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ORIGINAL ARTICLE

Character expression, reproductive barriers, and origin of the rare fern hybrid *Asplenium* ×*aran-tohanum* (Aspleniaceae)

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Abstract

Hybridization is a ubiquitous force in plant evolution. In ferns, hybrids are often easily recognized by their intermediate morphology and abortive spores and thus provide a good model for studying reproductive isolating barriers between species. *Asplenium* ×*aran-tohanum* is a rare fern hybrid, despite wide coexistence of its parental species (*A. billotii* and *A. trichomanes* subsp. *quadrivalens*) in Western Europe. We made a complete characterization of its three known individuals, including macro- and micromorphology, sporogenesis, gametophyte reproduction, and chloroplast DNA inheritance and evolution. The hybrid expressed morphological characters that were mostly intermediate between those of the parents, but some characters were more similar to one parent or the other. Sizes of both guard cells and spores indicate that the hybrid is tetraploid, as are both parents, and one parent (*A. billotii*) consistently acted as the female. A very small fraction of spores (~ 7%) were viable and the resulting gametophytes could not form sporophytes, either sexually or apogamously, suggesting that effective postzygotic barriers exist between the parents. The lineages of these taxa diverged about 35 million years ago, which may explain the strong reproductive isolation and rarity of the hybrid. *Asplenium* ×*aran-tohanum* appears to be an evolutionary dead end, probably formed recurrently at the places where it grows, but incapable of completing its life cycle or producing viable offspring.

Keywords *Asplenium* · Ferns · Hybrid · Iberian Peninsula · Phylogenetic analyses · Reproductive isolation · Speciation

Introduction

Hybridization is a prominent mechanism of evolution in plants, which gives these organisms huge evolutionary potential (Wissemann 2007; Hegarty and Hiscock 2008; Soltis and Soltis 2009). For example, interspecific hybridization in concert with whole genome duplication can give rise to new allopolyploid species that are reproductively viable and isolated from their parent taxa. Both hybridization and polyploidization are very common in ferns (Wood et al. 2009), probably due to the absence, or weakness, of prezygotic isolating barriers (Haufler 2008). Most non-polyploid fern hybrids are thought to be sterile, as a consequence of uneven chromosome segregation during sporogenesis (e.g., Stergianou and Fowler 1990) and are thus considered evolutionary dead ends. However, some hybrids produce minor proportions of apparently normal spores (Hornych and Ekrt 2017 and references therein). These spores have been shown to form F2 hybrids both in laboratory cultures and in nature, which suggests the existence of recombinant homoploid hybrid speciation in ferns (reviewed by Sigel 2016).

Fern hybrids are often easy to recognize because of their abortive spores and morphology that is intermediate between the parental species (Reichstein 1981). However, many F1 hybrids express some characters similar to one or the other of their parental species or even beyond the range

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of the parents (transgressive characters) (Rieseberg and Ellstrand 1993). The development and extension of molecular techniques in recent decades allow us to elucidate the ancestry of hybrids and also to explore new explanations about the evolutionary processes and dynamics involved in the formation of hybrid lineages (Hegarty and Hiscock 2005; Sigel 2016). It is now not complicated to assess whether hybridization is recurrent, i.e., whether the hybrid has arisen multiple times from different independent lineages of the parental taxa or, instead, has formed just once (Soltis et al. 2014). It is also possible to detect the directionality of the hybridization (which of the parents has acted as female in a given hybrid cross) and, in cases of recurrent hybridization, whether all hybrid contacts have had the same direction of parentage (non-reciprocal hybridization) or if both parents have acted as females (reciprocal hybridization). In the second case, the proportion of crossings in which the parent species have acted as females may be similar (symmetrical hybridization) or significantly different (asymmetrical hybridization) (e.g., Sigel et al. 2014; Testo et al. 2015; Yahaya et al. 2016). Asymmetry in reproductive isolation is common both in ferns and in other plant groups (e.g., van der Velde and Bijlsma 2004; Tiffin et al. 2001).

By applying molecular dating techniques, it is also possible to estimate the approximate date at which the parental lineages diverged and therefore the maximum age at which the hybridization could have first occurred (Sessa et al. 2012, 2018). Moreover, the probability of hybridization (Mallet 2005) and the fertility of the resulting hybrid (Sigel 2016) are inversely related to the genetic divergence between parent species. Understanding the biological processes that underlie the hybridization phenomenon itself remains a challenge in most cases, because successful cross-fertilization depends on many additional factors, including timing of gametangia formation, gamete viability and compatibility, and in ferns, whether male-inducing pheromones (i.e., antheridiogens) or apogamy are present (Vogel et al. 1998a; Regalado et al. 2010; Testo et al. 2015).

Asplenium L., the largest fern genus in terms of species-level diversity, with around 700 species (PPG1 2016), is prone to hybridization and polyploidization, and its species sometimes also adopt an apogamous life cycle. All together, these factors have led to the emergence of numerous convoluted mixed-ploidy lineages or complexes in *Asplenium* (Sleep 1983; Werth et al. 1985; Perrie et al. 2010; Dyer et al. 2012; Chang et al. 2013; Sessa et al. 2018). Based on the results of several studies that have investigated evolutionary dynamics in some of these groups, it seems that recurrent, non-reciprocal origins are the rule in the evolutionary formation of *Asplenium* hybrids. Hybridization in *Asplenium* has also been documented to occur between phylogenetically quite distant lineages within the genus (Sessa et al. 2018).

Asplenium ×*aran-tohanum* C.Alejandre & M.J.Escal is a rare hybrid, putatively derived from *A. trichomanes* subsp. *quadrivalens* D.E.Mey. and *A. billotii* F.Schultz. The involvement of these two species was suggested in the original description of the hybrid (Alejandre et al. 2005), based on the general morphology of the fronds and pinnae (Fig. 1). Despite the fact that both parents are very common in Western Europe (especially *A. trichomanes* subsp. *quadrivalens*) (Nogueira and Ormonde 1986; Moreno et al. 2015) and often grow in close proximity to one another in their rocky habitat, this hybrid has been detected in only three localities to date, all in the Iberian Peninsula (Fig. 2). Given that both parents are allotetraploids and that they do not share recent diploid ancestors (Liu et al. 2018; Sessa et al. 2018), the hybrid is likely a tetraploid formed by four different genome sets. This lack of homology between chromosomes likely causes irregular pairing behavior and uneven distribution to the forming spores. However, Alejandre et al. (2005) noted that some spores are apparently well-formed, which would suggest the potential for spore dispersal and subsequent gametophyte growth and reproduction. Conversely, the extreme rarity of *A.* ×*aran-tohanum* indicates the existence of effective reproductive isolation barriers. This hybrid therefore provides a suitable model for studying plant hybridization and interspecific isolating barriers. Here we report the results of a complete characterization of the hybrid's three known individuals, including macro- and micromorphology, gametophyte reproduction, and chloroplast DNA inheritance and evolution. Our specific objectives were

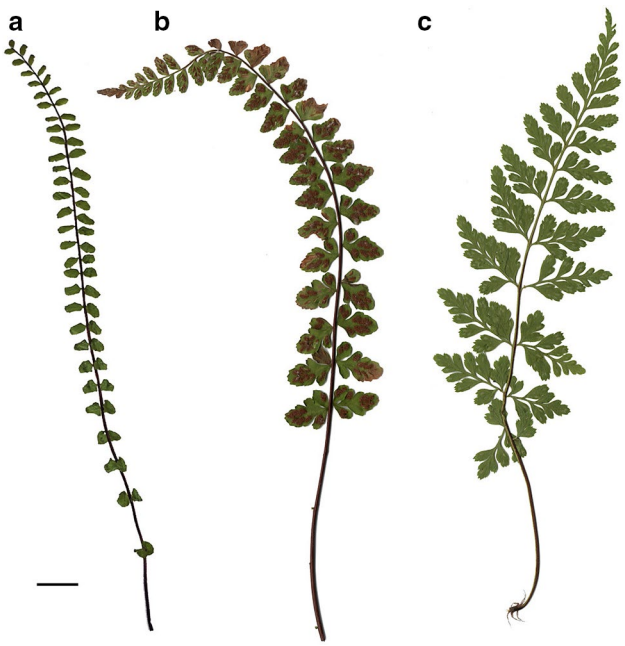

Fig. 1 Fronds of *Asplenium trichomanes* subsp. *quadrivalens* (**a**), *A.* ×*aran-tohanum* (**b**) and *A. billotii* (**c**). Bar = 1.3 cm in **a** and **c**; 1 cm in **b**

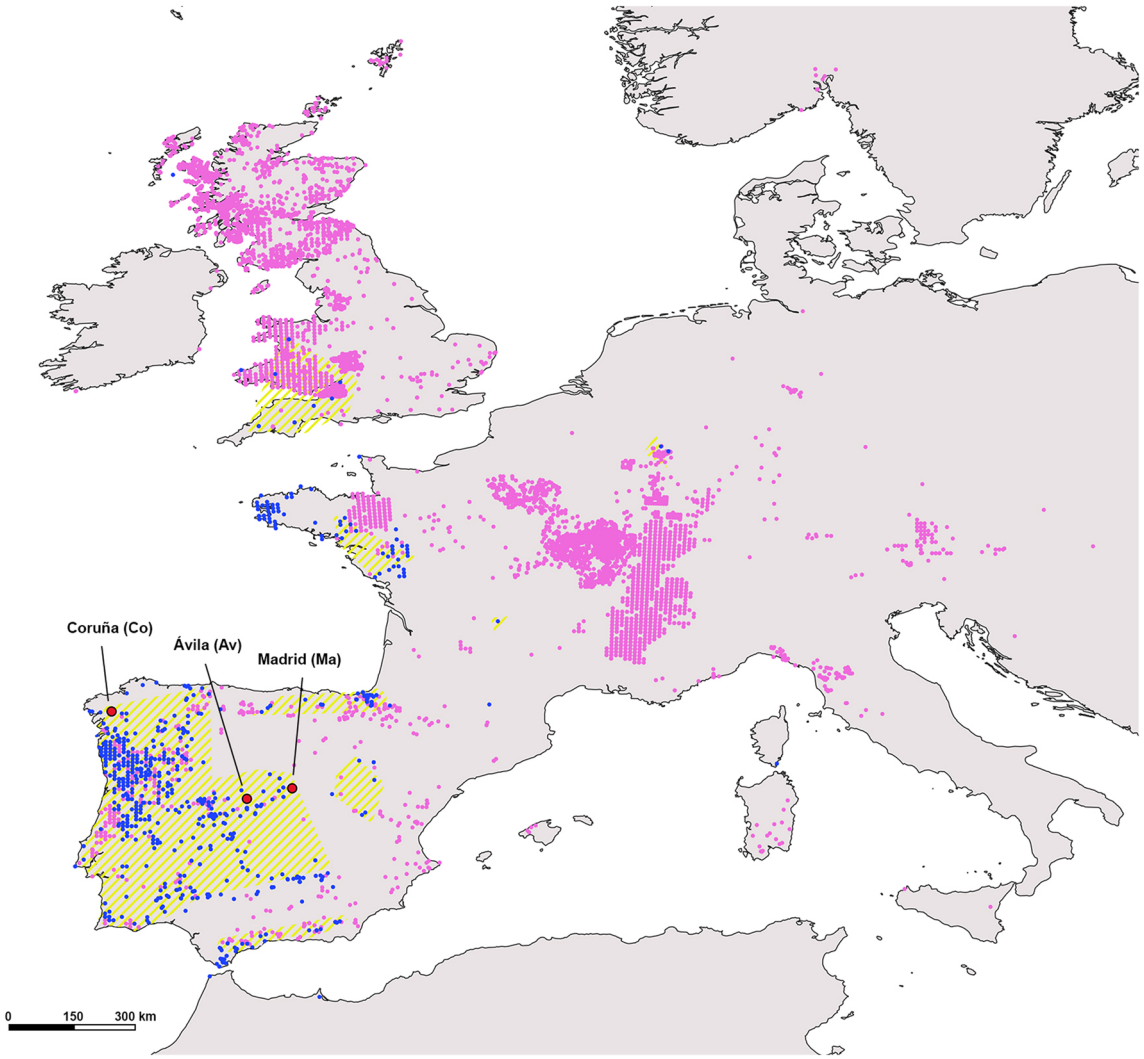

Fig. 2 Distribution of the three *Asplenium* taxa in Western Europe. Small purple dots: *Asplenium trichomanes* subsp. *quadrivalens*. Small blue dots: *A. billotii*. Yellow shaded area: potential area of hybridization, with coexisting populations of both taxa. The red circles represent the three localities in which the hybrid *A.* ×*aran-tohanum* has been located (the labels are the names of the Spanish provinces). Data about the parental species comes from GBIF (2019a, b)

to: (1) determine the expression of morphological characters by the hybrid; (2) quantify its success at sporogenesis and test whether any of its "normal" spores could give rise to F2 hybrids; and (3) infer whether the hybrid has originated recurrently and reciprocally and the earliest possible date of occurrence of hybridization. More generally, we investigate whether genetic divergence between parent species or reproductive barriers explain the extreme rarity of this hybrid.

Materials and methods

Plant material

We were able to study the three known individuals of *Asplenium* ×*aran-tohanum*, along with parents collected from the close vicinity of each ("Appendix 1"). Some

Spanish herbaria (MA, MACB, MAF) were searched in order to locate other hybrid specimens, but without success. The holotype of the hybrid is located in a private collection (Herbarium Alejandre), and we had available some extracted rhizomatic scales and pinnae with sporangia. The other hybrid samples (the two individuals from Madrid and A Coruña, and all the collections of *A. billotii* and *A. trichomanes* subsp. *quadrivalens*) have been deposited in the public herbarium of the Faculty of Biology, Universidad Complutense de Madrid (MACB).

Morphological data and analysis

For each of the available individuals, we measured the following characters with the aid of dissecting and light microscopes: frond (length, shape, division of the lamina, petiole length, number of pinna pairs), pinnae (length, outline, lobules, indument, guard cell length), rhizome scales (length, sclerosed rhizome scale, cell traits), sporangia (number of annulus cells, form of basal cells), and spores (abortion percentage, exospore length, form, macro-ornamentation, micro-ornamentation). "Sclerosed rhizome scale" was defined as the portion (%) of the length of rhizome scales with sclerosed middle cells (Fig. 3). Spores were considered aborted when they lacked a protoplast or were collapsed. Differences among taxa and individuals were tested with a hierarchical ANOVA for the following microcharacters: guard cell length, rhizome scale length, sclerosed rhizome scale, no. of annulus cells, exospore length and spore abortion percentage. For each taxon, microcharacters were determined in 2–3 individuals, on each of which 30 measurements were made when possible. The only exception was spore abortion percentage, which was counted in four random samples of 100 spores per individual. Taxon was treated as a fixed factor and individual as a fixed factor nested within taxon. Subsequent pairwise comparisons were made using Tukey tests ($P < 0.05$). For improving normality, sclerosed rhizome scale and spore abortion percentage were arcsine-transformed, while number of annulus cells was log-transformed. These statistical analyses were done with STATISTICA version 7.0 (Stat Soft, Tulsa, OK, USA).

Gametophyte development

Spores were extracted by hand from the three hybrid individuals. All abortive and well-formed spores of each individual were sown in a Petri dish 6 cm ∅ with mineral agar medium (Dyer 1979). Dishes were sealed with Parafilm, to ensure a constant environment of saturated humidity, and incubated in a growth chamber at 22 ± 2 °C, 12-h light photoperiod and constant light regimen from daylight fluorescent tubes at a photon irradiance of 30–45 µmol m^{-2} s^{-1}. Every 3 days, dishes were inspected under a compound microscope to detect spore germination and subsequent events of gametophyte development, including transition from filamentous to bidimensional growth, notch meristem initiation, and gametangia formation.

Molecular procedures

Total DNA was extracted from ~ 20 mg of dried leaf material, following the manufacturer's protocols of the NZY Plant/Fungus gDNA isolation kit (NZYTech, Lisboa, Portugal). We used polymerase chain reaction (PCR) to amplify three plastid regions: *trn*G–*trn*R, *rbc*–L, and *rps*4–*trn*S. These molecular markers have been widely applied in phylogenetic and evolutionary studies in ferns. We used the polymerase kit NZYTaq II 2x Green Master Mix (NZYTech,

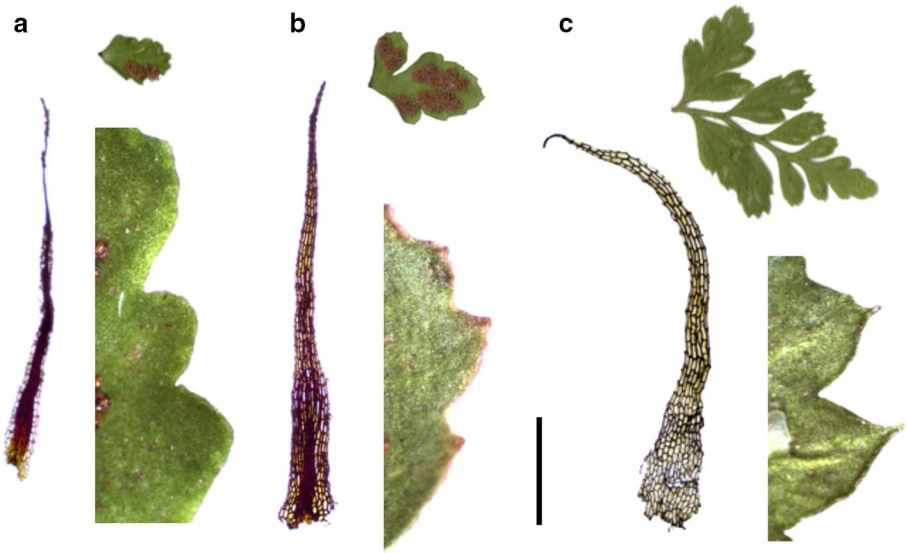

Fig. 3 Comparison of some interesting morphological traits between the taxa studied. For each taxon, the figure shows a rhizome scale (left), one medium pinna (right above) and detail of pinna margin (right below). **a** *Asplenium trichomanes* subsp. *quadrivalens,* **b** *A.* ×*aran-tohanum,* **c** *A. billotii*. Bar = 1.5 mm for all the rhizome scales and for the margin in **c**; 15 mm for all the pinnae; 0.7 mm for margins in **a**, **b**

Lisboa, Portugal), following the manufacturer's protocol: 25 µL Master Mix (0.2 U polymerase/µL), 3 µL of each primer (5 µM), 3 µL of DNA template, and dH_2O to a final volume of 50 µL. Primers and reaction conditions followed those of previous studies: for *trn*G–*trn*R: 1F/22R, 35 x [1 m 94 °C, 1 m 56 °C, 2 m 30 s 72 °C] + 10 m 72 °C (Gabriel y Galán et al. 2013); for *rbc*–L: 1F/1361R, 35 x [45 s. 94 °C, 1 min. 55 °C, 2 min. 72 °C] + 10 min. 72 °C (Schuettpelz and Pryer 2007); for *rps*4–*trn*S: F/R, 38 × [30 s 94 °C, 40 s 42 °C, 1 m 30 s 72 °C] + 7 m 72 °C (Li and Lu 2006). Agarose 1% gel electrophoresis was used to check PCR products. After purification (NZYGelPure, NZYTech, Lisboa, Portugal), samples were sequenced on an ABI3730XL sequencer (Eurofins Genomic, Ebersberg, Germany). We generated 27 new sequences that have been published in the European Nucleotide Archive (ENA; accession numbers are given in "Appendix 1").

Phylogenetic analyses

In order to place the sequences of our taxa of interest in a general context within the genus, we used accessions of 15 species of *Asplenium*, previously published in GenBank ("Appendix 2"). Included among these were sequences from other samples of *A. trichomanes* subsp. *quadrivalens* and *A. billotii* from other geographical locations. We used the species *Hymenasplenium unilaterale* (Lam.) Hayata as the outgroup, due to its known phylogenetic position as sister to the genus *Asplenium* (Schneider et al. 2004; Xu et al. 2019).

We assembled contigs using the software Geneious R9 (Kearse et al. 2012) and used MAFFT 1.3.5 (Katoh and Standley 2013) to build alignments, which were then checked manually. Because the chloroplast is considered a single non-recombining molecule (Wolf et al. 2010, 2011), we concatenated all three plastid loci into a single matrix (Online Resource 1).

We performed maximum likelihood (ML) and Bayesian inference (BI) analyses, applying models of nucleotide evolution previously identified using jModelTest v.2 (Darriba et al. 2012) and the Bayesian information criterion (BIC). Separate analyses were performed for the ML and BI analyses, since different sets of models are available in the software for each of these. ML analyses were conducted in RAxML 8.2.8 (Stamatakis 2015) using multiple cores and SSE3 vector instructions. We used the option to complete 1000 bootstrap replicates and a search for the ML tree in a single run. The BI analysis was performed using MrBayes 3.2.6 (Ronquist et al. 2012). We ran this analysis for 20,000,000 generations with four chains and assessed completion using Tracer 1.6 (Rambaut et al. 2014). We discarded the first 25% of trees as burn-in and used TreeAnnotator 2.4.2 (Bouckaert et al. 2014) to combine and summarize the post burn-in trees.

Results

Morphological characterization and spore abortion

Degree of lamina division is the best macrocharacter to distinguish the three taxa (Table 1), with *Asplenium* ×*aran-tohanum* showing an intermediate lamina dissection (1-pinnate-pinnatifid to 2-pinnate) between those of its parent species (Fig. 3). The shape of pinnae lobules was also intermediate in the hybrid and has diagnostic value (Table 1, Fig. 3). Although the hybrid tended to have intermediate traits for the other macrocharacters, there was some overlap between taxa (Table 1). All studied microcharacters differed significantly among taxa, and among individuals within each taxon (Table 2), the only exceptions being sclerosed rhizome scale and spore abortion, which showed no significant individual variation. Rhizome scale length of the hybrid was intermediate relative to the parents (Table 1). In the hybrid, only a short portion of the scale length was sclerosed (7 ± 2%), whereas *A. trichomanes* subsp. *quadrivalens* had almost all length sclerosed (97 ± 1%) and *A. billotii* lacked sclerosed cells (Fig. 3). At the apex of the scales, cells of *A.* ×*aran-tohanum* were similar (rectangular) to those of *A. trichomanes* subsp. *quadrivalens*, whereas at the scale base, cells in the hybrid were similar (polygonal) to those of *A. billotii* (Table 1). Guard cell length and number of annulus cells were both similar between *A.* ×*aran-tohanum* and *A. trichomanes* subsp. *quadrivalens* ($P > 0.05$, Tukey test), and larger in *A. billotii* ($P < 0.05$, Tukey test). Exospore length showed low among-taxa variation, which was not significant according to Tukey tests (Table 1). Spore abortion percentage was much higher in the hybrid (92.8 ± 0.9%, Table 1) than in the parents (both ~ 2%).

Gametophyte development and reproduction

Of the three hybrid individuals, only the most recently sampled (Madrid) showed some spore germination. Spores took about 15 days to germinate after sowing. The spore wall broke up and a first rhizoid emerged, followed by a green, prothallial cell. After a filamentous phase, longitudinal cell divisions originated planar, bidimensional prothalli. Finally, around 50 days after sowing, by means of meristematic activity at the apical pole of the prothalli, adult, winged gametophytes developed, without hairs, reaching about 2–3 mm in length. On the abaxial surface of these adult gametophytes, archegonia were continuously formed throughout the observational period (6 months), but we never observed antheridia nor vegetative proliferations. In consequence, no new sporophytes were detected (Online Resource 2).

Table 1 Distinctive characters of *Asplenium*×*aran-tohanum* and its parents

Character	*A. trichomanes*	*A.*×*aran-tohanum*	*A. billotii*
Frond			
Length (cm)	10–25	11–21	10–30
Outline	Linear-lanceolate	Linear-lanceolate	Ovate-lanceolate
Division of the lamina	1-pinnate	1-pinnate-pinnatifid to 2-pinnate	2-pinnate-pinnatifid to 3-pinnate
Petiole length	Shorter than the lamina	Shorter than the lamina	Similar to the lamina
Pinna pairs (no.)	10–30	12–27	12–20
Pinnae			
Length (cm)	0.4–1.2	0.8–1.3	2.0–6.0
Outline	Ovate-rectangular	Lanceolate	Lanceolate
Lobules	Shallow, rounded lobules	Acute, without shallow teeth	Acute, somewhat acuminate, with deep teeth
Indument	Short glandular hairs	Large glandular hairs	Large glandular hairs
Guard cell length (µm)	51.6 ± 0.9 a	53.1 ± 0.8 a	57.1 ± 0.9 b
Rhizome scales			
Length (mm)	5.7 ± 0.2 a	8.6 ± 0.2 b	9.7 ± 0.3 c
Sclerosed rhizome scale (%)	97 ± 1 a	7 ± 2 b	0 ± 0 c
Cell traits	Lightly clathrate, rectangular cells	Strongly clathrate, rectangular cells on the apex, polygonal at the base	Strongly clathrate, polygonal cells
Sporangia			
Annulus cells (no.)	19.2 ± 0.2 a	19.3 ± 0.2 a	20.3 ± 0.3 b
Basal cells form	Long, one strongly shorter	Medium sized, one shorter	Short, both of similar size
Spores			
Exospore length (µm)	33.6 ± 0.4 a	31.4 ± 0.7 a	32.7 ± 0.7 a
Form	Elipsoidal	Most of them abortive, otherwise variable between parents	Reniform
Macro-ornamentation	Reticulate	Most of them aborted, otherwise variable between parents	Long ridges
Micro-ornamentation	Verrucate	Most of them aborted, otherwise variable between parents	None
Abortion (%)	2.0 ± 0.5 a	92.8 ± 0.9 b	2.4 ± 0.5 a

Macro- and microcharacters are given as minimum–maximum and mean ± standard error, respectively. Different letters indicate taxa with significantly different means ($P < 0.05$, Tukey tests). See Table 2 for ANOVA results

Table 2 Hierarchical ANOVA for testing the differences in microcharacters among the three *Asplenium* taxa

Character	Source of variation					
	Taxon			Individual (Taxon)		
	df	*F*	*P*	*df*	*F*	*P*
Guard cell length	2	18.43	**< 0.0001**	5	35.45	**< 0.0001**
Rhizome scale length	2	68.10	**< 0.0001**	3	3.74	**0.013**
Sclerosed rhizome scale	2	1081.00	**< 0.0001**	3	0.53	0.66
No. of annulus cells	2	5.67	**0.0040**	4	10.09	**< 0.0001**
Exospore length	2	3.23	**0.041**	4	2.88	**0.024**
Spore abortion	2	1041.16	**< 0.0001**	6	1.15	0.36

Significant differences ($P < 0.05$) are indicated in bold. *df* degrees of freedom

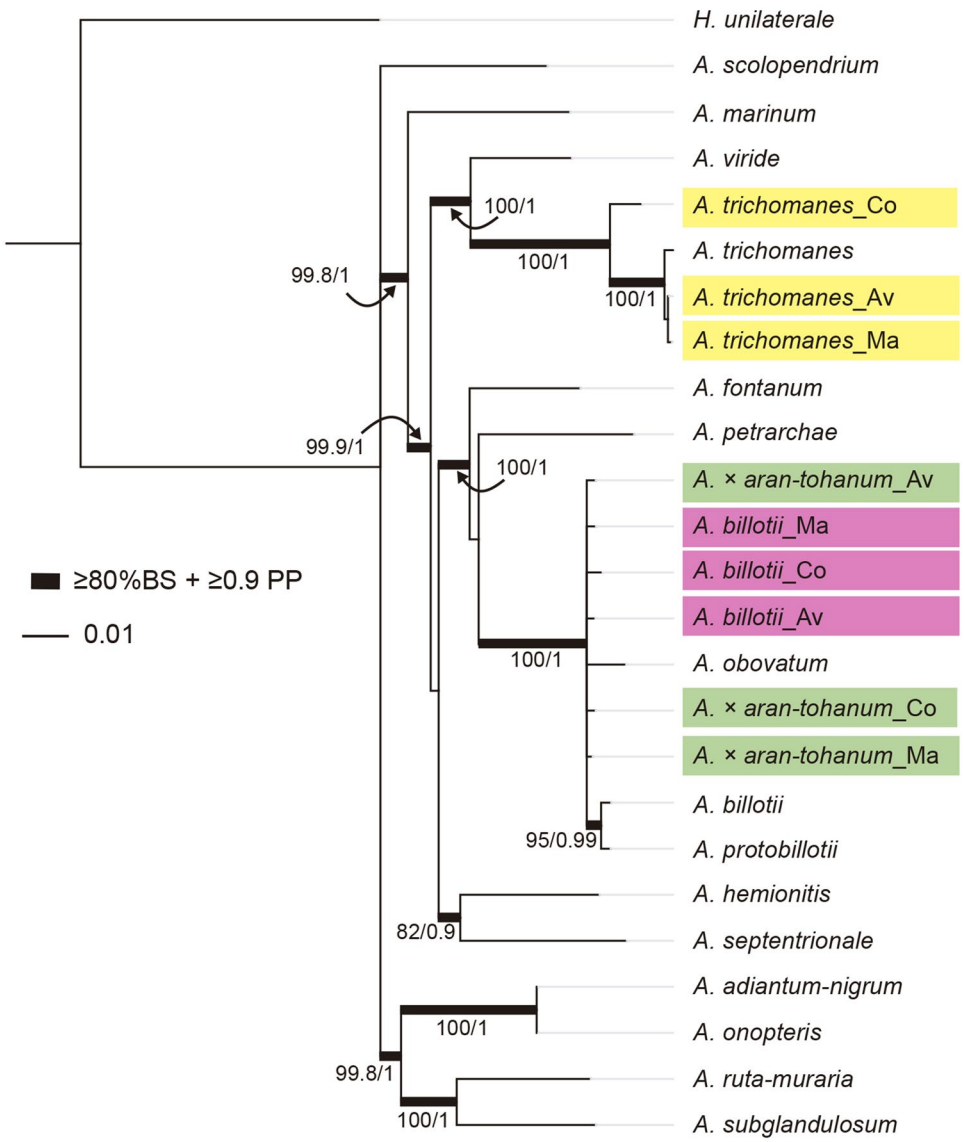

Fig. 4 Phylogeny of *Asplenium* based on cpDNA sequences, showing the highly supported position of *Asplenium* ×*aran-tohanum* within the *A. billotii* group (*A. billotii*, *A. obovatum* subsp. *obovatum* and subsp. *protobillotii*). Values below branches indicate the statistical support including, in this order, BS (bootstrap percentage) and PP (posterior probability). Branches with high support have been highlighted. After the names of the study taxa, the acronym of the collection locality is included: Av, Ávila; Co, A Coruña; Ma, Madrid (see Fig. 2)

Phylogenetic analyses

Both ML and BY analyses produced well-resolved trees, with the same basic topology, in which the majority of the clades were supported by high statistical values (Fig. 4). The only unresolved groups were those comprising the different individuals of the taxa of interest (*A. trichomanes* subsp. *quadrivalens*, *A. billotii*, *A.* ×*aran-tohanum*). The phylogenetic position of *A. scolopendrium* L. was also unclear. The sequences of the hybrid are closely related to those of *A. billotii*, intermixed with sequences of *A. obovatum* Viv. subsp. *obovatum* and *A. obovatum* subsp. *protobillotii* (Demiriz, Viane & Reichst.) Herrero, Pajarón & Prada, all of them forming a maximally supported clade (100 BS/1.00 PP). The closest relatives to these taxa are *A. petrarchae* DC. and *A. fontanum* (L.) Bernh. All the sequences of *A. trichomanes* subsp. *quadrivalens* are grouped in a maximally supported lineage sister to *A. viride* Huds. There is no evidence of geographical signal in the tree: for each taxon, accessions from the three different geographical locations were not resolved as sister to one other. Therefore, from our data, we have no significant evidence that the hybrid had multiple origins. The lack of resolution perhaps suggests very recent formation of each of the three individuals, and they cannot be assigned to any one parental population.

Given that plastid genomes in *Asplenium* are inherited via the female gamete (Vogel et al. 1998b), and that all the accessions of *A.* ×*aran-tohanum* were strongly supported as forming a clade with *A. billotii*, it is highly probable that *A. billotii* has been the only maternal parent of the hybrid in the three known instances of its occurrence.

Discussion

Character expression in the hybrid

Asplenium ×*aran-tohanum* showed many intermediate characters between those of *A. billotii* and *A. trichomanes* subsp. *quadrivalens* (Table 1). Our results thus support that these two taxa are the parents of the hybrid, as suggested by Alejandre et al. (2005). Some characters of *A.* ×*aran-tohanum* (e.g., number of annulus cells) had parental values rather than intermediate ones. Parental values may be the result of loci having a dominant (rather than codominant) effect (López-Caamal and Tovar-Sánchez 2014). The progenitors have marked morphological differences (Table 1), as a consequence of high genetic divergence (see below). Although some macrocharacters of the hybrid overlap those of the parents, the degree of lamina division and shape of pinnae lobules distinguish the three taxa. Two microcharacters of rhizome scales, length and portion with sclerosed cells, have also diagnostic value. The hybrid showed an exospore length similar to those of both parents and a guard cell length closer to that *A. trichomanes* subsp. *quadrivalens*. Given that sizes of spores and guard cells are proxies of ploidy level (Barrington et al. 1986; Gabriel y Galán et al. 2011), our data indicate that *A.* ×*aran-tohanum* is tetraploid like both of its parents, as expected.

We also considered whether other taxa than the presumed parents examined here could have been involved in the formation of *A.* ×*aran-tohanum*. Regarding the male lineage, some other *A. trichomanes* subspecies could have participated, such as subsp. *inexpectans* Lovis or subsp. *pachyrachis* (Christ) Lovis & Reichst. However, the former is a highly localized endemic from the Balearic Islands that occurs only in limestone substrates, while the latter, although more widespread in the Iberian Peninsula, is quite different in morphology and none of its primary traits are even partially exhibited by the hybrid, e.g., the hybrid's fronds are not decumbent, its pinnae are not coriaceous, spore size is not in the correct range, etc. (Nogueira and Ormonde 1986). Regarding the female lineage, some other possible candidates are *A. obovatum* subsp. *obovatum* or subsp. *protobillotii*, evolutionary related to *A. billotii* (Sessa et al. 2018), which could potentially transmit a somewhat "*billotii*-like" morphology to the hybrid. However, these plants have very limited populations in the northeast and southwest of the Iberian Peninsula, respectively, and are thus very far from the locations of the hybrid (Nogueira and Ormonde 1986). Only *A. billotii* and *A. trichomanes* subsp. *quadrivalens* coexist in the three locations in which the hybrid has been detected.

Hybrid sporogenesis and gametophyte reproduction

The hybrid produced a low proportion of "normal" spores (~7%) compared to the parents (~98% in both taxa). It is not unusual for fern hybrids to produce a number of non-abortive spores nor is it unusual for normal sexual species to produce abortive ones (Wagner et al. 1986; Hornych and Ekrt 2017). The proportion of "normal" spores we found in *A.* ×*aran-tohanum* is slightly higher than previous data from other *Asplenium* hybrids. For example, the triploid *A. trichomanes* nothosubsp. *lusaticum* (D. E. Meyer) Lawalrée (*A. trichomanes* L. subsp. *trichomanes* × subsp. *quadrivalens*) was found to produce 0.2% "normal" spores on average (Hornych and Ekrt 2017). Other hybrids within the genus are also known to form some small proportion of "normal" spores (Wagner et al. 1986), although often no precise quantification has been carried out. The mean spore abortion we found in *A. trichomanes* subsp. *quadrivalens* (2.0%) is almost identical to a previous report in the same taxon (1.5%, Hornych and Ekrt 2017).

We found that "normal" spores of *A.* ×*aran-tohanum* can germinate and the resulting gametophytes change from one-dimensional to two-dimensional growth and eventually develop an apical notch meristem. This vegetative growth is similar to those of the parents (Pangua et al. 1994; Herrero et al. 2002). Likewise, Wagner et al. (1986) found unexpectedly high spore germination in several American *Asplenium* hybrids and stated that "in spite of most abortive spores, many 'sterile' hybrids are capable of forming at least limited populations." However, although *A.* ×*aran-tohanum* gametophytes formed archegonia, we did not observe antheridia or sporophytes. This presumed inability to form male gametes could indicate that this hybrid is sterile, in agreement with the fact that only one hybrid individual was found at each of the three known localities (Alejandre et al. 2005 and our own observations). F2 hybrids seem to be exceptional in *Asplenium*. One F2 sporophyte has been produced artificially from the spores of *A.* ×*alternifolium* nothosubsp. *heufleri* Aizpuru, Catalán & Salvo (Reichstein 1981), which also has *A. trichomanes* subsp. *quadrivalens* as one of the parent species. In addition, *Asplenium murbeckii* Dörfl has been observed to form F2 hybrids in a few locations (Reichstein 1981). However, unlike *A.* ×*aran-tohanum*, this hybrid is formed from two autotetraploid parents, which allows occasional full chromosome pairing at sporogenesis (Reichstein 1981).

We do not know whether the archegonia we observed in *A.* ×*aran-tohanum* contain functional eggs that could fuse with sperm from progenitor species. These hypothetical backcrosses would allow exchange of genetic material between the parents (i.e., introgression). However, the result of introgression would be populations of hybrids

characterized by a continuum of morphological forms between those of the parent species (Sigel 2016), and we did not find these "hybrid swarms" in any of the three hybrid sites. Apogamy also allows some fern hybrids to form populations (DeBenedictus 1969), but this apparently is not the case for *A.* ×*aran-tohanum*, as we did not find apogamous sporophytes in the spore culture. It must be noted that spores of two of the three hybrid individuals did not germinate at all. This result was expected because these spores were obtained from very old (14 and 20 years) herbarium specimens and fern spores rapidly die at room-temperature dry conditions (Ballesteros et al. 2019). For example, other *Asplenium* species that frequently share habitat with our study taxa show germination decline after a few months at these conditions (Aragón and Pangua 2004).

Recurrence, reciprocity, and dating of hybridization

Although we could not satisfactorily resolve the phylogeny of the hybrid accessions from the three geographical locations, both the long distance between them (Fig. 2), which makes spore dispersal unlikely, and likely hybrid sterility (see above) support the hypothesis that hybrid formation was recurrent. Independent multiple origins of hybrids and derivative polyploids are considered to be common for interspecific crossings (Soltis et al. 2014), as is also known to occur in *Asplenium* (e.g., Werth et al. 1985; Chang et al. 2013). For example, a somewhat similar scenario to ours was found in a New Zealand *Asplenium* complex, with two tetraploid species crossing multiple times (Shepherd et al. 2008; Perrie et al. 2010). The resulting hybrids gave rise to several allooctoploid species via additional chromosome doubling, which restores homologue pairing and thus normal sporogenesis. By contrast, our studied hybrid is not known to form a fertile allopolyploid taxon.

Our data also support that *A. billotii* is one of the parents of *A.* ×*aran-tohanum* (Fig. 4), in agreement with our morphology data. Chloroplast DNA sequences of the three hybrid individuals are very close to those of the allotetraploid *A. billotii* and its diploid progenitors (*Asplenium obovatum* subsp. *obovatum* and subsp. *protobillotii*) (Sessa et al. 2018), whereas *A. trichomanes* subsp. *quadrivalens* is grouped in a different lineage within the genus *Asplenium*. Assuming chloroplast maternal inheritance (Vogel et al. 1998b), we could state that the origin of *A.* ×*aran-tohanum* is non-reciprocal, with one of the parents, in this case *A. billotii*, acting always as female. This conclusion is limited by the small number of hybrid individuals available. In any case, non-reciprocity in hybridization seems to be common in *Asplenium*, as for example the constant female role of *A. septentrionale* (L.) Hoffm. in the formation of *A.* ×*alternifolium* Wulfen with *A. trichomanes* s.l. as the second, always paternal parent (Vogel et al. 1998a). The causes of asymmetrical hybridization in ferns are poorly understood. Testo et al. (2015) suggested a combination of reproductive differences between hybridizing species, including selfing potential, antheridiogen responsiveness, sperm mobility, and gamete size. Although antheridiogen systems have been reported in *Asplenium* (Schneller and Hess 1995), as far as we know they are not known to occur in *A. billotii* or *A. trichomanes*. Our culture experiment also does not support antheridiogen effects in the hybrid, as we did not find male gametophytes despite the existence of females in close proximity. It must also be noted, however, that some examples are known of reciprocal origins for hybrids and polyploid derivatives in *Asplenium*: for example, *A. billotii* itself was formed multiple times by reciprocal crossings between its diploid ancestors (Sessa et al. 2018).

It is also interesting to note that both *A. billotii* and *A. trichomanes* s.l. are very prone to hybridization, and many hybrids and derivative polyploids have been described (Sleep 1983; Vogel et al. 1998a) apart from *A.* ×*aran-tohanum*. The existence of all these hybrids shows that *Asplenium* taxa have at least partially ineffective prezygotic reproductive barriers against congeneric species. However, in some cases, intriguing gametic barriers have been developed: *A. monodon* Liebm. is able to accept sperm from other taxa (*A. auritum* Sw., forming *A.* ×*lellingerianum* C. Sánchez & L. Regalado) but apparently is unable to accept its own sperm, surviving by means of an apogamous life cycle (Regalado et al. 2010).

Based on a moderately well-sampled, time-calibrated phylogenetic tree of the Aspleniaceae (Sessa et al. 2018), the two parent species *A. trichomanes* and *A. billotii* clearly belong to different lineages within the genus *Asplenium*. These two lineages diverged early in the evolutionary history of *Asplenium*, with a divergence point conservatively estimated at around 35 Ma (Paleogene), which is the maximum age at which the hybrid *Asplenium* ×*aran-tohanum* could have been formed. Thus, *A.* ×*aran-tohanum* represents the cross between two very anciently diverged clades. The scarce previous information on dates of hybrid formation in the genus *Asplenium* shows hybridization between lineages that separated even longer ago: *A. obovatum* subsp. *obovatum* and *A. onopteris* L., belonging to clades separated by 45 Ma (Sessa et al. 2018), form the hybrid *A.* ×*bouharmontii* Badré & R. Prelli, which in turn gave rise to the allotetraploid *A. balearicum* Shivas. Like *A.* ×*aran-tohanum*, that hybrid occurs infrequently, with only two known individuals (Badré et al. 1981; Nardi 1983), despite sympatry of the parent species. The rarity of both hybrids indicates that reproductive barriers are in place and is consistent with the positive correlation between degree of isolation and genetic divergence that has been found in both ferns (Sigel 2016) and flowering plants (Levin 1978). In addition, microhabitat differentiation between parent species may cause spatial segregation of their gametophytes and thus prevent hybridization, as has

been proposed for other *Asplenium* species of rocky habitats (Vogel et al. 1998a).

Conclusions

Asplenium ×*aran-tohanum* exhibits morphological characters that are generally intermediate between those of its parent species, with no evidence of transgressive expression. Prezygotic isolation (asynchrony in gametangia formation, gamete incompatibility, etc.) seems highly efficient, since hybrid sporophytes are infrequent despite wide sympatry between the parents. There are also strong postzygotic barriers, as the spores produced by the F1 hybrid are mostly inviable and the few resulting gametophytes cannot form F2 hybrid sporophytes, either sexually or apogamously. This level of reproductive isolation was expected, given the ancient divergence between the lineages of the parent species and the uncommonness of the hybrid. *Asplenium* ×*aran-tohanum* thus represents an evolutionary dead, which is probably formed de novo where it occurs, with one of the parent taxa predominantly acting as female. Future studies should further explore the role of reproductive isolating barriers in fern evolution and the causes of asymmetrical hybridization between species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This research did not involve any testing on humans or animals.

Information on Electronic Supplementary Material

Online Resource 1. Alignment used to produce the phylogeny.
Online Resource 2. Critical gametophytic events and structures of *Asplenium* ×*aran-tohanum*.

Appendix 1. List of material

Due to the existence of just three sampling spots, the material is ordered by location at the first level (alphabetical order of Spanish Province), with indication of geographical coordinates and altitude over the sea level. For each sample, basic collection data (collector, date and herbarium voucher) is given. ENA (European Nucleotide Archive) accesions are included for the markers *trn*G–*trn*R, *rbc*–L, and *rps*4–*trn*S, in this order.

ÁVILA: Solosancho, 40.52 -4.90, 1380 m a. s. l. ***Asplenium* ×*aran-tohanum*** Alejandre & M.J.Escal.: *Alejandre* and *Escalante*, 18 Dec 2004, Herbarium Alejandre 1407/04; LR588501, LR588504, LR588496. ***Asplenium billotii*** F.Schultz: *Gabriel y Galán*, 26 Feb 2018, MACB 112993; LR585233, LR585211, LR586053. ***Asplenium trichomanes* subsp. *quadrivalens*** D.E.Mey: *Gabriel y Galán*, 26 Feb 2018, MACB 112994; LR585230, LR585209, LR586055.

A CORUÑA: Santiago, 42.88 8.55, 230 m a. s. l. ***Asplenium* ×*aran-tohanum*** Alejandre & M.J.Escal.: *L.G. Quintanilla* 641 and *B. Pías*, 5 Nov 1998, MACB 112961; LR588499, LR588502, LR588498. ***Asplenium billotii*** F.Schultz: *L.G. Quintanilla* 643 and *B. Pías*, 5 Nov 1998, MACB 112959; LR585232, LR585212, LR586054. ***Asplenium trichomanes* subsp. *quadrivalens*** D.E.Mey: *L.G. Quintanilla* 642 and *B. Pías*, 5 Nov 1998, MACB 112960; LR585228, LR585210, LR586056.

MADRID: La Cabrera, 40.84 -3.67, 1080 m a. s. l. ***Asplenium* ×*aran-tohanum*** Alejandre & M.J.Escal.: *Molino* et al., 19 May 2016, MACB 112692; LR588500, LR588503, LR588497. ***Asplenium billotii*** F.Schultz: *Molino* et al., 19 May 2016, MACB 112958; LR585231, LR585213, LR586052. ***Asplenium trichomanes* subsp. *quadrivalens*** D.E.Mey: *Molino* et al., 19 May 2016, MACB 112957; LR585229, LR585208, LR586057.

Appendix 2. Additional sequences

GenBank accessions, ordered by taxa, for the markers *trn*G–*trn*R, *rbc*–L, and *rps*4–*trn*S, in this order, corresponding to the taxa used for constructing the phylogenetic tree.

Asplenium adiantum-nigrum L.: KP861371, KP899628, KR233955. ***Asplenium billotii*** F.Schultz: KC792645, KC792629, KF923967. ***Asplenium fontanum*** (L.) Bernh.: KC792642, KC792625, KC792642. ***Asplenium hemionitis*** L.: KU753794, KP899633, JQ364916. ***Asplenium marinum*** L.: KP861374, KU753804, AY549773. ***Asplenium obovatum*** Viv. **subsp. *obovatum***: KC792639, KC792628, KF923970. ***Asplenium obovatum* subsp. *protobillotii*** Demiriz, Viane & Reichst.: KC792638, KC792635, KF923973. ***Asplenium onopteris*** L.: KC792636, KC792632, KF923965. ***Asplenium petrarchae*** (Guérin) DC.: KP861366, KP899640, AY549804. ***Asplenium ruta-muraria*** L.: KP861361, KP899645, AY549763. ***Asplenium scolopendrium*** L.: KP861370, KP899647, AY459169. ***Asplenium septentrionale*** (L.) Hoffm.: KP861377, KP899649, AY549777. ***Asplenium subglandulosum* subsp. *hispanicum*** (Coss.) Salvo, Prada & T.E.Díaz: KU753793,

KP899651, JX068786. ***Asplenium trichomanes* subsp. *quadrivalens*** D.E.Mey.: KP861388, KP899654, AY549794. ***Asplenium viride*** Huds.: KP861387, KP899656, EF645627. ***Hymenasplenium unilaterale*** (Lam.) Hayata: JF832224, EF452140, AY459170.

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