Phylogenetic Relationships between *Asplenium bourgaei* (Boiss.) Milde and *A. jahandiezii* (Litard.) Rouy Inferred from Morphological Characters and *rbc*L Sequences

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Received: December 14, 2000; Accepted: May 25, 2001

Abstract: Micromorphological analysis reveals that *A. jahandiezii*, which occurs as a local endemic taxon in the Grand Canyon du Verdon in southern France, shows substantial differences to *A. bourgaei*, from the eastern Mediterranean region, in the surface structure of perispores, sporangial indehiscence and the size of stomatal subsidiary cells. Nucleotide sequences and genetic distances (0.9% nucleotide substitutions) of the *rbc*L gene support the view that both taxa are related but distinct sibling species. Both species form a monophyletic group together with *Asplenium fontanum* and *A. majoricum*.

Key words: *Asplenium*, *rbc*L sequences, sibling species, molecular phylogeny.

Introduction

The worldwide distributed genus *Asplenium* is represented in Europe by 43 taxa (31 species and 12 subspecies) and constitutes the largest fern genus of this continent (Crabbe et al., 1993[4]). Species differ considerably in morphology (e.g., undivided or 1- to 3-pinnated leaves), size, ecological demands (either on calcareous, or siliceous, sandstone, dolomite, or serpentine rocks), distribution range (wide versus local distribution), and chromosome numbers (diploid, tetraploid, or triploid).

Among the European *Asplenium* species, the diploid *A. bourgaei* and *A. jahandiezii*, which occur only on calcareous rocks, are remarkable in that they have very similar morphology and very disjunct and partly strongly limited distribution. Whereas *A. bourgaei* occurs in the Near East and locally on Aegean islands (Karpathos, Rhodes, Chalki and Kastellorizon), *A. jahandiezii* has only been found on rock faces of the 25-km long gorges of the Grand Canyon du Verdon (Haute Provence). No populations of either *A. bourgaei* or *A. jahandiezii* have been found between these distribution areas. Thus, both taxa are completely allopatric and species ranges are separated by more than 2000 km.

Both ferns might constitute glacial relics or palaeoendemics, a view already mentioned by Greuter et al. (1983[6]) and Kramer and Viane (1990[7]). The conspicuous morphological similarities induced Meyer (1962[9]) to consider both taxa to be closely related or even identical. This argument was exclusively based on macromorphological features.

In this communication we have analysed some relevant micromorphological characters (which are useful in fern taxonomy; van Cotthem, 1970[2], 1973[3]; Rasbach et al., 1994[10]) of both taxa, especially morphology of sporangia (indurated annulus cells, dehiscence) and spores (length of exospore, structure of perispore), as well as the stomatal type. In addition, we have amplified and sequenced 1060 nucleotides of the *rbc*L gene of both taxa and those of other members of the genus *Asplenium* and other genera in order to analyse the genetic relatedness of these taxa and how they are positioned in a broader phylogenetic framework.

Materials and Methods

Plant material

The origins of the analysed fern taxa are listed in Table **1**. Voucher specimens are deposited in the herbarium of G. Schulze and that of the Institute of Systematic Botany and Plant Geography (Heidelberg University); DNA is deposited in the collection of the Institute for Pharmaceutical Biology, Heidelberg University. The identity of fern taxa collected in the wild or kept in the Botanical Garden of Heidelberg was thoroughly examined (by G. S.) and it was verified that the identifications were correct.

Micromorphology

Three to four individuals of *A. bourgaei* and *A. jahandiezii* were analysed to determine intraspecific variation. Sporangia: The number of the indurated annulus cells was determined from 50 fully mature sporangia. Spores: The length of exospore was measured from 150 untreated spores in chloral hydrate–glycerol. Light microscopy was carried out with SM-LUX (Leitz); SEM analysis: spores were coated with gold and analysed with the SEM Leitz 1200 B.

Plant biol. 3 (2001) 364–371

ISSN 1435-8603

Table 1 Origin of samples

Taxon	Date of collection	Origin of plant material
Asplenium adiantum-nigrum L.	17.4.1994	Germany: Gimmeldingen (Rheinland-Pfalz), SE slope of the Platten-Berg, 230 m above sea level (a.s.l.), sandstone.
Asplenium bourgaei (Boiss.) Milde	May 1998	S Turkey: Sample 1: Sillyon (*ca.* 25 km NW Serik), calcareous rocks at the summit of ruin hill, 150 m a.s.l., crevice exposed to S. Sample 2 has been found in Çirali ruin walls near Chimaera (about 60 km SW of Antalya). Sample 3: Çirali, walls on the way to Olympos, 10 m a.s.l.; AF318591. Sample 4: Perge (18 km NE Antalya), walls on the E side of the arena, 50 m a.s.l.
Asplenium fontanum (L.) Bernh.	18.7.1988	Switzerland: St. Maurice (Canton Wallis), calcareous rock on the way to the chapel "Notre Dame du Scex", 500 m a.s.l., E exposed.
Asplenium jahandiezii (Litard.) Rouy	4.7.1998	Samples 1 and 2: S France: Grand Canyon du Verdon, calcareous rocks near Clue de Carejuan, 700 m a.s.l., SW exposed (14 km SW of Castellane).
Asplenium majoricum Litard.	22.3.1964	Spain: Mallorca, Soller, W exposed terrace wall on an olive tree culture above the river Barranc, 200 m a.s.l.
Asplenium ruta-muraria L.	6.6.1996	Germany: Rheinland-Pfalz, Bann (5 km SE of Landstuhl) sandstone wall at the church, 380 m a.s.l., NW exposed.
Asplenium septentrionale (L.) Hoffm.	18.5.1981	Germany: Rheinland-Pfalz, Dannenfels on the Donnersberg, SE side of the "Hirten-Fels", Rhyolith, 600 m a.s.l.
Asplenium trichomanes ssp. *trichomanes* L.	16.7.1975	Germany: Rheinland-Pfalz, Eisenschmelz (2 km N of Winnweiler), Porphyrite rock in the valley of the Bichelbach, 280 m a.s.l., S exposed.
Asplenium trichomanes ssp. *quadrivalens* D. E. Meyer	15.9.1981	Germany: Ludwigswinkel (S-Pfalz), sandstone rock on the Lindels-Kopf, 340 m a.s.l., NW exposed.
Asplenium trichomanes ssp. *coriaceifolium* Rasbach, Reichstein and Bennert	16.10.1986	Spain: Mallorca, Soller, N exposed wall in and olive tree culture on the road to Ses Tres Creus near the sanctuary, 170 m a.s.l., Collectors: H. and K. Rasbach.
Asplenium trichomanes ssp. *hastatum* S. Jenssen	6.11.1984	Germany: Schwäbische Alb (Baden-Württemberg), Bära valley (N Fridingen), calcareous rock on the road between Bärenthal and Gnadenweiler, 800 m a.s.l., SE exposed.
Asplenium viride Hudson	7.9.1979	Switzerland: Schwyz (30 km E of Luzern), calcareous rock in the forest on the SE end of the Lauerzer See, 500 m a.s.l., N exposed.
Asplenium hispanicum (Cosson) Greuter and Burdet (*Pleurosorus hispanicus* [Cosson] Morton)	16.5.1999	Spain: Grazalema (Prov. Cadiz), calcareous rock near the road to Ronda, 900 m a.s.l., NE exposed.
Asplenium bulbiferum G. Forst.	25.1.1999	Heidelberg Botanical Garden
Asplenium serratum L.	3.1.1999	Heidelberg Botanical Garden

* if not otherwise mentioned, ferns were collected by G. Schulze.

DNA sequence analysis

DNA was isolated from dried leaves using the CTAB method (Doyle and Doyle, 1990[5]). The marker genes were amplified by polymerase chain reaction (PCR) using the following primers: *rbc*L-N (forward) 5′-ATG TCA CCA CAA ACA GAR ACK AAA GC-3′; *rbcL*-1R (reverse) 5′- GGG TGC CCT AAA GTT CCT CC-3′.

PCR conditions: A final volume of 50 µl contained 0.5 – 1 µg DNA, 5 µl 10 × Taq buffer (500 mM KCl, 100 mM Tris-HCl, 1 % Triton × 100, pH 9.0), 3 µl 25 mM $MgCl_2$, 12.5 pmol primer, 1.5 µl dNTPs (10 mM), 0.75 U Taq-Polymerase (Amersham-Pharmacia Biotech) and 1 µl 20 mg/ml BSA. PCR cycle for *rbcL:* 2 min at 94 °C, then 30 cycles with 45 sec at 94 °C, 90 sec at 45 °C and 90 sec at 72 °C, and finally 5 min at 72 °C.

Most of the sequences were obtained by cloning the PCR products into plasmid vector (Promega p-GEM T – Vector system I) and sequenced using fluorescence labelled M13 primers flanking the polycloning site. In a few taxa the PCR products were sequenced directly.

Cloned *rbc*L fragments or PCR products were further amplified by cycle-sequencing using the "ThermoSequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Life Science), according to the protocol of the manufacturer. Products were sequenced with an automatic sequencer ALFexpress (Amersham-Pharmacia Biotech).

Sequences are deposited with GenBank (Table **2**). The *rbc*L sequences of *Blechnum occidentale* (U05909), *Polypodium australe* (U21140), *Pteris fauriei* (U05647), *Angiopteris evecta*

Table 2 GenBank accession numbers of the taxa sequenced

Taxon	GenBank Accession Number
Asplenium septentrionale	AF318586
Asplenium majoricum	AF318587
Asplenium fontanum	AF318588
Asplenium jahandiezii 1	AF318589
Asplenium jahandiezii 2	AF318590
Asplenium bourgaei 1	AF318591
Asplenium bourgaei 2	AF318592
Asplenium viride	AF318593
Asplenium trichomanes. trichomanes	AF318594
Asplenium trichomanes quadrivalens	AF318595
Asplenium trichomanes hastatum	AF318596
Asplenium trichomanes coriaceifolium	AF318597
Asplenium ruta-muraria	AF318598
Pleurosorus hispanicus	AF318599
Asplenium adiantum-nigrum	AF318600
Asplenium bulbiferum	AF318601
Asplenium serratum	AF318602

(L11052), *Marchantia polymorpha* (U87079) were taken from the EMBL nucleotide sequence database. Sequences were aligned manually or with use of CLUSTAL V (1.6) (gap-penalty 10).

Aligned sequences were analysed using the phylogeny program PAUP* 4.0b4a (Swofford, 1998[11]). Methods employed were unweighted Maximum Parsimony (MP), Neighbour-Joining (NJ) with Tamura-Nei distance algorithm, and Maximum Likelihood (MLE). For Maximum Parsimony a heuristic search with the "tree-bisection-and-reconnection" swapping algorithm and the stepwise addition option "closest" was applied. For Maximum Likelihood the following parameters were specified: number of substitution types = 6, estimated transition/transversion ratio, estimated base frequencies, substitution rates = gamma, estimated shape parameter. *Marchantia* and *Angiopteris* were employed as outgroup. A bootstrap analysis (1000 replicates) was performed to estimate how good the bifurcations were supported by the data. The program package MEGA (Kumar et al., 1993[8]) was used to calculate sequence statistics.

Results and Discussion

Morphology

At the morphological level we have only compared *A. bourgaei* and *A. jahandiezii*. Variation within each taxon was very small but substantial differences could be discovered between *A. bourgaei* and *A. jahandiezii*. Besides an overall morphological similarity (Fig. **1**), both taxa have special leaf characters in common. Both show more or less toothed leaflets which are broadly adnate to the rachis and more or less distinctly auriculate at the upper margin. The lower margins of the leaflets run down narrowly along the rachis as small wings. This morphological character cannot be found in any other European *Asplenium* species. Smaller differences can be seen in the colouration of the rachis and the petiole; in *A. bourgaei* the petiole and the lower half of the rachis are dark brown, whereas *A. jahandiezii* has a green rachis and petiole (which is brown only at its base).

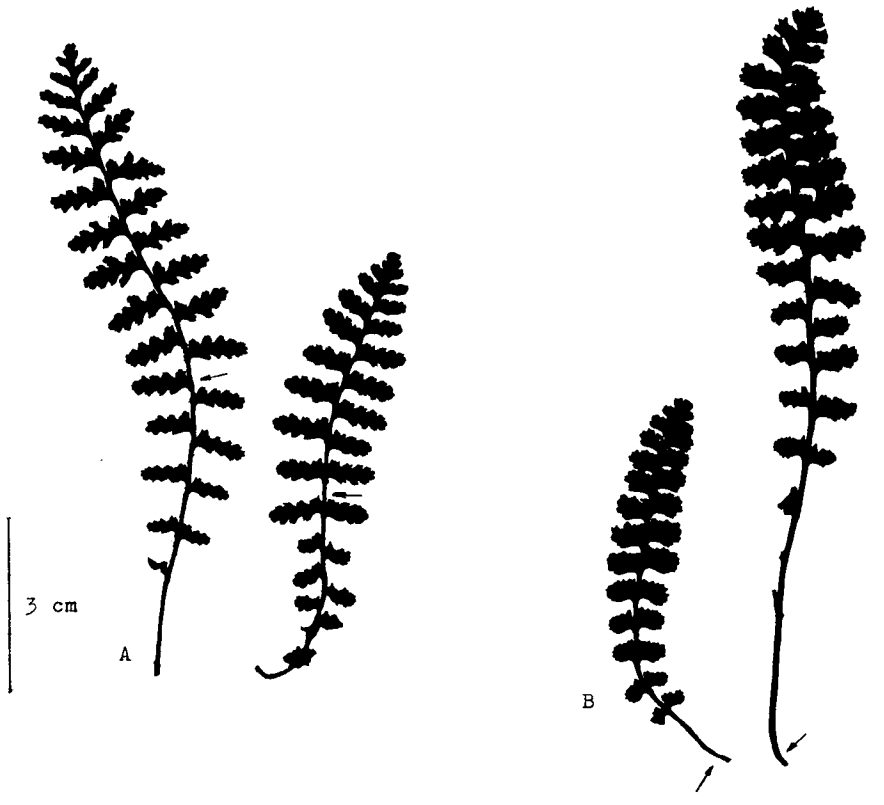

Fig. 1 Leaves of *Asplenium bourgaei* (**A**) and *A. jahandiezii* (**B**). Arrows indicate the upper limit of brown colouration of the petiole and rachis.

The number of annulus cells in sporangia was slightly higher in *A. bourgaei* than in *A. jahandiezii* (Table **3**). Another important character concerns the indehiscence of mature sporangia. A detailed analysis of our material revealed that even mature sporangia of *A. jahandiezii* are completely indehiscent, whereas *A. bourgaei*, collected at 4 different localities in Turkey, all had either partly or completely opened sporangia (Fig. **2**). The phenomenon of sporangial indehiscence in ferns was first mentioned by Brownsey (1977[1]). He had observed that sporangia of *Asplenium lepidum* do not open to release their spores, but dropped as indehiscent sporangia. Sporangia of a related species, *A. haussknechtii* (both taxa are tetraploid, occurring only on calcareous rocks), open normally to release their spores.

The length of exospores is almost identical in both species (Table **3**), but the structure of the perispores differed markedly (Fig. **3**). SEM analysis has shown that the spores of *A. bourgaei* possess an echinate surface with spines of different size and length, whereas the spores of *A. jahandiezii* are covered with an irregular network of small ledges or ridges. As the perispore sculpture has a considerable taxonomic value in ferns, this difference may also indicate that these fern taxa represent distinct species.

Table 3 Comparison of micromorphological characters

Character	*A. bourgaei*	*A. jahandiezii*
Sporangia (n = 50)*		
Number of indurated annulus cells	23 ± 5	18 ± 4
Spores (n = 150)*		
Length of exospore (µm ± s.d.)	29.3 ± 2.6	27.4 ± 2.9
Size of subsidiary cells (n = 50)*		
Length (µm)	59 ± 4	73 ± 5
Width (µm)	70 ± 5	110 ± 3
Length × Width	4130	8030

* Characters were evaluated for 3 different individuals from different locations

Stomatal type and size of the subsidiary cells can also be a valuable tool for fern taxonomy (van Cotthem, 1970[2], 1973[3]). The dorsal epidermis of both *Asplenium* species shows polycytic stomata, which are typical for the genus *Asplenium* (Fig. **4**). A strong difference can be seen in the size of the subsidiary cells, which are significantly smaller in *A. bourgaei* than in *A. jahandiezii*. Rasbach et al. (1994[10]) have used the same characters to differentiate *Asplenium cuneifolium* and *A. onopteris* as distinct species. The reported size difference (the subsidiary

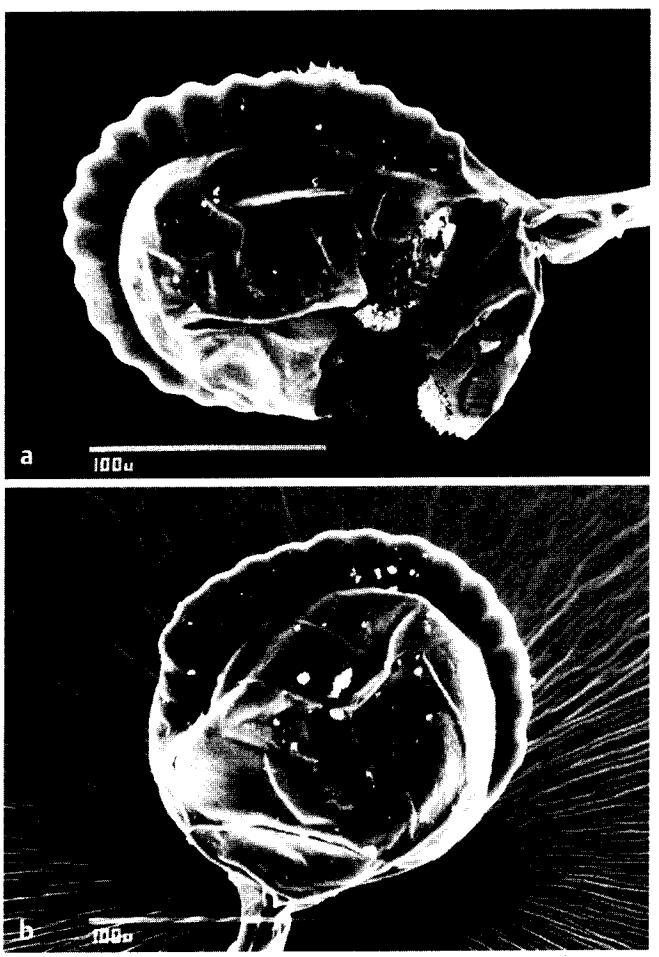

Fig. 2 Mature sporangia of *Asplenium bourgaei* (**a**) and *A. jahandiezii* (**b**).

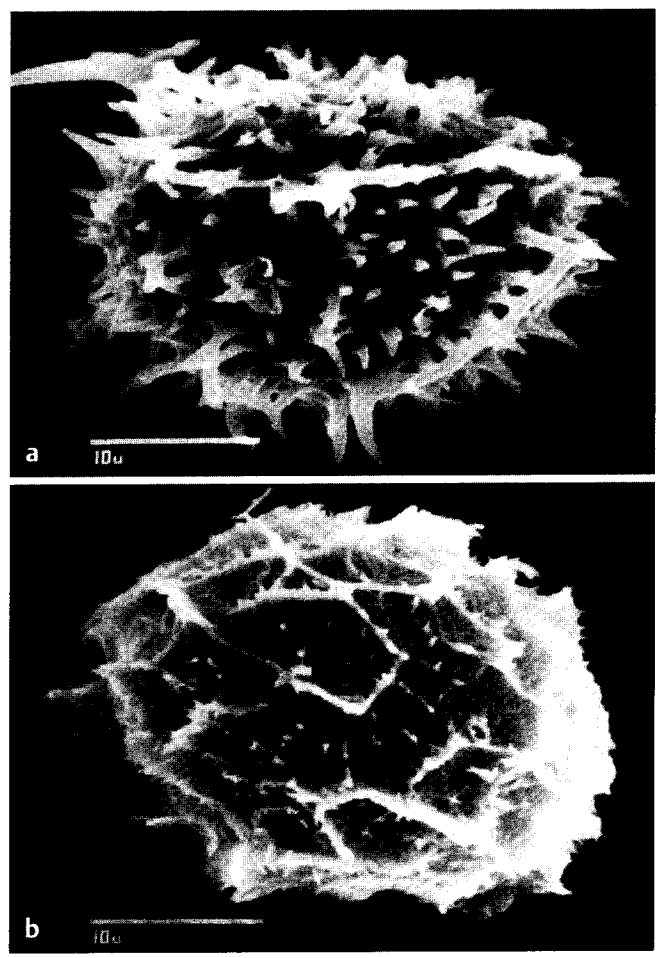

Fig. 3 SEM photographs of spores from *A. bourgaei* (**a**) and *A. jahandiezii* (**b**); bar = 10 µm (preparation and photos C. Erbar).

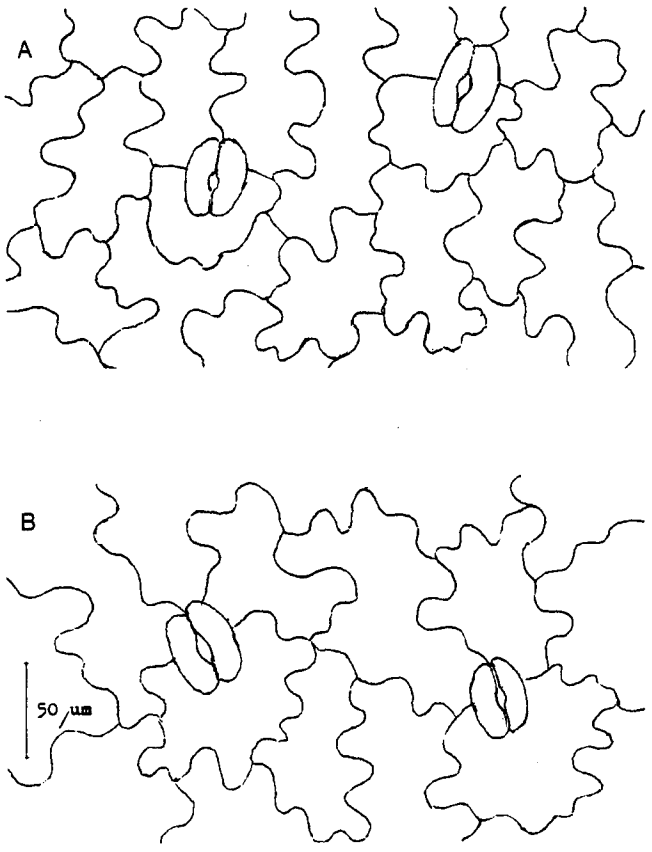

Fig. 4 Cell pattern of the dorsal leaf epidermis of *Asplenium bourgaei* (**A**) and *A. jahandiezii* (**B**).

cells of *A. onopteris* are 39% smaller than those of *A. cuneifolium*) is distinctly smaller than that found for the species pair *A. bourgaei/A. jahandiezii.* The subsidiary cells of *A. bourgaei* are 49% smaller than those of *A. jahandiezii* (Table **3**). This would be further evidence that *A. bourgaei/A. jahandiezii* are distinct species.

RbcL *Sequences*

*Rbc*L sequences were generated from *A. bourgaei, A. jahandiezii* and 13 other (mainly European) *Asplenium* species. In the dataset of 1060 characters (position 101 to 1160 of the *rbc*L gene), 145 sites were variable and 71 sites parsimony informative. The abundance of each nucleotide (adenine: 26.47%; cytosine: 21.17%; guanine: 24.77%; thymine: 27.57%) was in agreement with other published studies, indicating that the nucleotides adenine and cytosine were more and guanine less abundant than the theoretically expected frequency of 25% (if all 4 bases were equally abundant).

Molecular phylogenies were reconstructed using both character state and distance methods. MP, MLE and NJ trees are almost congruent in topology, indicating that the phylogenies are well supported by the sequence data (Fig. **5**). Minor differences concern the relative positions of *Blechnum, Polypodium* and *A. bulbiferum* and *A. serratum* which are not relevant for our specific question of differentiation of the *A. bourgaei* and *A. jahandiezii* pair.

Two individuals each were analysed for both *A. bourgaei* and *A. jahandiezii.* Sequences were identical within each taxon indicating no intraspecific variation. Both taxa form a pair of sibling species in all reconstructions with bootstrap support of 100%. Both taxa differ by 9 nucleotide substitutions (including 2 transversions) within position 101 to 1160 of the *rbc*L gene (genetic distance = 0.9%) (Table **4**). The genetic distance between *A. bourgaei/jahandiezii* is much higher than between the 4 subspecies of *A. trichomanes* which were included in the study, indicating that the genetic distances found for *A. bourgaei/jahandiezii* clearly exceed those of intraspecific diversity.

A. bourgaei/jahandiezii form a monophyletic clade with *A. fontanum* and *A. majoricum.* Among the taxa examined, three further monophyletic groups are resolved, independent from the method of phylogeny reconstruction: *A. viride* clusters together with *A. trichomanes, Pleurosorus hispanicus* is found to be connected to *A. ruta-muraria* and *A. adiantum-nigrum,* and *A. bulbiferum* shares a common ancestor with *A. serratum.* As *Pleurosorus* would create a paraphyletic group, its taxonomic classification apparently needs re-evaluation; our data implicate a merger of *Pleurosorus* with *Asplenium.*

Conclusions

Whereas morphology of leaves, number of indurated annulus cells and spore size are similar in *A. bourgaei* and *A. jahandiezii,* other decisive characters, such as the surface structure of perispores, sporangial indehiscence and the size of stomatal subsidiary cells, clearly show that these taxa differ substantially. As these characters separate species in other taxon pairs (van Cotthem, 1970[2], 1973[3]; Rasbach et al., 1994[10]), we assume that *A. bourgaei* and *A. jahandiezii* represent distinct species and are not identical as suggested by Meyer (1962[9]). Furthermore, genetic differences between *A. bourgaei* and *A. jahandiezii* (genetic distance 0.9%) clearly exceed those usually found within species (distances between subspecies are below 0.2%), as indicated from the differences found for the 4 subspecies of *A. trichomanes* (Fig. **5**). The genetic differences indicate that both taxa must have become separated a considerable time ago because *rbcL* represents a rather slowly evolving gene.

A. bourgaei shows its main distribution at present in the northeastern Mediterranean region, but might have occurred further west during glacial periods. It is likely that its spores had drifted into the steep Grand Canyon du Verdon by long-distance dispersal (recall that sporangia in *A. bourgaei* open normally and release their spores) and since no gene flow connected these plants with the original populations they could develop distinct genetic lineages in an allopatric speciation process. As the range of both species is separated by more than 2000 km, they are reproductively isolated, and to our knowledge, natural hybrids and hybrid zones do not exist. It was outside the scope of our study to try artificial crosses between these two taxa in order to see whether fertile or sterile hybrids would result.

In conclusion, both morphological and genetic characters indicate that *A. jahandiezii* is a distinct species which appears to have been derived from *A. bourgaei* or from an ancestor common to both taxa.

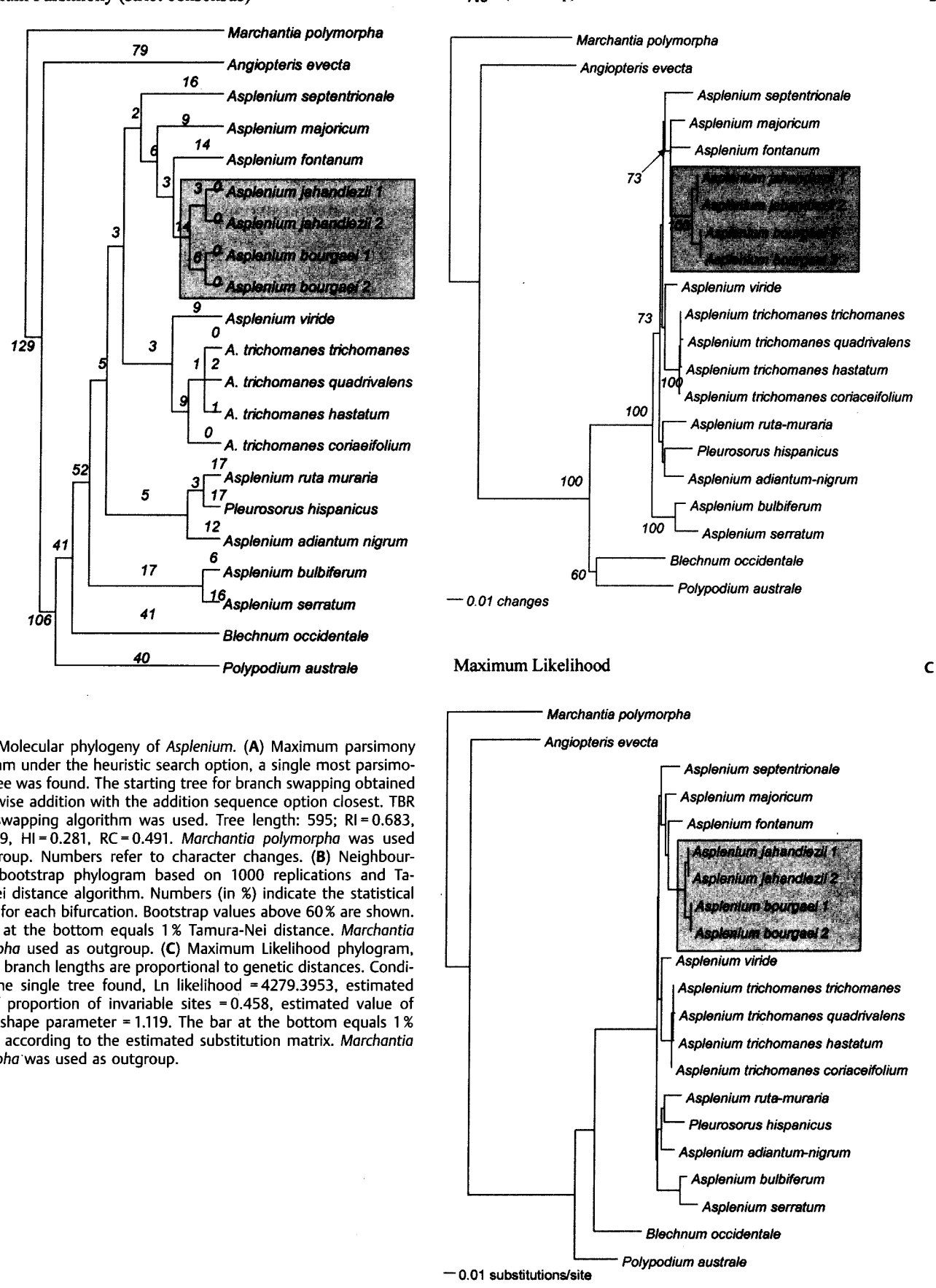

Fig. 5 Molecular phylogeny of *Asplenium*. (**A**) Maximum parsimony cladogram under the heuristic search option, a single most parsimonious tree was found. The starting tree for branch swapping obtained by stepwise addition with the addition sequence option closest. TBR search swapping algorithm was used. Tree length: 595; RI = 0.683, CI = 0.719, HI = 0.281, RC = 0.491. *Marchantia polymorpha* was used as outgroup. Numbers refer to character changes. (**B**) Neighbour-joining bootstrap phylogram based on 1000 replications and Tamura-Nei distance algorithm. Numbers (in %) indicate the statistical support for each bifurcation. Bootstrap values above 60% are shown. The bar at the bottom equals 1% Tamura-Nei distance. *Marchantia polymorpha* used as outgroup. (**C**) Maximum Likelihood phylogram, in which branch lengths are proportional to genetic distances. Conditions: one single tree found, Ln likelihood = 4279.3953, estimated value of proportion of invariable sites = 0.458, estimated value of gamma shape parameter = 1.119. The bar at the bottom equals 1% distance according to the estimated substitution matrix. *Marchantia polymorpha* was used as outgroup.

Table 4 Distances between *Asplenium* taxa studied. Above diagonal: % of pairwise nucleotide substitutions (1.0 = 100 %); below diagonal: number of pairwise nucleotide substitutions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *Asplenium septentrionale*	–	0.030	0.033	0.038	0.038	0.041	0.041	0.029	0.028	0.030	0.029	0.029	0.037	0.041	0.036	0.044	0.053
2 *Asplenium majoricum*	31	–	0.022	0.027	0.028	0.028	0.028	0.022	0.027	0.029	0.028	0.026	0.034	0.038	0.030	0.039	0.048
3 *Asplenium fontantanum*	34	23	–	0.030	0.030	0.032	0.033	0.028	0.029	0.031	0.030	0.029	0.034	0.041	0.033	0.043	0.050
4 *Asplenium jahandiezii 1*	39	29	31	–	0.000	0.009	0.009	0.029	0.037	0.039	0.038	0.036	0.042	0.042	0.037	0.051	0.060
5 *Asplenium jahandiezii 2*	39	29	31	0	–	0.009	0.009	0.029	0.037	0.039	0.038	0.036	0.042	0.043	0.037	0.052	0.060
6 *Asplenium bourgaei 1*	42	30	34	9	9	–	0.000	0.032	0.040	0.042	0.041	0.039	0.044	0.043	0.040	0.052	0.063
7 *Asplenium bourgaei 2*	42	29	34	9	9	0	–	0.032	0.040	0.042	0.041	0.039	0.044	0.044	0.040	0.052	0.063
8 *Asplenium viride*	30	23	29	31	31	34	34	–	0.018	0.020	0.019	0.017	0.028	0.034	0.026	0.037	0.047
9 *Asplenium trichomanes* ssp. *trichomanes*	29	29	30	39	39	42	42	19	–	0.002	0.001	0.001	0.026	0.037	0.030	0.037	0.044
10 *Asplenium trichomanes* ssp. *quadrivalens*	31	31	32	41	41	44	44	21	2	–	0.003	0.003	0.027	0.038	0.031	0.039	0.046
11 *Asplenium trichomanes* ssp. *hastatum*	30	30	31	40	40	43	43	20	1	3	–	0.002	0.027	0.036	0.031	0.038	0.044
12 *Asplenium trichomanes* ssp. *coriaceifolium*	30	28	30	38	38	41	41	18	1	3	2	–	0.027	0.036	0.029	0.036	0.043
13 *Asplenium ruta-muraria*	38	36	36	44	44	47	46	30	28	29	29	29	–	0.032	0.029	0.045	0.050
14 *Pleurosorus hispanicus*	43	40	43	45	45	46	46	36	39	40	38	38	34	–	0.030	0.045	0.049
15 *Asplenium adiantum-nigrum*	37	32	35	39	39	42	42	28	32	33	33	31	31	32	–	0.039	0.048
16 *Asplenium bulbiferum*	45	41	45	54	54	55	54	39	39	41	40	38	47	47	41	–	0.021
17 *Asplenium serratum*	55	51	52	64	63	67	66	50	47	49	47	46	53	52	51	22	–

Acknowledgements

We thank Prof. Dr. C. Erbar (Institut f. Systematische Botanik u. Pflanzengeographie, Universität Heidelberg) for recording the SEM photographs.

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