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The origin, status and distribution of *Asplenium presolanense* spec. nov. (Aspleniaceae, Pteridophyta) *[1]

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Abstract

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The origin, status and distribution of the allotetraploid *Asplenium presolanense* have been investigated. This taxon was described as a calcicole subspecies of *A. adulterinum*, discriminated as much by its ecology as its morphology. *Asplenium adulterinum* s. str. is a small allotetraploid rock fern with a scattered, but widespread, distribution in Europe growing nearly exclusively on serpentine and other ultrabasic rocks. *Asplenium presolanense* was reported from three sites, one population of c. 30 plants on limestone at Presolana in the Bergamo Alps, Italy, one population of five plants on micaschist in Val di Gag (Poschiavo, Switzerland, some 35 km north) and several populations on limestone on Vancouver Island, Canada. Our evidence from morphological comparison, allozyme electrophoresis and length polymorphism in a non-coding spacer on the chloroplast DNA suggests that these three populations of putative *A. presolanense* represent two different taxa. The plants from Switzerland and Canada are shown to be *A. adulterinum* s. str., the allotetraploid derived from diploid *A. viride* and diploid *A. trichomanes* subsp. *trichomanes*. The taxon from Presolana is considered to merit specific status as *A. presolanense* as it is of distinct origin. Calcifuge *A. trichomanes* subsp. *trichomanes* is not involved but, instead, the calcicole diploid *A. trichomanes* subsp. *inexpectans* is postulated to be the other parental taxon. Genetic evidence suggests that *A. presolanense* is an apoendemic taxon.

Key words: *Asplenium*, biosystematics, endemic, Alps, biogeography, genetic variation in small populations, molecular methods, Pteridophyta

* This paper is dedicated to our dear friend the late Prof. Tadeus Reichstein.

1. Introduction

1.1. Historical remarks

Asplenium presolanense spec. nov. (Aspleniaceae, Pteridophyta) was originally described as *A. adulterinum* Milde subsp. *presolanense* Mokry, Rasbach & Reichst., discriminated as much by its ecology as its morphology. The first discovery of a plant of *A. adulterinum* on limestone was made by Mokry in 1979 on Presolana in the Bergamo Alps in northern Italy (Presolana) (Figure 1). Later, in 1983, another colony was found by Göldi in Val di Gag in the Kanton Graubünden in Switzerland (Val di Gag), ca. 30 km north of the first site (Mokry et al. 1986). Investigations in the northwest of Vancouver Island, Canada (Vancouver Island), in autumn 1982 led to the discovery of further colonies of *A. adulterinum* on limestone (Ogilvie & Ceska 1984). All these plants have been referred to as *A. adulterinum* subsp. *presolanense* (Mokry et al. 1986). The type RAS-369 was collected from the northern slope of Presolana above Colere (Mokry et al. 1986). Recognised on the basis of its morphology and ecology, it was described as a subspecies of *A. adulterinum*. The populations of *A. adulterinum* subsp. *presolanense* from Presolana and Vancouver Island were reported from limestone, the third population from Val di Gag was reported to be from micaschist in Mokry et al. (1986), but more recently the rock where the plants grow in the Val di Gag has been described as serpentine or magnesite by Göldi (in Moser & Palese 1995).

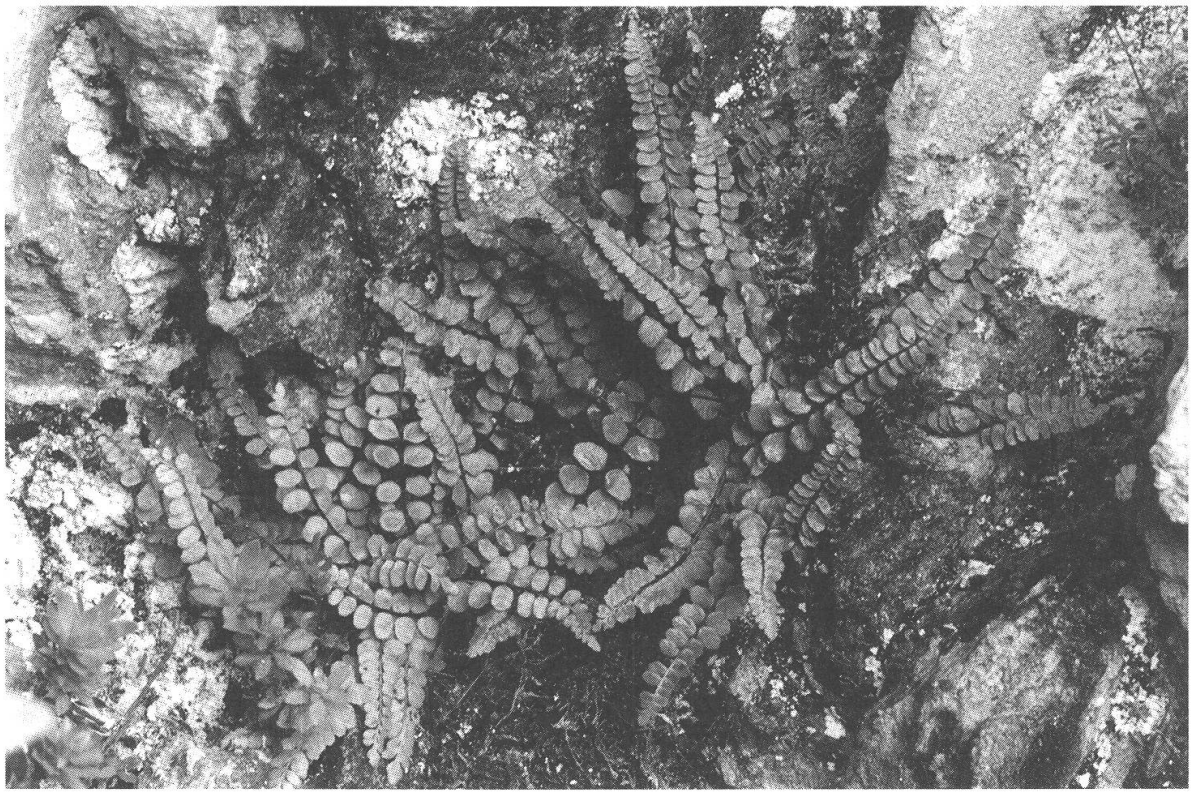


Fig. 1. *Asplenium presolanense* at the type locality at Presolana, Bergamo Alps, Italy. (Photo: Vogel)

1.2. The biosystematics of Asplenium presolanense

Lovis (1958, 1968a) proved, based on hybridisation studies, that *A. adulterinum* s. str. is an allotetraploid derived from diploid *A. trichomanes* L. subsp. *trichomanes* and diploid *A. viride* Huds., whereas Mokry et al. (1986) postulated that *A. adulterinum* subsp. *presolanense* had originated from diploid *A. trichomanes* L. subsp. *inexpectans* Lovis and *A. viride*. *Asplenium adulterinum* s. str. has been regarded as a European endemic taxon normally associated with serpentine outcrops. Serpentine is one of the few substrates where calcifuge *A. trichomanes* subsp. *trichomanes* can grow together with calcicole *A. viride*. However, *A. adulterinum* has been reported from other substrates, such as paragneiss and magnesite, especially in Austria (Melzer 1986, Justin 1993).

Asplenium trichomanes subsp. *inexpectans* is a diploid calcicole sister taxon to the calcifuge *A. trichomanes* subsp. *trichomanes*. Only two diploid hybrids between *A. viride* and *A. trichomanes* subsp. *trichomanes* are known from the wild (Reichstein 1981), whereas about 20 diploid hybrids of *A. trichomanes* subsp. *inexpectans* and *A. viride* have been discovered. This latter hybrid, *A.* × *bavaricum* D. E. Mey. nothosubsp. *adulteriniforme* (Lovis, H. Melzer & Reichst.) Muñoz Garm. (Figure 2), was described from Austria (Lovis et al. 1965). Lovis demonstrated that diploid hybrids between *A. viride* and diploid *A. trichomanes* produce diplospores from which fertile tetraploid plants can be raised (Lovis et al. 1965, Lovis & Reichstein 1968a, 1968b). Such plants were found to be cytologically and morphologically similar to *A. adulterinum* from the wild (Lovis 1968a).

Mokry et al. (1986) assumed that *A. adulterinum* subsp. *presolanense* had been derived from diplospores of *A.* × *bavaricum* nothosubsp. *adulteriniforme*, thus the tetraploid offspring of *A.* × *bavaricum* nothosubsp. *adulteriniforme* (TR-1526 = JDL-A6), experimentally produced from spores, was labelled *A. adulterinum* subsp. *presolanense* in the legend to Figure 4A in Mokry et al. (1986). This hypothesis was supported by the following evidence:

Morphological similarity

- in order to demonstrate the close morphological similarity between the artificially raised and wild tetraploid plants silhouettes of pressed fronds were illustrated together in Figure 4 in Mokry et al. (1986);
- all three populations show a distinct prostrate growth habit and have imbricate arrangements of pinnae, thus distinguishing all of them from *A. adulterinum* subsp. *adulterinum*.

Ecology

- the diploid hybrid had also been found on limestone;
- in culture, the diploid hybrid showed a copious production of diplospores from which tetraploid plants had been raised (Lovis et al. 1965, Lovis & Reichstein 1968a);
- it had been demonstrated (Lovis 1958) that *A. trichomanes* subsp. *trichomanes* was involved in the origin of the serpentine *A. adulterinum* s. str. and, therefore, the limestone tetraploid must be the "missing" natural offspring of *A.* × *bavaricum* nothosubsp. *adulteriniforme*.

In their recently published account of the genus *Asplenium* in the "Flora of North America", Wagner et al. (1993) suggested that the genetics of American and European *A. adulterinum* should be compared in order to determine to which subspecies the American plants belong. Evidence for the discrimination of *A. adulterinum* subsp. *presolanense* by Mokry et al. (1986) was derived mainly from morphological comparison and the ecology of this taxon. In this paper we re-examine the evidence presented by Mokry et al. (1986) and present additional evidence derived from molecular methods to elucidate the origin, status and distribution of this taxon.

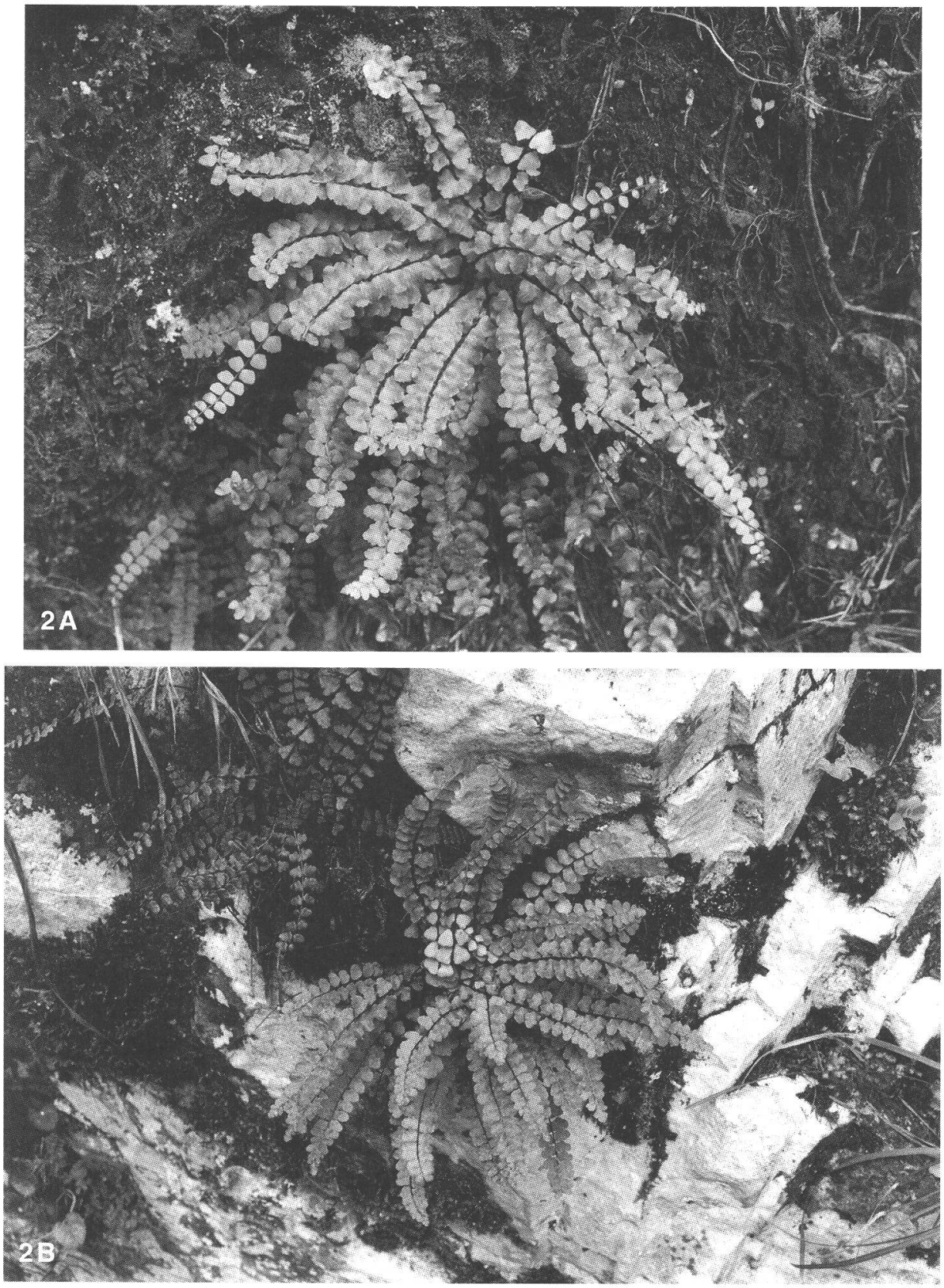


Fig. 2. Diploid *Asplenium* × *bavaricum* D. E. Mey. nothosubsp. *adulteriniforme* (Lovis, H. Melzer & Reichst.) Muñoz Garm. from the *locus classicus* near Gutenstein (A) and from the Weizklamm north of Graz (B), together with *A. trichomanes* subsp. *inexpectans*. Both of these sites are in Austria. An offset of the hybrid from the Weizklamm was included in the allozyme electrophoresis. (Photos: Vogel)

2. Material

Material for morphological comparison and for both allozyme electrophoresis and chloroplast DNA (cpDNA) investigation was obtained by the collection of single fronds of *Asplenium* plants in the wild, and vouchers from populations investigated have been deposited in BM.

Material of *A. presolanense* was collected from the type locality. At Presolana the plant is confined to a very restricted area (ca. 75×40 m) and only 23 plants in four subpopulations (n=9, n=8, n=4, n=2) were large enough to allow the removal of a single frond or part of a frond. About thirty individuals were counted in total. Putative *A. presolanense* from Presolana, Val di Gag and Vancouver Island were compared with *A. adulterinum* from all over Europe, but especially with *A. adulterinum* from Insubria (the area around the Great Lakes shared by Italy and Switzerland, Figure 4), and with an offset of *A.* × *bavaricum* nothosubsp. *adulteriniforme* from the Bärenschützklamm in Austria. In order to investigate the origin of the polyploids, material of the diploid ancestral taxa *A. viride*, *A. trichomanes* subsp. *trichomanes* and *A. trichomanes* subsp. *inexpectans* from regions from all over Europe was included.

One plant, now in the Arktisch-Alpiner Garten (Chemnitz, Germany), and kindly made available to us by its Curator Stefan Jeßen as SJ 2358 (Figure 3), is an off-set of a plant col-

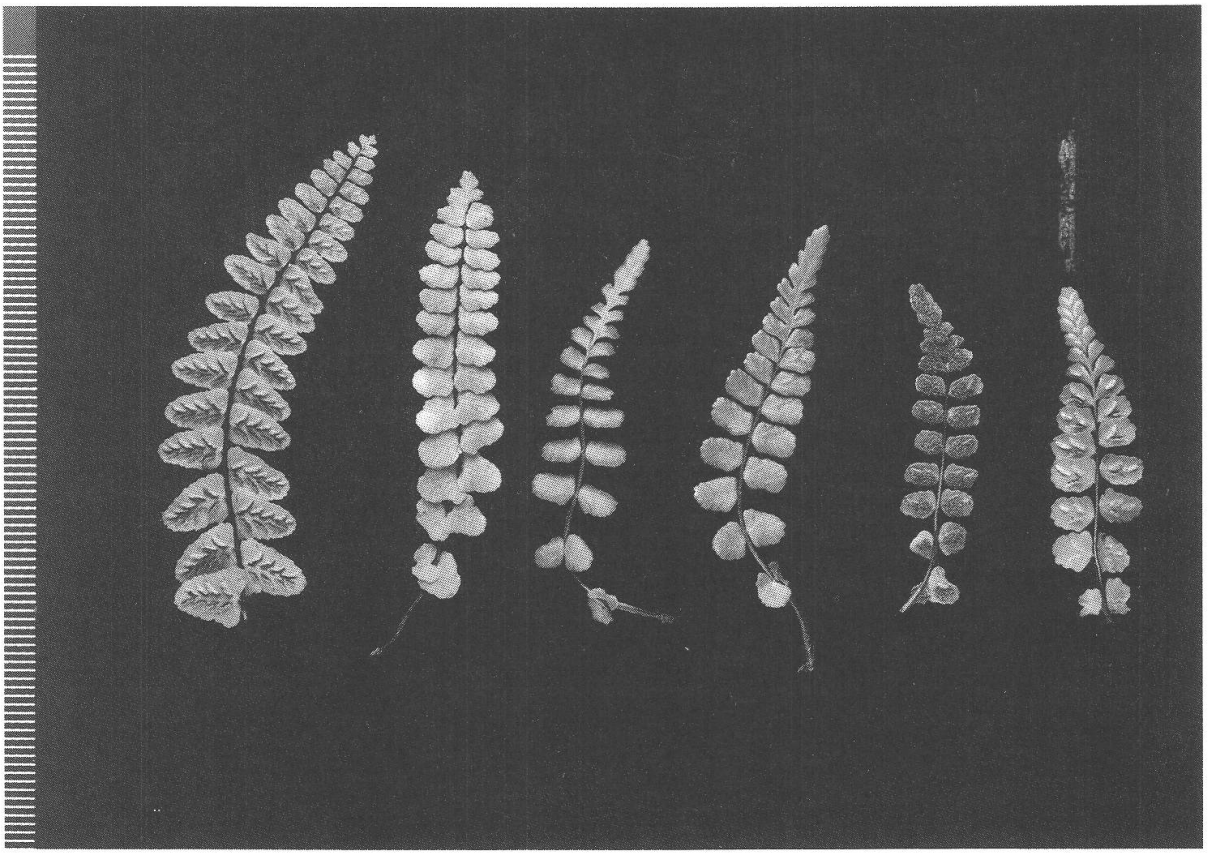


Fig. 3. Morphological variation in *Asplenium trichomanes* L. subsp. *hastatum* (Christ) S. Jess. at Presolana, Bergamo Alps, Italy. The first two fronds (from the left) are from a typical plant. The other four fronds are from offspring of SJ 2358. This plant is characterised by a long green tip (c. top upper-third of the fronds). This morphological feature may have been instrumental for the plant having been mistaken for *A. presolanense*. (Scale: 1mm, Photo: Harry Taylor, Photostudio NHM).

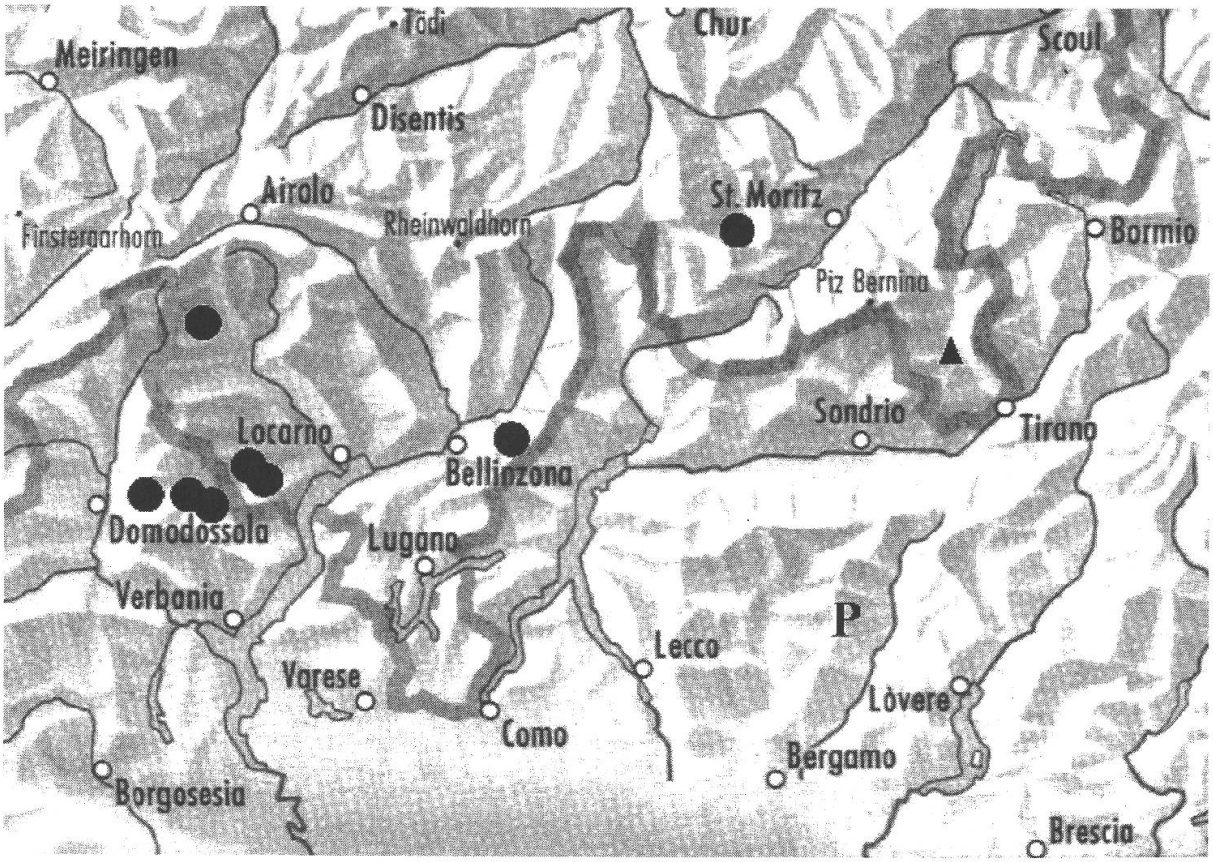


Fig. 4. Distribution of populations of *Asplenium adulterinum* and *A. presolanense* from the southern Alps investigated for this study. ● populations of *A. adulterinum* on serpentine, ▲ putative *A. presolanense* from Val di Gag, **P** *A. presolanense*.

lected as *A. adulterinum* subsp. *presolanense* by H. W. Bennert (SP 1/87, Kalkfelsen ob Colere, ca. 1200 m, loc. classicus, 19. 8. 87). SJ 2358 (Figure 3) is morphologically very different from the other plants of *A. presolanense* (Figure 1). Spores from this offset were sown and grown in London. In May 1997 this taxon was re-discovered as a population of less than 15 plants in the wider vicinity at Presolana. Samples from the offset SJ 2358, spore sowings from the offset and collections from twelve plants in the wild were analysed. From the direct neighbourhood of *A. presolanense* at Presolana 45 plants of *A. viride* in two populations, as well as 128 plants in nine populations of *A. trichomanes* s.l., have been investigated.

Fronds from one plant each of *A. presolanense* and of *A. viride*, as well as from four plants of *A. trichomanes* were made available to us for investigation from the vicinity of putative *A. presolanense* at the Val di Gag by R. Göldi in 1997. Furthermore, material of putative *A. presolanense* from Vancouver Island and Val di Gag was investigated from spore sowings. Spore material from Val di Gag and Vancouver Island was kindly supplied by the late Prof. Reichstein and H. & K. Rasbach. Material from Val di Gag was investigated from two spore sowings from two independent collections: a) TR 5851 (from the original collection by Göldi, see Mokry et al. 1986), b) from a collection by H. & K.Rasbach (Ras s.n.). Material from Vancouver Island was investigated from spore sowings from three plants, the original material collected by Ceska & Ogilvie No. 13983 and from two plants supplied by Reichstein (TR 5754, TR 5759, both raised from the Ceska & Ogilvie collection). The method described by Lovis (1968b) was used to raise material from spores.

3. Methods

In a wide-ranging study of the biosystematics of the genus *Asplenium*, fragment length polymorphism in a non-coding spacer of the chloroplast DNA was investigated for many European taxa (Vogel 1995; Vogel et al. 1996). The intragenic spacer was amplified using the polymerase chain reaction (PCR) (Taberlet et al. 1991). The detailed methodology is described in Vogel et al. (1996, 1998a).

Allozyme electrophoresis has been carried out on *Asplenium* plants using established methods (Soltis et al. 1983, Haufler 1985, Wendel & Weeden 1989, Schneller & Scheffrahn 1989). The following enzyme systems were informative and could be analysed for locus and allelic variation: Phosphoglucoisomerase (PGI, E.C. 5.3.1.9), 6- Phosphogluconate dehydrogenase (6-PGD, E.C. 1.1.1.44), Isocitrate dehydrogenase (IDH, E.C. 1.4.1.42), Hexokinase (HEX, E.C. 2.7.1.1), Malate dehydrogenase (MDH, E.C. 1.1.1.37), Diaphorase (DIA, E.C. 1.6.99), Aconitase (ACON, E.C. 4.2.1.3), Leucine aminopeptidase (LAP, E.C. 3.4.11.1), Shikimate dehydrogenase (SkDH, E.C. 1.1.1.25), Triose-phosphate isomerase (TPI, E.C. 5.3.1.1), Phosphoglucomutase (PGM, E.C. 5.4.2.2), Asparate amino-transferase (AAT, E.C. 2.6.1.1) and Glutamate dehydrogenase (GDH, E.C. 1.4.1.2). Enzyme systems were resolved on a combination of several gel/buffer systems using either 12.8% starch gels or 1% agarose gels. The enzyme systems TPI and IDH resolved best with tris-citrate buffer pH 7.2 on agarose gels as described by Schneller & Scheffrahn (1989). PGI, PGM and MDH can also be resolved with this system. The enzyme systems IDH, MDH, 6-PGD, HEX, SkDH, ACON, GDH were resolved with morpholine-citrate buffers over the pH range of 6.4–7.4 (Wendel & Weeden 1989), or on System 5 (Soltis et al. 1983), while LAP, PGI, PGM, TPI, AAT, DIA were resolved on a modified System 7 (Soltis et al. 1983) over the pH range of 8.3–9.3., and on the modified System 8 (Haufler 1985). Band homologies were determined by running samples side-by-side on the same gel. Allelic variants within loci were distinguished from products of different loci by assuming that the enzymes in *Asplenium* conform to established models of organellar compartmentalisation (Gastony & Darrow 1983, Weeden & Wendel 1989) and by analysing patterns of bands from natural, interspecific hybrids and allotetraploids. The most anodally migrating locus was labelled "*1*", and alleles were designated alphabetically. For the *A. adulterinum* complex some 5000 tetraploids and their diploid ancestral taxa have been investigated so far (Vogel, unpubl.). By determining which alleles are present, and perhaps unique to each diploid, and identifying these alleles in "(fixed) heterozygous" banding patterns of polyploids, it was possible to investigate the origins of the polyploids.

4. Results

4.1. Chloroplast DNA

Chloroplast DNA is inherited maternally in *Asplenium* (Vogel et al. 1998a, b). Length polymorphism in the PCR fragments generated by the chloroplast primers E and F (Taberlet et al. 1991, Vogel et al. 1996) can be used to distinguished the diploid taxa involved in the *A. adulterinum* complex (Figure 5). *Asplenium viride* has a cpDNA fragment length of ca. 450 base pairs (bp), *A. trichomanes* subsp. *trichomanes* has a cpDNA fragment length of ca. 280 bp and *A. trichomanes* subsp. *inexpectans* has cpDNA fragment length of either ca. 350 bp or ca. 360 bp. All plants of *A. adulterinum* (Figure 5, lanes 3–9) and *A. presolanese* (Figure 5, lanes 10–12) have the cpDNA fragment length of *A. viride* with ca. 450 bp. Six diploid wild hybrids between *A. trichomanes* subsp. *inexpectans* and *A. viride* from Aus-

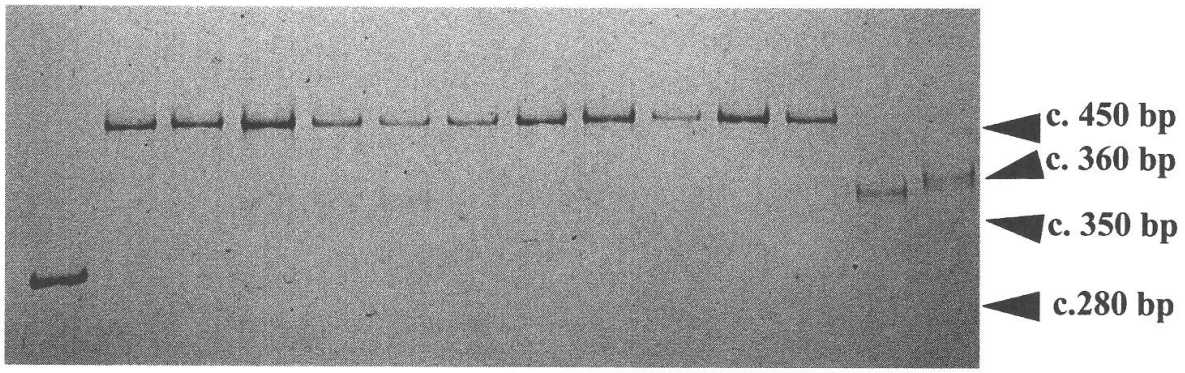


Fig. 5. Variation of chloroplast fragments generated by Primers E and F in the *Asplenium adulterinum* complex. *A. viride*, *A. adulterinum* and *A. presolanense* have a cpDNA fragment size of ca. 450 bp, indicative of *A. viride* being the female parent in hybrids leading to the formation of the two allotetraploid taxa. Lane 1: *A. trichomanes* subsp. *trichomanes*, serpentine, west of Helsinki, Finland; lane 2: *A. viride*, paragneiss, Mirnock, Austria; lane 3: *A. adulterinum*, serpentine, west of Helsinki, Finland; lane 4: *A. adulterinum*, serpentine, Bjørkedalen, Norway; lane 5: *A. adulterinum*, paragneiss, Mirnock, Austria; lane 6: *A. adulterinum*, serpentine, Druogno, Italy; lane 7: *A. adulterinum*, serpentine, Mt Aiona, Italy; lane 8: *A. adulterinum*, serpentine, Sedlice, Slovakia; lane 9: *A. adulterinum*, magnesite, Eichberg, Austria; lane 10: *A. adulterinum*, limestone, Vancouver Island, Canada; lane 11: *A. adulterinum*, serpentine/micaschist, Val di Gag, Switzerland, lane 12: *A. presolanense*, limestone, Presolana, Italy; lane 13: *A. trichomanes* subsp. *hastatum*, limestone, Presolana, Italy – SJ 2358, originally identified as *A. presolanense* (see Figure 3); lane 14: *A. trichomanes* subsp. *inexpectans*, limestone, Weizklamm, Austria. Lane 1 has a fragment of ca. 280 bp for *A. trichomanes* subsp. *trichomanes*. Lanes 2 to 12 have a fragment of ca. 450 bp, representing or being derived from *A. viride*. Lane 13 has a fragment of ca. 350 bp. Lane 14 is *A. trichomanes* subsp. *inexpectans* from Austria with a cpDNA fragment size of ca. 360 bp.

tria all have the cpDNA fragment length of *A. viride*. These results from the diploid hybrids and their tetraploid derivatives indicating a strong bias in the direction of hybridisation, with only *A. viride* acting as the female parent. A similar strong bias has been observed in plants of *A.* × *alternifolium* Wulfen at all three ploidy levels, where *A. septentrionale* acts predominantly as female parent (Vogel et al. 1998b). The plant SJ 2358, formerly believed to be *A. presolanense*, has a cpDNA fragment length of ca. 350 bp (Figure 5, lane 13), compatible with *A. trichomanes* subsp. *inexpectans* as female parent.

4.2. Allozyme electrophoresis

Thirteen enzyme systems coding with a total of 18 loci were studied to investigate genetic variation in *A. adulterinum* and its ancestral diploids from serpentine and other substrates from different regions from all over Europe. At the majority of loci, the diploid taxa had different alleles, or were polymorphic with one or more shared alleles. The allozyme patterns observed in the tetraploid could in all cases be reconstructed from the alleles present in the putative diploid ancestors ("additive" patterns). Our allozyme data demonstrated that *Asplenium adulterinum* s. str. has additive banding patterns of alleles observed in *A. trichomanes* subsp. *trichomanes* and *A. viride* in all enzyme systems tested (Vogel, unpubl.). These same "additive" banding patterns were also found in *A. adulterinum* from substrates other than serpentine (such as material from Austria growing on paragneiss and magnesite), as well as for the populations of putative *A. presolanense* from Val di Gag and Vancouver Island (Figure 6, Table 1). In contrast, *A. presolanense* from the type locality at Presolana in the Bergamo Alps

Table 1. Enzyme phenotypes and inferred genotypes in the most informative loci (MDH-1, MDH-2, MDH-3, MDH-4; ACON-1, ACON-2; DIA-2; IDH: 6-PGD-2; PGI-2) to discriminate *Asplenium adulterinum* and *A. presolanense*. This analysis is based on the patterns observed in *A. viride*, *A. trichomanes* subsp. *trichomanes* and *A. trichomanes* subsp. *inexpectans*. Patterns in *A. viride* from "elsewhere" are only given where they represent alleles which are represented in *A. adulterinum* from Vancouver Island, Val di Gag or the Centovalli. **ACON-1:** The apparent monomorphism in ACON-1 with 2–4 bands has been interpreted as evidence of duplicate loci. However, in the absencee of segregation data, alleles cannot be assigned to specific loci.

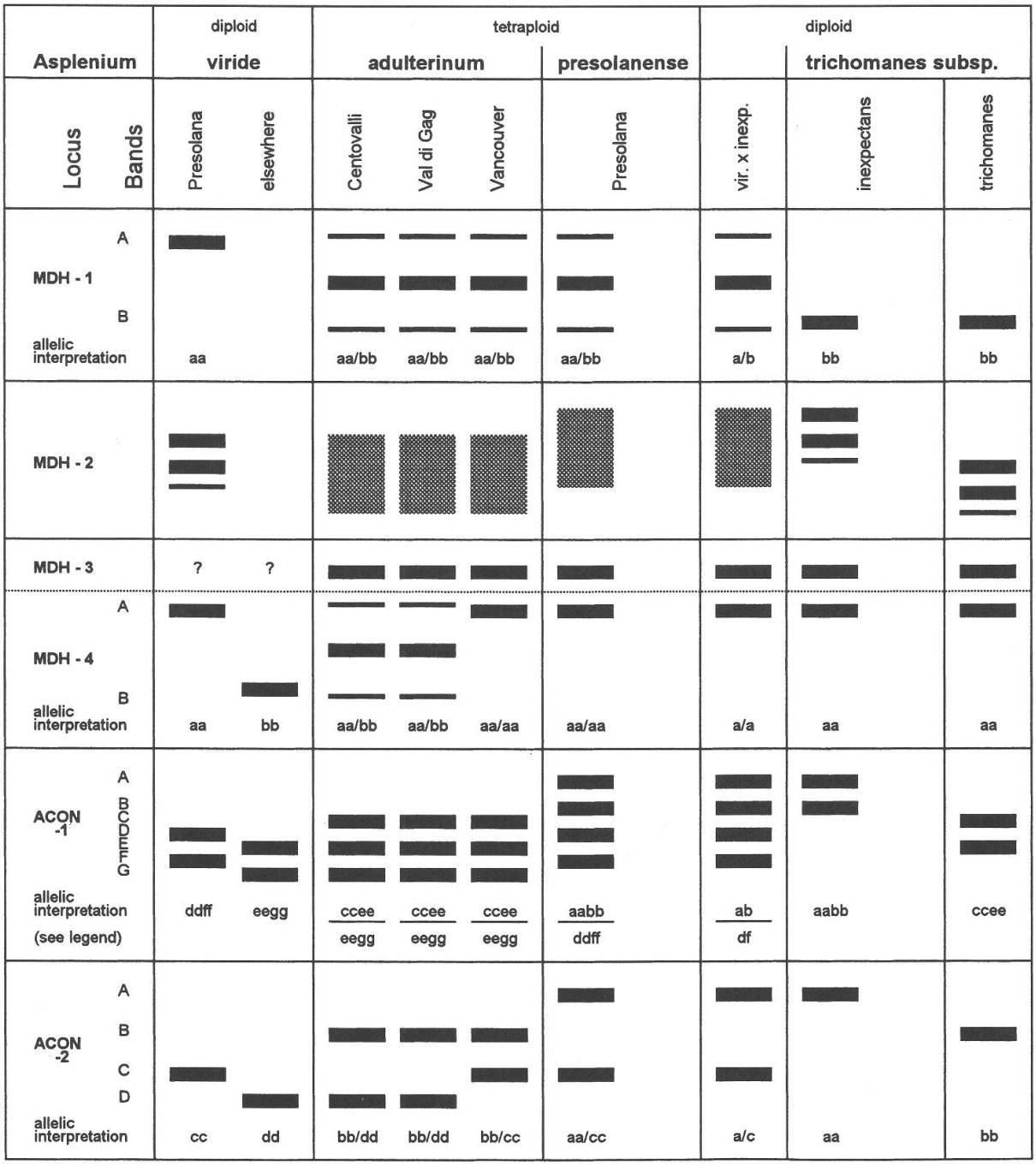
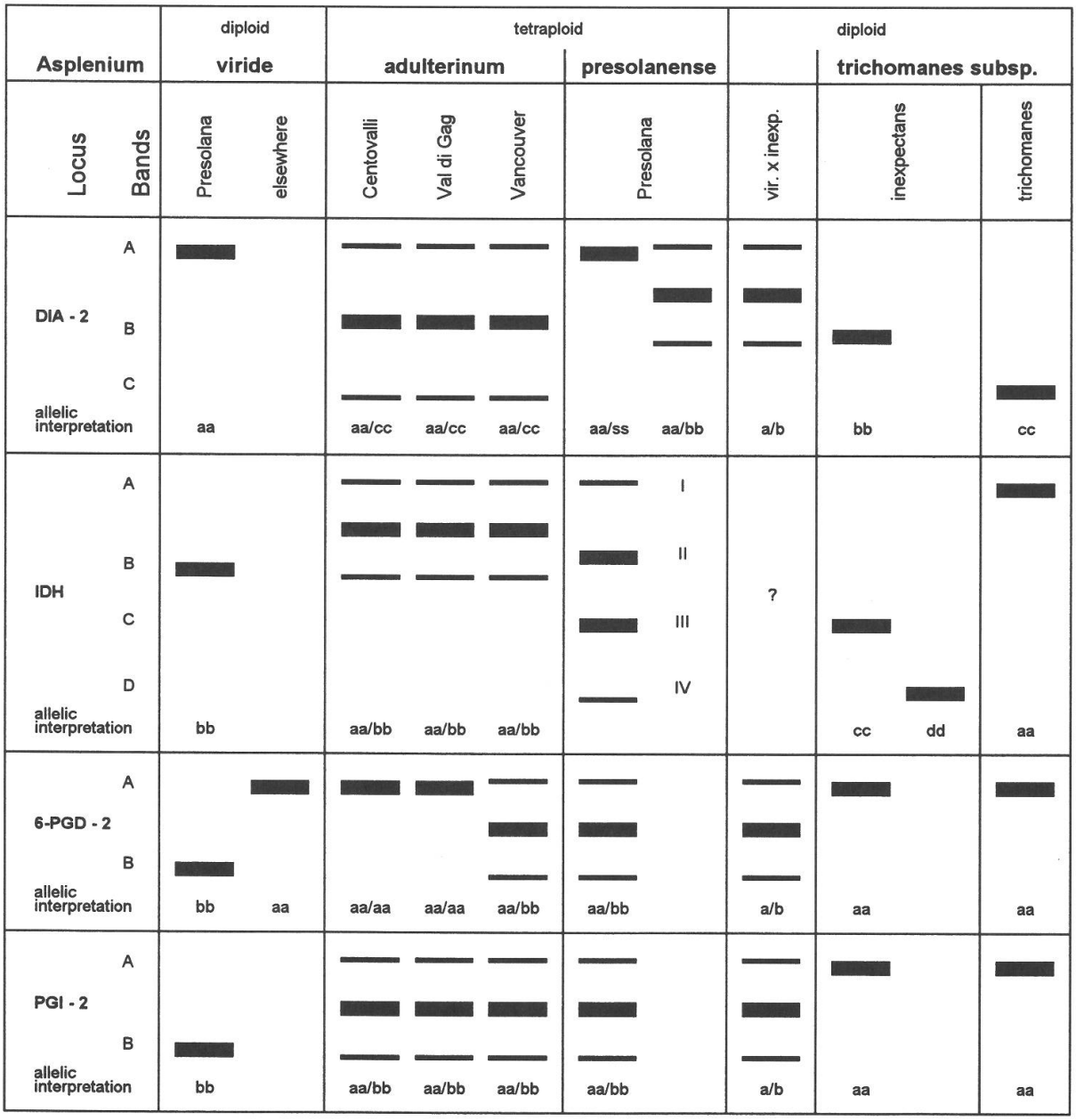


Table 1. (cont.)



has additive banding patterns of *A. viride* and *A. trichomanes* subsp. *inexpectans* (Figure 6, Table 1). Twenty-three plants of *A. presolanense* investigated from Presolana revealed no allelic variation at any of the 18 loci scored with the exception of DIA-2, where a one-banded pattern was found in 19 plants (from three subpopulations) and a three-banded pattern was observed in four plants in two subpopulations. All 45 plants of *A. viride* from Presolana were monomorphic for all 18 loci analysed.

The enzyme profile of offspring of the plant SJ 2358, collected at Presolana by H. W. Bennert as *A. adulterinum* subsp. *presolanense*, and those of the similar population of twelve plants found at Presolana by two of the authors (JCV & FJR) show no evidence of any involvement of *A. viride*, i.e. no alleles were found which were unique to *A. viride*. SJ 2358 has

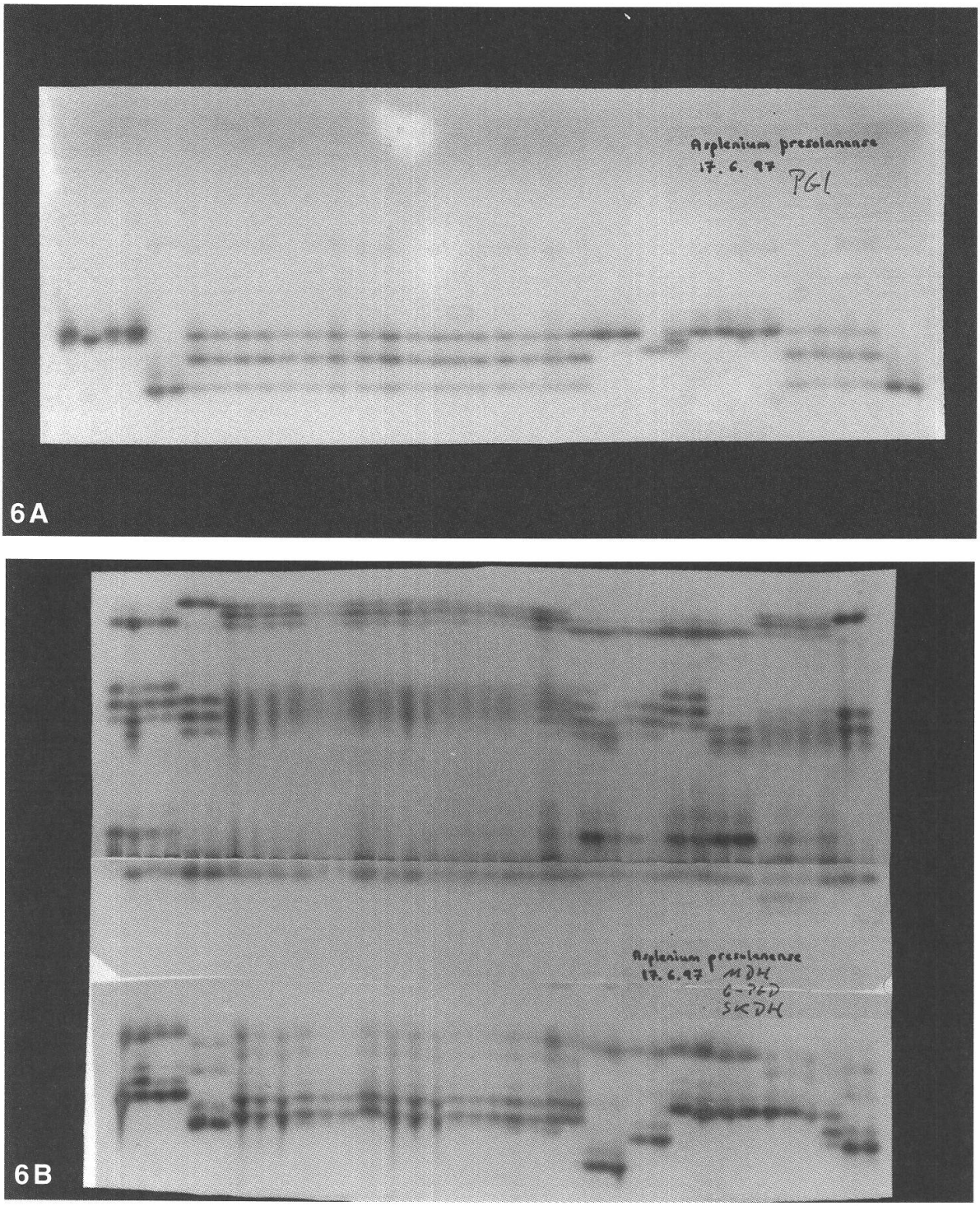


Fig. 6. Zymograms showing electrophoretic banding patterns in PGI (A), MDH and 6-PGD (B). For explanation of banding patterns see Table 1 and text. All plants, unless indicated, from Presolana, lane 1: *Asplenium trichomanes* subsp. *hastatum*; lane 2: *A. trichomanes* subsp. *quadrivalens*; lanes 3–4: *A. trichomanes* subsp. *hastatum* SJ 2358-form; lanes 5–6: *A. viride*; lanes 7–21: *A. presolanense*; lane 22: *A.* × *bavaricum* nothosubsp. *adulteriniforme* (Weizklamm); lanes 23–28: *A. trichomanes* subsp. *inexpectans* (23–26: France; 27–28 Austria); lanes 29–30: *A. trichomanes* subsp. *trichomanes* (S-Sweden); lanes 31–34: *A. adulterinum* (31: Ravecchia; 32: Bordei, both from serpentine from the Centovalli; 33: Val di Gag; 34: Vancouver Island, the latter two had been reported as being *A. presolanense*); lanes 35–36: *A. viride*.

a long green tip (Figure 3), which is probably why it was mistaken for *A. presolanense* by its first collector and by Vogel (Vogel 1995). The plant has been cytologically confirmed to be tetraploid and evidence from cpDNA (*A. trichomanes* subsp. *inexpectans* size fragment) and allozymes suggests it to be a peculiar morphological form of *A. trichomanes* L. subsp. *hastatum* (Christ) S. Jess. (Figure 3; Figure 6: lanes 1, 3+4; Vogel, unpubl.). *Asplenium trichomanes* subsp. *hastatum* is the most common *Asplenium* on the rock faces at Presolana (for determination see Jeßen 1995, p. 114), and is depicted in Mokry et al. (1986, Figure 4G= Ras-392), but identified as subsp. *quadrivalens* in the footnote.

Populations of diploid *A. trichomanes* s.l. show a considerable amount of genetic variation at all loci in all enzyme systems investigated (Vogel, unpubl.). However, some of this variation is present as "private alleles" restricted to a particular taxon or to a taxon in a specific geographic region. Such private alleles (or patterns) may help to clarify the origins of all three populations of putative *A. presolanense*. The additive patterns at four loci, MDH-2, ACON-1, ACON-2 and DIA-2, exclude *A. trichomanes* subsp. *trichomanes* as an ancestral taxon of *A. presolanense* from Presolana, because the alleles or patterns which have contributed to this allopolyploid have not been observed in *A. trichomanes* subsp. *trichomanes* (Figure 6, Table 1). On the other hand, these four loci provide evidence of the involvement of *A. trichomanes* subsp. *trichomanes* in putative *A. presolanense* from Vancouver Island and Val di Gag (Figure 6, Table 1). Neither diploid subspecies of *A. trichomanes* has been found at Presolana, but two plants of *Asplenium trichomanes* subsp. *trichomanes* have been identified amongst the collection by Göldi from Val di Gag in Switzerland.

Allozymes are codominantly inherited and in many cases polyploid taxa show additive banding patterns for the alleles derived from their putative ancestral taxa. Most alleles expressed in the allopolyploid *A. presolanense* had identical mobilities to those found in the putative parental diploids (Table 1). Furthermore, the alleles and patterns observed in *A. presolanense* at Presolana can be attributed to genetic variation observed in *A. viride* at Presolana (in 17 loci) and *A. trichomanes* subsp. *inexpectans* from Austria (in 17 loci). However, genetic variation in IDH was more difficult to explain. While the heterozygous pattern for IDH in *A. adulterinum* was resolved satisfactorily on both agarose and starch gels the pattern in *A. presolanense* was not. We interpreted the observed pattern in *Asplenium presolanense* as representing four bands (Table 1). The slowest band (IV) has the same mobility as an allele observed in *A. trichomanes* subsp. *inexpectans* in Austria, while the fastest band (I) has the same mobility as the allele observed in *A. trichomanes* subsp. *trichomanes*. Band II is close to, but not identical with alleles observed in *A. viride*, while band III had the same mobility as the allele observed in *A. trichomanes* subsp. *inexpectans* from France.

4.3. Morphology

The following features were stressed by Mokry et al. (1986) to discriminate *A. presolanense* from *A. adulterinum*: 1. the arrangement of pinnae, 2. pinna margins, 3. the shape of the terminal pinna, 4. the growth habit and 5. the ecology of the plants. However, a re-examination of these features revealed that they are not reliable characters for such a discrimination as the variation expressed in *A. adulterinum* s. str. from different populations all over Europe encompasses the variation and features attributed solely to *A. presolanense* by Mokry et al. (1986) (Table 2).

As shown in Figure 1 plants of *A. presolanense* at Presolana have an imbricate arrangement of pinnae with entire margins, and a broad terminal pinna. However, imbrication of pinnae is not confined to this taxon but can also be observed in *A. adulterinum* s. str., e.g. at the Eichberg or Mt. Mirnock, Austria (Figure 7). On the other hand, the two populations of pu-

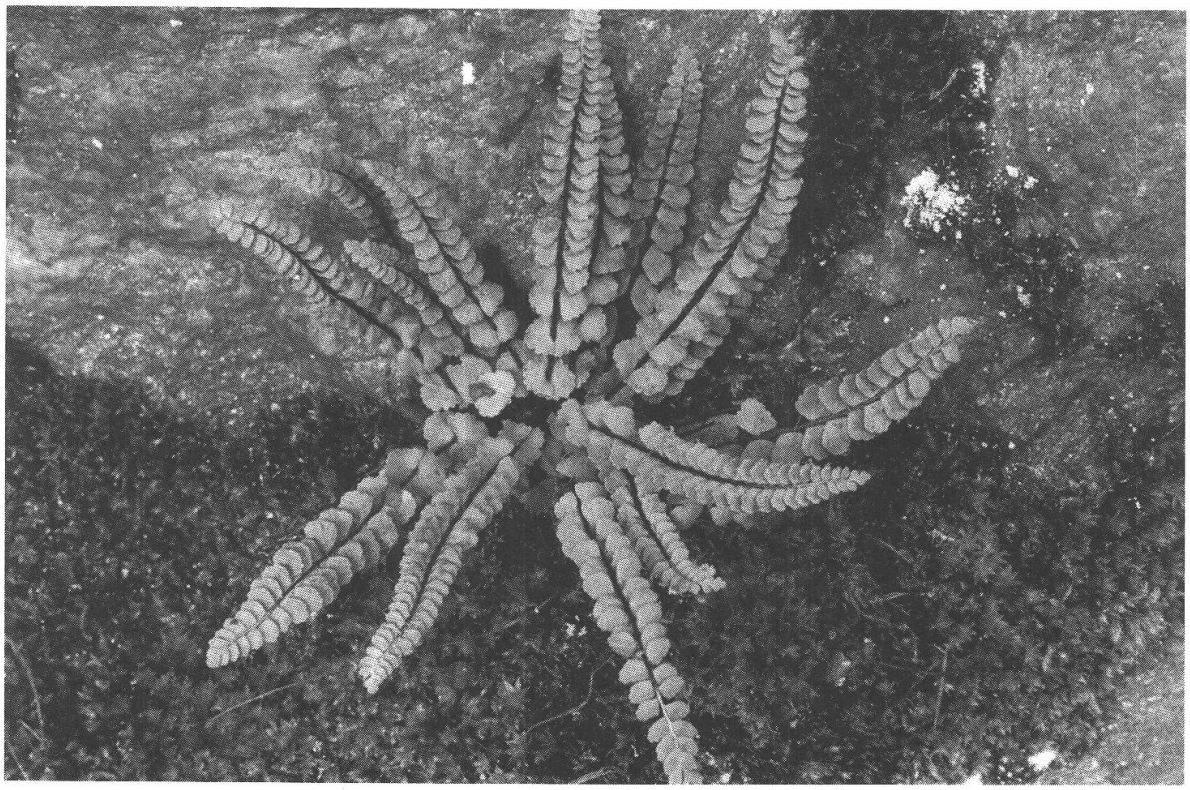


Fig. 7. *Asplenium adulterinum* Milde on paragneiss at the Mirnock in Austria. This plant exhibits the imbricate arrangement of pinnae and has a wide terminal pinna, characters which have been used to discrimate *A. presolanense* from *A. adulterinum*. (Photo: Vogel)

Table 2. Characters which have been used by Mokry et al. (1986) to differentiate *A. adulterinum* and *A. presolanense*. The morphological and ecological variation expressed in *A. adulterinum* s. str. from different populations from all over Europe and from Vancouver Island encompasses the variation and features attributed solely to *A. presolanense* by Mokry et al. (1986). Furthermore, the presence, absence and expression of a character or characteristic differ certainly between, sometimes even within, populations of *A. adulterinum* s. str..

Character	Presolana	Val di Gag	Vancouver	*A. adulterinum* s. str.
Arrangement of pinnae	imbricate to just touching	not imbricate, well spaced	not imbricate, well spaced	widely spaced to imbricate
Pinna margin	entire to slightly crenate	crenate	crenate	distinctly crenate to slightly crenate
Frond habit	prostrate	erect	prostrate?	prostrate to erect
Substrate	limestone	micaschist/ serpentine	limestone	serpentine, magnesite, paragneiss

tative *A. presolanense* from Val di Gag and Vancouver Island do not possess imbricate pinnae. The character of a broad final pinnae is shared by populations of *A. presolanense* at Presolana and Vancouver Island, but is also present in *A. adulterinum* s. str. from magnesite at the Eichberg in Niederösterreich (Lämmermayr 1930). While different populations of *A. adul-*

terinum s. str. can have all degrees of crenation from entire to distinctly crenate, *A. presolanense* from Presolana has entire pinnae margins, while plants of both populations at Val di Gag and from Vancouver Island have distinctly crenate pinnae margins.

The description of taxa is normally based on morphological characters. Morphological variation may not accompany speciation or reflect evolution. In reticulate polyploid complexes such as the *A. adiantum-nigrum* or *A. trichomanes* aggregates the putative ancestral diploid taxa are closely related **and** morphologically variable. Therefore the tetraploid derivatives can exhibit considerable phenotypic plasticity, making the discrimination of taxa using (micro-) morphological characters, at best, difficult (Vogel et al. 1996). While the entire pinnae margin, in conjunction with an imbricate pinnae arrangement is found in most plants of *A. presolanense* at Presolana and would discriminate it from *A. adulterinum* some plants show a weak crenation and, where shaded, less overlapping pinnae. We therefore conclude that, due to the great morphological variation in *A. adulterinum* s. str., an unequivocal discrimination between *A. presolanense* and *A. adulterinum* s. str is not always possible on morphological characters. On the other hand, using molecular methods such as allozyme electrophoresis, a multilocus phenotype profile can be established which separates the two taxa. This methodology furthermore demonstrates that *A. adulterinum* and *A. presolanense* represent distinct phylogenetic lineages.

4.4. The origin and status of putative Asplenium presolanense from Val di Gag and Vancouver Island

Our evidence from allozyme electrophoresis demonstrates that the plants from Val di Gag (Switzerland) are *A. adulterinum* s. str. and are indistinguishable on morphological and genetic evidence from populations from serpentine outcrops at Mulengs (Switzerland, 50 km north-west) or from populations in Centovalli near Lago Maggiore (ca. 100 km west). This is further supported by morphology, as shown in Figure 4 in Mokry et al. (1986) and by its ecology. Originally the plants from Val di Gag were reported to be growing on micaschist (Mokry et al. 1986), but now the rock type is believed to be serpentine or magnesite (Göldi, in Moser & Palese 1995).

In a review of the distribution of *A. trichomanes* subsp. *inexpectans* (Bennert et al. 1989), H. & K. Rasbach reported a single plant of diploid *A. trichomanes* (apparently morphologically resembling subsp. *inexpectans*) growing next to *A. adulterinum* in Val di Gag in Switzerland. They also report *A. viride* from there. The diploid *A. trichomanes* was inferred to be subsp. *inexpectans* because it was growing on the same substrate as putative *A. presolanense*. We have been able to demonstrate that the *A. adulterinum* in Val di Gag is not *A. presolanense* and have identified two plants of diploid *A. trichomanes* from there as subsp. *trichomanes*. This interpretation is also more compatible with the current geological findings in that area. As a result, the records of *A. presolanense* and *A. trichomanes* subsp. *inexpectans* for the Swiss flora are no longer deemed to be valid.

Vancouver Island is a large island on the northern Pacific coast of North America. During a study of the mountain flora of the island in 1982, *A. adulterinum* was discovered on a ridge between Lime Creek and Fault Creek at 1250 m altitude (Ogilvie & Ceska 1984). Lime Creek Ridge is composed of limestone of the Upper Triassic Quatsino Formation which is in contact with granite and granodiorite of the middle Jurassic Vancouver Island intrusions and with basaltic rocks of the Karmutsen Formation (Muller et al. 1974). The site is dominated by alpine heath and rock outcrops and the species was found infrequently on walls of sinkholes. Of all the mountains examined, the highest species diversity was described from Lime Creek Ridge (Ogilvie & Ceska 1984), including the calcicole fern *Polystichum lonchitis* (L.) Roth. Ceska

(1991) reported three *Asplenium* species from Vancouver Island: *A. viride* (for its distribution see Map 148 in Cody & Britton 1989), *A. trichomanes* and *A. adulterinum*. The mixture of rocks on the island could provide suitable habitats for both diploid taxa of *A. trichomanes*, subsp. *trichomanes* and subsp. *inexpectans*. Moran (1982) reported diploid *A. trichomanes* subsp. *trichomanes* from the southern part of Vancouver Island but the presence of *A. trichomanes* subsp. *inexpectans* has not been confirmed. From the evidence of allozyme electrophoresis the plants of *A. adulterinum* from Vancouver Island appear to represent true *A. adulterinum* s. str., with no evidence of any involvement of *A. trichomanes* subsp. *inexpectans* (Table 1). *Asplenium adulterinum* from Canada has a multilocus phenotype so far not observed in Europe, but which can be reconstructed additively from alleles present in European populations of *A. trichomanes* subsp. *trichomanes* and *A. viride*. This is compatible with the hypothesis of an independent origin for Canadian material of *A. adulterinum*, rather than long-range spore dispersal from a European source (Vogel, unpubl.). However, material from wild collections of *A. trichomanes*, *A. viride* and *A. adulterinum* from Vancouver Island would be desirable to test this hypothesis, and to determine whether *A. trichomanes* subsp. *inexpectans*, a taxon at present believed to be confined to Europe, does grow there.

4.5. The origin of Asplenium presolanense

Asplenium presolanense is known so far only from one population of about 30 plants at Presolana in the Bergamo Alps. An investigation of rock faces in the vicinity with similar exposure and at similar altitude failed to reveal a second population. As demonstrated, *A. presolanense* at Presolana is of unique and distinct origin in comparison with serpentine-dwelling *A. adulterinum* s. str.. The evidence presented from ecological and genetic investigations shows that the taxon from Presolana in the Bergamo Alps in northern Italy is distinct from *A. adulterinum* and that it merits specific status.

> *Asplenium presolanense* (Mokry, Rasbach & Reichst.) J. C. Vogel & Rumsey spec. nov. Basionym: *Asplenium adulterinum* Milde subsp. *presolanense* Mokry, Rasbach & Reichst. in Botanica Helvetica, 96/1: 8 (1986).
> Type: Italy, Prov. Bergamo, northern slope of the Presolana, tributary valley of the Valle di Scalve, shady limestone rocks above Colere, at ca. 1200 m alt., 9. 9. 1983, leg. H. & K. Rasbach, Ras-369 (Holotype: **G**).
> Paratypes raised as progeny from spores of F. Mokry s.n., 31. 7. 1979, under the accession number T. Reichstein 5082, are deposited in **G**, **BM**!, **FI**, **OAC**, **RO** and **Z**.

5. Discussion

The work by Mokry et al. (1986) concentrated on the population at Presolana; the later findings of the plants from the Val di Gag and Vancouver Island are treated as an annex in that publication. However, plants from all three populations were described as one taxon. No attempt was made to demonstrate the origin of *A. presolanense* from *A. viride* and *A. trichomanes* subsp. *inexpectans* by controlled hybridisation experiments.

The proposed origin and distribution of *A. presolanense* by Mokry et al. (1986) has to be viewed critically for the following reasons:

- No direct experimental evidence was provided to support the hypothesis that the diploid *A. trichomanes* subsp. *inexpectans* is a parent of *A. presolanense*,
- all three populations of putative *A. presolanense* are morphologically and/or ecologically distinct from each other,

- the diploid "precursor" *A.* × *bavaricum* nothosubsp. *adulteriniforme*, and its artificially raised tetraploid derivative, are morphologically distinct from *A. presolanense* (see Fig. 4: A–E, in Mokry et al. 1986; Figure 1 and 2),
- this diploid precursor has been found only in the eastern Alps, some 400 km away from the two putative populations of *A. presolanense* in Presolana and Val di Gag,
- diploid *A. trichomanes* subsp. *inexpectans* is probably under-observed, but so far there are no confirmed records from Italy, Switzerland and the central and western Alps.

One of the taxa ancestral to *A. presolanense*, diploid *A. viride*, is widespread in the Alps and is present at the type locality while the other diploid ancestor, *A. trichomanes* subsp. *inexpectans*, has its nearest populations some 300–400 km away, either to the west in southern France or in eastern Austria. Despite the fact that both putative ancestral diploids of *A. presolanense* grow together currently in southern France and in Austria, no allotetraploid plants derived from them have been reported from either area. However, about twenty plants of *A.* × *bavaricum* nothosubsp. *adulteriniforme*, with copious production of fertile diplospores, have been recorded from Austria since the 1960s (Melzer 1966; Reichstein 1981, 1984; personal observations). Three possible modes of origin of *A. presolanense* at Presolana can be postulated,

i) *A. presolanense* is an apoendemic (=narrow endemic polyploid of local origin, according to the definition of Favarger & Contandriopoulos, 1961) of considerable age,
ii) a remnant isolated population of an old, formerly more widespread taxon, or
iii) the result of a recent long-range (diplo-) spore dispersal event, perhaps from Austria.

Asplenium presolanense occupies a very special micro-ecological niche at its type locality, growing on vertical rock faces and under overhangs. Apparently suitable sites in the vicinity are not colonized, but it is a difficult terrain to explore and impracticable to do so exhaustively. Our experiments showed that spore germination is difficult and spores may take several months before germination. This may influence the ability of the species to colonize new sites. A poor dispersal capacity for *A. presolanense* and a restricted range of suitable habitats in which it can grow may account for the current rarity of the species. We suspect that it may have a more widespread distribution in the Bergamo Alps, but it is unlikely ever to have been as widespread in Europe as its sister taxon *A. adulterinum*.

Using allozymes from *A. presolanense* as biosystematic markers, the genetic make-up of the ancestral *A. trichomanes* subsp. *inexpectans* can be reconstructed (Werth, 1989). Eighteen loci in 13 enzyme systems were studied, of which alleles present in 17 loci can be found in *A. trichomanes* subsp. *inexpectans* from Austria. This supports the hypothesis of a recent long-range (diplo-) spore dispersal from Austria, the only area from where the diploid precursor has been recorded. Despite the fact that the complex four-band pattern in IDH cannot be explained at the moment, it appears that alleles could be involved that share the same mobility as alleles observed exclusively in subsp. *trichomanes* and in geographically separated populations of subsp. *inexpectans* from Austria and France. This complex pattern would exclude an origin of *A. presolanense* out of Austria and would increase the possibility that a common ancestor of the diploid taxa of *A. trichomanes* was involved in the formation of *A. presolanense*.

Allozyme electrophoresis revealed that *A. presolanense* is monomorphic for all enzyme systems observed, with the exception of Diaphorase (DIA). Here, *A. presolanense* is genetically variable at one locus. Up to four isozymes are reported for Diaphorase and the quaternary structure can vary between monomers, dimers to tetramers. In *Asplenium* the upper locus DIA-1 is not always well resolved, but DIA-2 can be analysed, as it is normally well

resolved as sharp blue bands. A wide three-banded pattern is found in *A. adulterinum*, the fast allele having the mobility of the allele present in *A. viride* and the slow allele having the same mobility as that in *A. trichomanes* subsp. *trichomanes* (Table 1). The presence of a three-banded pattern in *A. adulterinum* and other polyploid *Asplenium*, would indicate that the quaternary structure of alleles in DIA-2 in *Asplenium* is dimeric. The variation at the locus DIA-2 in *A. presolanense* is discussed under this premise. Two phenotypes were observed. Four plants in two subpopulations had a three-banded pattern, compatible with a combination of the alleles from *A. viride* at Presolana and *A. trichomanes* subsp. *inexpectans* from Austria (Table 1), while 19 plants in three subpopulations had a single band with the same mobility as the allele present in the local *A. viride*. The two subpopulations of eight and nine plants were monomorphic for the one-band pattern, the smallest population of two plants was monomorphic for the three-band pattern and a small population of four plants, situated between the two larger subpopulations, had two plants with each pattern. Such genetic variation can be explained in three ways:

i) independent origins for the two forms;
ii) genetic segregation in a population with both allelic forms and their heterozygote;
iii) a "loss of function" mutation (in an originally fixed heterozygous pattern) of the allele derived from *A. trichomanes* subsp. *inexpectans*.

As the mobility of the alleles present matches that of the ancestral diploid taxa, and no homozygotes for the slow allele were observed, the hypothesis that the patterns represent genetic segregation in a population with both allelic forms and their heterozygote is not well supported. While multiple origin cannot entirely be excluded it appears to be a most unlikely explanation. *Asplenium presolanense* is so far only known from this one population of less than 30 plants, and we do not believe that two ancient clades could persist in such a small community. All of these considerations point to the possibility of a mutation producing a loss of function at one locus in a previously monomorphic fixed heterozygous pattern, which could then be fixed by intra-gametophytic selfing. No recombinants between the two patterns have been observed. While conclusions are limited by the small sample size, the substructuring of genetic variation between the four subpopulations would indicate that intra- or inter-gametophytic selfing is taking place. Whatever conclusion might be reached to explain the origin of genetic diversity in this small population of *A. presolanense*, its presence might, in conjunction with the complex IDH pattern, be indicative of a considerable age for the population.

In close proximity to *A. presolanense*, plants such as *Campanula raineri* Perpenti, *Physoplexis comosa* (L.) Schur and *Telekia speciosissima* (L.) Less are present. These three, and others, e.g. the narrow endemics *Moehringia dielsiana* Mattf. or *Saxifraga presolanensis* Engler, are reported to represent old relicts (Pitschmann et al. 1965, Ravazzi 1988). This part of the southern slope of the Alps may never have been completely covered by ice during the several glaciation periods since the Tertiary (Hantke 1993). Investigations by Emmert-Straubinger (1991, cit. in Hantke 1993) revealed that the interglacial flora in the Bergamo Alps was characterised by a high percentage of south-east European, thermophilous and submediterraean floristic elements growing in a humid, winter-mild and sub-oceanic climate. These conditions in this area would have supported populations of *A. trichomanes* subsp. *inexpectans*, that may have been involved in the formation of *A. presolanense* in the Bergamo Alps.

Taken together, the genetic, ecological and palaeo-ecological evidence supports the hypothesis that *A. presolanense* is a narrow endemic palaeopolyploid taxon that has survived *in situ* in this refugium during the last glaciation cycles.

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Zusammenfassung

Die Abstammung, Verbreitung und der taxonomische Status von *Asplenium presolanense* wurden untersucht. Dieses Taxon wurde ursprünglich als Unterart zu *Asplenium adulterinum* beschrieben. *Asplenium adulterinum* Milde ist eine allotetraploide Art, die selten, aber weitverstreut, auf Serpentin und anderen, meist ultrabasischen Gesteinen in Europa vorkommt. Mokry et al. (1986) beschrieben *A. adulterinum* subsp. *presolanense* als eine Unterart, die auf Kalk und Glimmerschiefer wächst. *Asplenium presolanense* wurde für drei Stellen angegeben: eine Population mit rund 30 Pflanzen in der Presolana in den Bergamasker Alpen (Italien), wenige Pflanzen im Val di Gag im Puschlav (Südschweiz, etwa 35 km nördlich des *locus classicus*) und von mehreren Populationen auf Vancouver Island vor der Westküste Kanadas. Mokry et al. (1986) führten die Unterscheidung der beiden Unterarten hauptsächlich auf morphologische und ökologische Merkmale zurück.

In der hier vorliegenden Untersuchung werden die Ergebnisse eines erneuten morphologischen und ökologischen Vergleichs durch molekulare Methoden ergänzt. Es kann gezeigt werden, daß die drei oben genannten Populationen von *A. presolanense* zwei verschiedene Taxa darstellen. Die Vorkommen im Val di Gag (Schweiz) und auf Vancouver Island (Kanada) sind auf Grund ihrer Morphologie und ihres genetischen Ursprungs aus *A. viride* und *A. trichomanes* subsp. *trichomanes* zu *A. adulterinum* s. str. zu stellen. *Asplenium trichomanes* subsp. *inexpectans* ist an der Entstehung der allotetraploiden Pflanzen in der Presolana beteiligt, und dieses Taxon wird als *A. presolanense* neu beschrieben. Molekulare Untersuchungen deuten darauf hin, daß *A. presolanense* eine apoendemische Art ist.

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