotropical fern *Hemionitis pinnatifida* (Adiantaceae) and the reconstruction of an ancestral genome. *Systematic Botany* 14: 439–447.

REICHSTEIN, T. 1981. Hybrids in European Aspleniaceae (Pteridophyta). *Botanica Helvetica* 91: 89–139.

RIVAS-MARTÍNEZ, S. 1967. Une espèce nouvelle d'*Asplenium* (*Aspleniaceae*) d'Espagne. *Bulletin du Jardin Botanique National de Belgique* 37: 329–334.

ROHLF, F. J. 1994. NTSYS-pc. Numerical taxonomy and multivariate analysis system, v. 1.80. Setauket, NY: Exeter Software.

ROTHMALER, W. 1937. Criptògames vasculares o Pteridóphites. Pp. 315–373 in *Flora de Catalunya*, eds. J. Cadevall and P. Font i Quer. Barcelona: Institut de Ciències.

SCHNELLER, J. J. and R. HOLDEREGGER. 1996. Colonisation events and genetic variability within populations of *Asplenium ruta-muraria* L. Pp. 571–580 in *Pteridology in Perspective,* eds. J. M. Camus, M. Gibby, and R. J. Johns. Kew: Royal Botanic Gardens.

SLEEP, A. 1983. On the genus *Asplenium* in the Iberian Peninsula. *Acta Botanica Malacitana* 8: 11–46.

SOLTIS, D. E. and P. S. SOLTIS. 1989. Polyploidy, breeding systems, and genetic differentiation in homosporous pteridophytes. Pp. 241–258 in *Isozymes in Plant Biology,* eds. D. E. Soltis and P. S. Soltis. Portland, OR: Dioscorides Press.

———, C. H. HAUFLER, D. C. DARROW, and G. J. GASTONY. 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. *American Fern Journal* 73: 9–27.

SOLTIS, P. S. and D. E. SOLTIS. 1990. Genetic variation within and among populations of ferns. *American Fern Journal* 80: 161–172.

———,———, and P. G. WOLF. 1990. Allozymic divergence in North American *Polystichum* (Dryopteridaceae). *Systematic Botany* 15: 205–215.

SUTER, M., J. J. SCHNELLER, and J. C. VOGEL. 2000. Investigations into the genetic variation, population structure, and breeding systems of the fern *Asplenium trichomanes* subsp. quadrivalens. *International Journal of Plant Sciences* 161: 233–244.

VAN DEN HEEDE, C. J., R. L. L. VIANE, and M. W. CHASE. 2003. Phylogenetic analysis of *Asplenium* subgenus *Ceterach* (Pteridophyta: Aspleniaceae) based on plastid and nuclear ribosomal ITS DNA sequences. *American Journal of Botany* 90: 481–495.

VIANE, R. L. L., A. C. JERMY, and J. D. LOVIS. 1993. *Asplenium*. Pp. 18–23 in *Flora Europaea. Volumen I: Psilotaceae to Platanaceae,* 2nd edition, eds. T. G. Tutin, N. A. Burges, A. O. Chater, J. R. Edmonson, V. H. Heywood, D. M. Moore, D. H. Valentino, S. M. Walters, and D. A. Webb. Cambridge: Cambridge University Press.

VOGEL, J. C., F. J. RUMSEY, J. J. SCHNELLER, J. A. BARRET, and M. GIBBY. 1999a. Where are the glacial refugia in Europe? Evidence from Pteridophytes. *Biological Journal of the Linnean Society* 66: 23–37.

———,———, S. J. RUSSELL, C. J. COX, J. S. HOLMES, W. BUJNOCH, C. STARK, J. A. BARRET, and M. GIBBY. 1999b. Genetic structure, reproductive biology and ecology of isolated populations of *Asplenium csikii* (Aspleniaceae, Pteridophyta). *Heredity* 83: 604–612.

WERTH, C. R., S. I. GUTTMAN, and W. H. ESHBAUGH. 1985. Electrophoretic evidence of reticulate evolution in the Appalachian *Asplenium* complex. *Systematic Botany* 10: 184–192.