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Molecular data Confirm the Presence of *Anacolia menziesii* (Bartramiaceae, Musci) in Southern Europe and Its Separation from *Anacolia webbii*

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ABSTRACT. ISSR (Inter-Simple Sequence Repeat) fingerprint data and nrITS sequences confirm the presence of *Anacolia menziesii* in Europe. The species is more variable genetically in North America than in Europe. The data show only minor differentiation between the North American and European populations of *A. menziesii. Anacolia webbii* is morphologically and genetically very similar to *Anacolia menziesii*, but can be separated from *Anacolia menziesii* with high confidence based on the ITS and ISSR data. Long distance dispersal is the most likely explanation for the disjunction of *Anacolia menziesii* between North America and Europe.

Anacolia menziesii (Turner) Parihar was described by Turner (1805) from the west coast of North America. The species grows in rock crevices and on soil from Alaska to Baja California and eastward to Colorado, northern Idaho and Montana (Flowers 1952; Griffin 1994). It is also known from Nepal (Gangulee 1969–1980) and was recently reported from Spain and Cyprus in the Mediterranean region (García-Zamora et al. 1998). *Anacolia webbii* (Mont.) Schimp. is distributed from the Canary and Madeira Islands to Morocco and Algeria in Africa, Portugal, Spain, Corsica and Sicily in Europe, and Iraq and Turkey in Asia (Agnew and Vondráček 1975; Düll 1985; 1992). The two species have quite similar gametophytes, the main differences being the wider upper laminal cells, prominent leaf papillae, uniformly bistratose leaf apices and margins, and the short setae of *Anacolia webbii*. But these characters are sometimes difficult to assess, and because of the dioicous sexual condition of these plants, sporophytes rarely develop. Townsend (1965), for example, classified plants from Cyprus lacking sporophytes as *A. webbii*, although he commented on their similarity to *A. menziesii*. Indeed, these plants were later identified as *A. menziesii* (García-Zamora et al. 1998) based on their leaf morphology. According to Flowers (1952), sporophytes of the two species can be distinguished by the length of the setae (5–12 mm in *A. menziesii* and approximately 1 mm in *A. webbii*) and the peristome (present, although often fragile and broken off in *A. menziesii* and always lacking in *A. webbii*).

In the absence of sporophytes, *A. menziesii* and *A. webbii* are distinguished primarily by leaf cell papillosity and thickness of the lamina (unistratose throughout or bistratose in part). These characters are generally good species-specific markers in mosses, but recent morphological and molecular data indicate that they may not always be fixed in closely related species (Shaw et al. 2003; O. Werner, unpubl. data). In *Dicranoweisia cirrata* (Hedw.) Lindb. and *Barbula indica* (Hook.) Spreng, for example, marked infraspecific variability in these characters can be observed. Therefore, we use a molecular approach to test the hypothesis that *A. menziesii* and *A. webbii* are genetically distinct, and that *A. menziesii* occurs in Mediterranean regions of Europe. Furthermore, the molecular data might give insight in the origin of the western North American-Mediterranean disjunction of *Anacolia menziesii*, especially whether it is the result of long distance dispersal, or vicariance. The ITS1 and ITS2 region of the nrRNA gene arrays were chosen for sequencing due to their high variability in many mosses (Shaw et al. 2002). Furthermore, we used ISSR- (Inter-Simple Sequence Repeat) (Gupta et al. 1994) markers as an independent approach with high resolving power. By comparing patterns of relationship suggested by these two quite different sorts of molecular markers, we were also able to determine if they yield congruent inferences. Nucleotide sequencing provides detailed information about the history of a small genomic region, while ISSR data provide a (presumably) random ''snapshot'' of the whole genome. If genealogical patterns are congruent, this implies that each provides independent information about a common underlying (organismal) phylogeny.

MATERIAL AND METHODS

Plant Material. A total of 16 accessions were used in this study, six of *A. webbii*, four of *A. menziesii* from Europe, five of *A. menziesii* from North America and one *A. laevisphaera* (Taylor) Flowers. *Anacolia webbii* and *A. menziesii* are very rare species. The European samples of these species include all collections made during the last decade known to the authors. The plants were classified corresponding to leaf characters (papillosity, morphology of leaf margin and leaf apex). Details on geographic origin, voucher data and GenBank accession numbers are given in Table 1.

DNA Extraction. Total DNA of one gametophore tip was extracted by the SDS protein precipitation method described by Milligan (1998).

TABLE 1. A list of the used specimens for the analysis. Taxon, geographic origin, voucher number and GenBank accession number of the ITS sequences are given.

Anacolia menziesii Almería (Spain), MUB 6595 (AF525783). Granada (Spain), MUB12960 (AF525791). Cyprus-1, Blockeel 26/104 (AF525792). Cyprus-2, Blockeel 26/125 (AF525793). Oregon (USA), NYBG00406643 (AF525785). Utah (USA), NYBG00406698 (AF525794). California (USA), NYBG00406667 (AF525795).
Anacolia menziesii var. *baueri* Idaho-1 (USA), NYBG00406637 (AF525796). Idaho-2 (USA), NYBG00406636 (AF525797).
Anacolia webbii Sicily (Italy), MUB12541 (AF525784). Rif Mountains (Morocco), MUB6873 (AF525786). Salamanca-1 (Spain), MUB6203 (AF525787). Salamanca-2 (Spain), MUB6204 (AF525790). La Palma-1, Canary Islands (Spain), MUB11120 (AF525788). La Palma-2, Canary Islands (Spain), MUB11127 (AF525789).
Anacolia laevisphaera Colombia, NYBG17246 (AF525798).

DNA Sequencing. PCR reactions were performed in an Eppendorf Mastercycler using 1 μl of the DNA solution in 50 μl final volume. The reaction mix contained the primers 18F and 25R (Stech and Frahm 1999), which are slightly modified versions of the primers ITS4 and ITS5 of White et al. (1990), at a final concentration of 400 μM, in the presence of 200 μM of each dNTP, 2 mM $MgCl_2$, 2 units Taq polymerase (Oncor Appligene) and the buffer provided by the supplier of the enzyme. Amplification started with 3 min denaturation at 94°C, followed by ten cycles of 15 s at 94°C, 30 s at 65°C lowered by 1°C at each cycle, and 1 min at 72°C (touch down procedure). This was followed by 25 more cycles with the annealing temperature set at 55°C. A final extension step of 7 min at 72°C completed the PCR. 5 μl of the amplification products were visualized on a 6% polyacrylamide gel and successful amplifications were cleaned with the QIAquick purification kit (Qiagen). The amplification primers were used in the sequencing reactions with the Big Dye sequencing kit and separated on a ABI-Prism 3700 sequencing machine using standard protocols.

The sequences were aligned manually using BioEdit (Hall 1999) and analysed using PAUP* (Swofford 1998) with maximum parsimony as the optimality criterion. Gaps were coded as present (1) or absent (0) but not as a fifth character state. When gaps of different length occurred at the same site, they were treated as different mutational events. For example, a deletion of two bases at a site where other sequences had a deletion of one base was coded as a deletion of one base absent (0) and deletion of two bases present (1). All characters were given equal weight. The heuristic search used the default settings of PAUP* 4.0b10. Bootstrap analysis were carried out with 1000 replicates and identical settings. An aligned matrix is available on request from the first author.

ISSR. ISSR is similar to RAPD (random amplified polymorphic DNA; Williams et al. 1990) in that it uses only a single primer in the PCR reaction, but it differs in that it does not use arbitrary primers. ISSR-primers are designed to match microsatellite sequences, short repeated sequence motifs that are found many times throughout the genome. If two identical microsatellites are found near one another and in opposite orientation, the ISSR-primer amplifies the region between the two microsatellites. This technique was introduced by Gupta et al. (1994) and Zietkiewicz et al. (1994) and is widely used by plant breeders, but has only recently been adopted by investigators interested in plant population genetics or taxonomy (Wolfe and Liston 1998). Its main advantage in comparison with RAPD is that longer primer sequences allow more stringent reaction conditions to ensure high specificity (and therefore reproducibility) of the amplification products.

For ISSR, 1 μl of DNA-extract was used in a 20 μl PCR reaction with 0.8 units Taq polymerase (Oncor Appligene), 2 mM $MgCl_2$, 200 μM of each of the dNTPs, 400 μM of ISSR-primer and the buffer supplied by the manufacturer of the enzyme. The thermocycler conditions were as follows: 3 min at 94°C followed by 30 cycles of 15 sec at 94°C, 30 sec at 45°C and 1 min at 72°C. These cycles were followed by a final extension step of 7 min at 72°C. 5 μl of the amplification reaction were separated on 6% Polyacrylamide gels. The DNA bands were visualized by silver staining and the gels scanned with a TWAIN compatible computer scanner with a transilumination module. Bands were scored as present (1) or absent (0) and the resulting data matrix was entered in the RAPDistance programme (Armstrong et al. 1995). The indices of Nei and Li (1979) and Jaccard (1901) were used to calculate genetic distances. The resulting distance matrices were used to infer Neighbor Joining and UPGMA trees using PHYLIP 3.6 (Felsenstein 1989). Support was estimated by bootstrap analysis with 1,000 replicates.

Estimation of Diversity Indices and of Molecular Variance. Haplotype diversity from ISSR markers and nucleotide diversity of the ITS data were estimated using DnaSP 3.5 (Rozas and Rozas 1999). Sites with alignment gaps or missing data were excluded. In both cases, the measures of Nei (1987) were computed.

The analysis of molecular variance (AMOVA), (Excoffier et al. 1992) was inferred using ARLEQUIN (Schneider et al. 2000). An exact test of population differentiation was performed as described by Raymond and Rousset (1995). This test gives the probability of observing less or equally likely data under the null hypothesis of panmixia.

RESULTS

ISSR. Initially, 19 ISSR primers were surveyed, eight of which gave variable amplification products. All assays were repeated twice and gave highly reproducible results. We tested the ISSR method with decreasing amounts of DNA. At lower DNA concentrations there were no bands that had not occurred previously at higher concentrations, but the band pattern did progressively become fainter (data not shown).

The band pattern of *A. laevisphaera* was extremely different from that of the other two species and was therefore excluded from further analyses. For *A. menziesii* and *A. webbii*, a total of 147 loci were identified. Twenty of these were constant among all specimens, nine were found exclusively in all samples of *A. webbii*, six exclusively in all samples of *A. menziesii*, and nine exclusively in all plants from *A. menziesii* of European origin. Details are listed in Table 2. Interestingly, the highest values for pairwise genetic distance using the index of Nei & Li (1979) (0.53) was between one plant of *A. menziesii* from Cyprus and the two specimens of *A. webbii* from Salamanca in Spain. Genetic distances among samples within *A. webbii* lay in the range of 0.06 and 0.15, within the European specimens of *A. menziesii* between 0.04 and 0.15, and within the American plants of *A. menziesii* between 0.14 and 0.34. Comparing European and American samples of *A. menziesii*, a minimum genetic distance of 0.22 and a maximum distance of 0.50 were found.

Cluster analyses of banding pattern using the indi-

TABLE 2. ISSR primer sequences and amplification products. Primers UBC-813 (CTCTCTCTCTCTCTCTT), UBC-814 (CTCTCTCTCTCTCTA), UBC-824 (CTCTCTCTCTCTCTG), ISSR1 (CAGCAGCAGCAGCAG), ISSR3 (GATAGATAGATAGATA), OW6 (GTGTGTGTGTGTRG), OW7 (CTCTCTCTCTCTCTTG), OW8 (CACACACACARY), OW9 (GTGTGTGTGTGTGTAY), OW10 (CTCTCTCTCTCTCTRA), and OW11 (CATACATACATACATA) gave no amplification product.

Name	Sequence 5′→3′	Number of loci	Loci monomorphic	Loci exclusively *A. webbii*	Loci exclusively *A. menziesii* Europe	Loci exclusively *A. menziesii*
UBC-840	GAGAGAGAGAGAGAGYT	11	4	—	1	—
ISSR2	CAACAACAACAACAA	22	—	5	3	2
ISSR4	GACAGACAGACAGACA	16	7	—	—	—
OW1	GAGAGAGAGAGAGAGAA	17	—	—	3	—
OW2	GAGAGAGAGAGAGAGAC	28	1	3	—	2
OW3	GAGAGAGAGAGAGAGAT	15	4	—	—	—
OW4	GAGAGAGAGAGAGAGAYA	15	1	1	2	—
OW5	GAGAGAGAGAGAGAGAYC	23	3	2	—	2
total		147	20	11	9	6

ces of Nei & Li (1979) and Jaccard (1901) resulted in identical topologies. Similarly, there were only minor differences when alternative clustering methods (NJ or UPGMA) were used in analyses of the distance matrices. In every case, a cluster of six samples of *A. webbii* was clearly separated from *A. menziesii*, with a bootstrap support of 100%. Within the clade formed by *A. webbii*, the two specimens from Salamanca in Spain are separated from other populations with 94% bootstrap support. Within the cluster of *A. menziesii*, the European specimens were always grouped together and this is supported by a bootstrap value of 100%. Figure 1 shows an UPGMA tree based on the ISSR data and the index of Nei & Li (1979), with corresponding bootstrap support values for the clades.

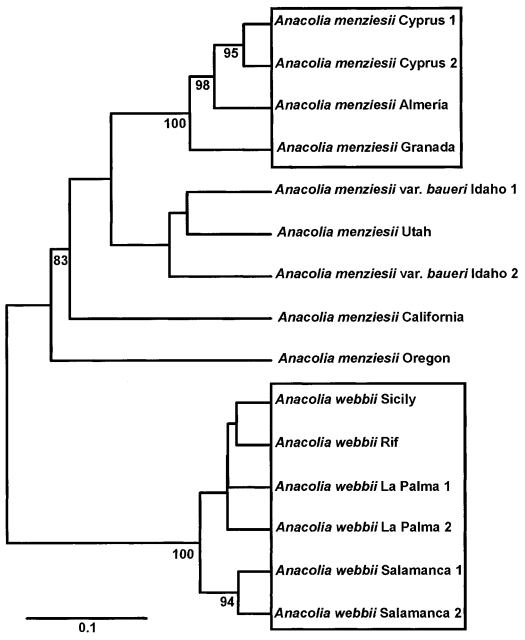

FIG 1. UPGMA tree for the ISSR data of *A. menziesii* and *A. webbii* calculated from distance values based on a Nei and Li coefficient. Bootstrap values (1000 replicates) are given. The bar indicates the genetic distance. The Mediterranean specimens are marked by a box.

The analysis of the molecular variance (AMOVA) revealed that 62.9% of the differences was found between the two species and 37.1% was found within the species (Table 3).

DNA Sequencing. The combined length of the ITS1–5.8S rRNA–ITS2 region was 759 to 766 base pairs (bp) for *A. menziesii* and 762 to 763 bp for *A. webbii*. 275 to 282 bases corresponded to the ITS1 region, 159 base pairs to the 5.8S rRNA gene, and 325 base pairs to the ITS2 region. In some cases, the last 16 base pairs of ITS2 could not be read and this part of the sequences was therefore excluded from further analyses. The 5.8S rRNA gene and the ITS2 region were constant for all accessions of *A. menziesii* and *A. webbii* with the exception of a sample of *A. menziesii* from Oregon, which had two base substitutions near the 3′-end of the ITS2 region. *Anacolia laevisphaera* had an ITS1 sequence so different from the other sequences that an alignment was impossible and it was excluded from further analyses. The ITS2 region showed a greater similarity between *A. laevisphaera* and other plants, but since there were no parsimony informative sites with respect to relationships within *A. menziesii* and *A. webbii*, a cladistic analysis of the relationships between *A. menziesii* and *A. webbii* based on the ITS2 region alone

TABLE 3. AMOVA results based on the ISSR data. The species *A. webbii* and *A. menziesii* were compared. Nearly two thirds of the observed variance correspond to differences between the two species. The chance to observe random values for Va and F_{ST} greater or equal than the found values is below 0.001.

Source of variation	Sum of squares	Variance components	Percentage
Among species	188.02	17.02 Va	62.9
Within species	120.38	10.03 Vb	37.1
Total	308.40	27.05	100.0
Fixation Index:	F_{ST} = 0.629		

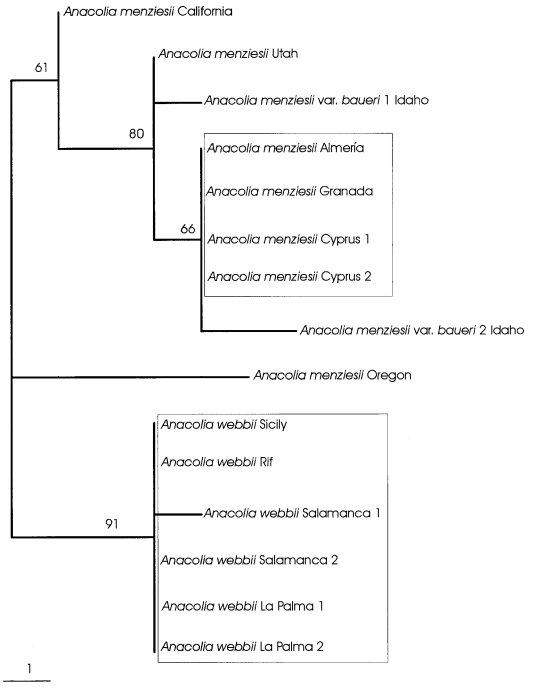

FIG 2. The most parsimonious tree reconstructed by PAUP* (tree length = 16, CI excluding noninformative characters = 0.9575; RI = 0.9697; RC = 0.901) based on the ITS data. The bar indicates a branch length corresponding to one mutation. Numbers at the nodes are bootstrap values (1000 replicates). The Mediterranean specimens are marked by a box.

was not possible. Within the species overall, there were 12 variable sites, including gaps, seven of them parsimony informative.

All samples of *A. webbii* with the exception of one plant from Salamanca, which had an insertion of one base, had identical sequences. They differed by at least four mutational steps from all of the samples of *A. menziesii* and seven mutational steps from the European plants of this latter species. All four European specimens of *A. menziesii* had identical sequences.

Based on these data, the haplotype diversity and the nucleotide diversity were calculated for the two species and for the North American and European populations of *A. menziesii*. *A webbii* and the European populations of *A. menziesii* had a haplotype and nucleotide diversities of zero (excluding gaps). The North American populations of *A. menziesii* alone had a haplotype diversity of $h = 0.900$ and a nucleotide diversity $P_i = 0.00412$. These values were $h = 0.806$ and $P_i = 0.00354$ when all populations of *A. menziesii* were included.

The single most parsimonious tree had a total length of 16 steps and is shown in Fig. 2. A bootstrap analysis with 1,000 replicates supported the clade formed by the six representatives of *A. webbii* with a support of 91%.

TABLE 4. AMOVA based on the haplotype frequencies of the nrITS sequences. The species *A. webbii* and *A. menziesii* were compared. The chance to observe random values for Va and F_{ST} greater or equal than the found values is below 0.001.

Source of variation	Sum of squares	Variance components	Percentage of variation
Among species	1.767	0.20085 Va	38.52
Within species	4.167	0.32051 Vb	61.48
Total	5.933	0.52137	100
Fixation Index:	$F_{ST} = 0.38525$		

38.52% of the ITS sequence variation was attributable to differences among the two species and 61.48% to differences within the species (Table 4). The null-hypothesis that *A. menziesii* and *A. webbii* form a single panmictic population was evaluated with an exact test of sample differentiation (Raymond and Rousset 1995). Significant differentiation between the two species was found at $p=0.00635$. We therefore rejected the null hypothesis. ITS sequence data, like the ISSR markers, indicate that populations of *A. menziesii* and *A. webbii* form genetically differentiated population systems.

DISCUSSION

The Usefullness of ISSR as a Molecular Marker in Bryophyte Taxonomy and Population Biology. In plants, ISSR markers have generally been used to study cultivated species, especially in cultivar identification and genomic mapping projects (Wolfe and Liston 1998). Only recently has it been applied to natural populations and relationships among closely related species (Ayres and Strong 2001; Camacho and Liston 2001; Deshpande et al. 2001; Wolfe and Randle 2001). To our knowledge, this is the first time that this technique has been applied to bryophytes and our results clearly encourage the use of this technique. Our study indicates that the method can be used with material stored up to eleven years in herbaria. This is an important point, because especially with rare species, as in the present case, or with specimens from remote sites, it may sometimes be impossible to use fresh material. There seemed to be no problem with the reproducibility of banding patterns, a problem often observed using RAPD. One problem with the RAPD technique is that the banding pattern becomes erratic when the DNA concentration decreases below a certain level. That is, new bands appear that were not visible at higher target DNA concentrations (Williams et al. 1993; O. Werner, unpubl. data). With the ISSR technique, we observed a decreasing band intensity when the DNA concentration was lowered, but no additional bands appeared. In our study, primers with GA-repeats worked especially well, but this may depend on the study species. In some organisms, at least, ISSR mark-

ers are more polymorphic than AFLPs (Blair et al. 1999).

Our conclusion is that ISSRs provide valuable markers for the estimation of relationships within and among closely related species of bryophytes (and other plants). The congruence we observed between ISSR and ITS results is noteworthy. Wolfe and Randle (2001) obtained similar results when they studied four species of *Hyobanche* L. using both ISSR and ITS. ISSR might be used in population studies or as a cost-efficient alternative to sequencing variable gene regions or as a complement to sequence data. It has the advantage over targeted sequencing that it surveys the complete genome and not just one gene. Nevertheless, congruence between genealogical inferences derived from ITS sequencing and ISSRs suggests that both approaches extract information about organismal relationships. The relationship between gene genealogies and organismal phylogenies are not always simple and congruence among markers cannot be assumed. Even different classes of ISSR markers (i.e., tracking different underlying microsatellite motifs) may give incongruent inferences about relationships if motif types are nonrandomly distributed in the genome, and different parts of the genome have had different genealogical histories (Blair et al. 1999).

Taxonomic Implications. The overall variability of ITS sequences within the two species of *Anacolia* is relatively low. Previous studies at the population level have shown higher variability within some bryophyte species, e.g. *Hylocomium splendens* (Hedw.) Schimp. (Chiang and Schaal 1999), *Dicranoweisia cirrata* and *Claopodium whippleanum* (Sull.) Renauld & Cardot (Shaw et al. 2002). Nevertheless, *A. webbii* is clearly separated from *A. menziesii* with high bootstrap support and by both methods used here. Additionally, the exact test of population differentiation clearly shows that the two species can be separated with confidence based on the sequence data. Therefore, separation of the two species is supported by our molecular data. Variability within *A. webbii* is very low. On a morphological basis, Flowers (1952) came to the conclusion that this species is uniform in its basic characters and that the collections he examined show little morphological variation. This might be due to the fact that this species is very rare and that it might have passed through a population bottleneck. In this case, morphological uniformity may reflect genetic uniformity.

Plants of *A. menziesii* from Europe are somewhat differentiated from North American plants. European samples share a point mutation in the ITS1 region that is only found in one of the *A. menziesii* var. *baueri* collections from North America, and the ISSR data place them in a clade nested within the North American group of populations. But these are minor differences that are probably due to geographic isolation. North American and European plants of the morphospecies, *A. menziesii*, are more similar to one another than are *A. menziesii* and *A. webbii*.

Since it was not possible to root the trees, there remains the possibility that *A. menziesii* is actually a paraphyletic species. In fact, that would not be surprising as most geographic and demographic scenarios for speciation initially result in paraphyletic taxa when reproductive isolation forms the basis for species definition. As time progresses, population extinctions and the fixation of different genetic variants in isolated population systems would give rise to mutually monophyletic taxa (Avise 2000). Although we cannot reject the possibility that *A. menziesii* is paraphyletic, the taxon is morphologically distinct and is differentiated genetically. On this basis, it warrants taxonomic recognition.

At the taxonomic level of varieties within *A. menziesii*, the present data do permit clear conclusions. The var. *baueri*, included as two specimens in our data set, is genetically quite similar to another population from Utah, and these three are genetically closest to European *A. menziesii*. Neither the sequence nor ISSR data indicate that this variety is clearly differentiated from typical specimens of the species. According to Lawton (1971), they differ in sporophyte characters, with the var. *baueri* having longer setae (up to 15 mm) than the var. *menziesii* (5–10 mm), oblong to short-cylindric capsules instead of subglobose to ovoid, and spores smaller (15–22 μm in var. *baueri* and 25–32 μm in var. *menziesii*).

Biogeographical Implications. The Mediterranean-western North American or so-called Madrean-Tethyan disjunction is a pattern found in some other bryophyte species. Recently, three species with this distribution pattern, *Dicranoweisia cirrata*, *Claopodium whippleanum*, and *Scleropodium touretii* (Brid.) L.F. Koch, were studied using ITS data (Shaw et al. 2002, Shaw et al. 2003). In each species, genetic diversity was higher in North America than in the Mediterranean region. The same is true in *A. menziesii*. This suggests that there might be a general pattern such that genetic diversity is higher in North American than in Mediterranean plants of disjunct moss taxa showing this distributional pattern. This might be the result of a general population bottleneck, perhaps during glacial periods, when geological barriers could have affected the Mediterranean populations more severely than those in North America.

The Mediterranean-western North American distribution pattern is often thought to reflect range fragmentation dating some 25 million years in the past (see Shaw et al. 2002 for a review). But the minimal degree of sequence differentiation between intercontinental disjuncts makes this explanation difficult to sustain. Frey et al. (1999) discuss the possibility of an extremely

low mutation rate in the case of the palaeoaustral moss *Lopidium concinnum* (Hook.) Wilson. But in the case of *Anacolia menziesii*, an extremely low mutational rate seems an unlikely explanation. The high levels of divergence in ITS sequence between *A. menziesii* and *A. webbii*, versus *A. laevisphaera*, indicate that in this genus the mutation rate within ITS is not extremely low. Indeed, we were unable to align ITS1 sequences between *A. laevisphaera* and the other two taxa. It has been suggested that in some studies, ITS pseudogenes were erroneously used to infer phylogenetic relationships between species (Mayol and Roselló 2001). This seems not to be the case here, however, because sequences for the 5.8S rRNA gene are identical for all *Anacolia* collections. Furthermore, the ISSR data also show a high differentiation between *A. laevisphaera* and the other two species. The fact that *A. laevisphaera* is so strongly divergent from both *A. menziesii* and *A. webbii* relative to divergence within or between these latter two species, supports the interpretation that divergence between *A. webbii* and *A. menziesii*, and between disjunct populations of *A. menziesii*, is relatively recent. We thus conclude the long distance dispersal hypothesis is favoured by our data, although alternative explications can not be ruled out completely with the available data. The most probable scenario is a dispersal from North America to the Mediterranean region. This could also explain the low level of genetic diversity of the Mediterranean samples of *A. menziesii* (as opposed to the glaciation-population bottleneck hypothesis).

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