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ORIGINAL ARTICLE

Inferring the potential of plastid DNA-based identification of derived ferns: a case study on the *Asplenium trichomanes* aggregate in Europe

Hong-Mei Liu[1] · Stephen R. Russell[2] · Johannes Vogel[3] · Harald Schneider[2,4]

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Abstract

The utility of three plastid DNA regions to identify fern species was explored with focus on the European representatives of the *Asplenium trichomanes* aggregate. The sampling included representatives of the three diploid and the four tetraploid taxa recognized in the European flora plus Macaronesia. Besides European samples, the compiled data set comprised specimens of a putative Hawaiian endemic and one species occurring in Southeast Asia. By combining the sequences of three non-coding plastid regions, 13 haplotypes were recovered of which four were found in more than one taxon. Evidences for four distinct diploid lineages were found that correspond to *Asplenium anceps*, *A. inexpectans*, *A. trichomanes s.s.*, and *A. tripteropus*. The four tetraploids occurring in Europe shared haplotypes with *A. inexpectans*. Thus, DNA barcoding can successfully identify the diploids, but fail to separate the tetraploids from their diploid ancestors. As a consequence, barcoding analyses of ferns need to take into account the differences of ploidy level measured by evidence independent from the DNA barcode. Evidence for uneven accumulation of intra-species DNA variation was recovered by comparing all species. Furthermore, the study provided evidence that the current taxonomy of these ferns requires to be revised. The two European diploids form well-separated clades and need to be recognized as *A. inexpectans* and *A. trichomanes* s.s. To keep name consistency for all European tetraploids, a new name *Asplenium jessenii* is introduced to replace *A. trichomanes* subsp. *hastatum*.

Keywords Barcoding gap · DNA barcode · Molecular species identification · Polyploidy · Species concepts · Species differentiation

Introduction

DNA barcoding is now widely used to identify plants using either DNA barcodes or related approaches (e.g. Valentini et al. 2009; Kress et al. 2010; Garcia-Robledo et al. 2013; Zhang et al. 2013; Braukmann et al. 2017; Ghorbani et al. 2017). The currently preferred DNA barcode of land plants is based on two regions of the usually uniparental inherited plastid genome (CBoL Plant Working Group 2009; Fazekas et al. 2012), but discrimination of species at the lower level has been found to be a major challenge utilizing these regions (Fazekas et al. 2009; Clement and Donoghue 2012; Maia et al. 2012). To improve the resolution, additional regions such as *nr*ITS or single-copy nuclear genes have been discussed as supplements to improve the discrimination power of DNA barcode-based identifications (Li et al. 2010; Fazekas et al. 2012; Liu et al. 2012; Pang et al. 2012; Zhang et al. 2013; Borisjuk et al. 2015; Hollingsworth et al. 2016; Wang et al. 2016). The observed differences in the utility of barcodes to identify plant species reflect arguably differences

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✉ Harald Schneider
harald@xtbg.ac.cn

[1] Gardening and Horticulture Department, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla County 666303, Yunnan, China

[2] Department of Life Sciences, The Natural History Museum, London SW7 5BD, UK

[3] Museum für Naturkunde, 10115 Berlin, Germany

[4] Centre for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla County 666303, Yunnan, China

in the evolutionary histories of plant genera that shaped the accumulation of genetic disparity such as the continuation of gene flow among sister species and/or slow lineage sorting, frequent reticulate evolution, and frequent speciation via polyploidization (Piredda et al. 2011; Arca et al. 2012; Clement and Donoghue 2012; Parmentier et al. 2013; van Velzen et al. 2012; Caetano Wyler and Naciri 2016). Lack of differentiation of DNA barcodes may be especially common in plant groups in which polyploidization is a frequent process contributing to the species diversity assembly.

The vast majority of these studies have focused on angiosperms, but some studies gave attention to other plant lineages such as ferns (Nitta 2008; Ebihara et al. 2010, 2013; de Groot et al. 2011; Li et al. 2011; Chen et al. 2013) and bryophytes (Hollingsworth et al. 2009; Liu et al. 2011; Bell et al. 2012; Hassel et al. 2013; Stech et al. 2013). In ferns, most studies using DNA-based identification have addressed either the assembly of barcodes for selected floras (Nitta 2008; Ebihara et al. 2010; de Groot et al. 2011; Nitta et al. 2017), identification of barcodes (Li et al. 2011), or the identity of gametophytes, cultivated specimens, or population of interest (Schneider and Schuettpelz 2006; Li et al. 2009; Pryer et al. 2010; Chen et al. 2013; Ebihara et al. 2013; Williams et al. 2016). To date, little attention has been given to two important issues relating to barcoding: firstly, to consider the effects of DNA variation and the occurrence of DNA barcoding gaps among closely related diploid species; secondly, the assignment of polyploidy taxa and the impact of reticulate evolution on plastid DNA-based species identification. A recent study stressed the need to expand the DNA barcode by including nuclear gene regions (Wang et al. 2016), but this approach will face considerable challenges as a result of technical challenge and the complexities of genome evolution in diploid and their polyploid offspring, such as incomplete lineage sorting and hybridization. Another issue is the challenge to taxonomic approaches by species complexes formed not only by recent cladogenesis events, but also by speciation events involving auto- and allo-polyploidy (Soltis and Soltis 1987; Barrington et al. 1989).

Here, we explore the accumulation of plastid DNA sequence variation in the *Asplenium trichomanes* aggregate with focus on taxa occurring in Europe. The selected plastid gene regions—*psb*A-*trn*H intergenic spacer (IGS), *rps*4-*trn*S IGS, and the *trn*L-*trn*F region—have been previously used both in DNA barcoding studies and/or phylogeographic studies on ferns (e.g. Kress and Erickson 2007; Shepherd et al. 2007; Hunt et al. 2009; Ebihara et al. 2010; de Groot et al. 2011; Wang et al. 2011, 2012; Chen et al. 2013). To explore the discrimination of closely related diploids, we collected three diploid taxa that occur in Europe and Macaronesia: (1) the Macaronesian endemic *Asplenium anceps* Hook. & Grew.; (2) the nearly globally distributed *A. trichomanes* subsp. *trichomanes* [=*A. trichomanes s.s.*]; and (3) the limestone endemic *A. trichomanes* subsp. *inexpectans* Lovis [=*A. inexpectans* (Lovis) Landolt] (Lovis 1964, 1977; Lovis et al. 1977; Tigerschiold 1981; Moran 1982; Reichstein 1984; Manton et al. 1986; Hou and Wang 2000; Tindale and Roy 2002; Rumsey et al. 2004). To detect the discrimination of polyploidy taxa, we included the tetraploids occurring in continental Europe: *A. trichomanes* subsp. *coriaceifolium* Rasbach, K. Rasbach, Reichst. & Bennert [=*A. azomanes* Rossello, Cubas & Rebassa], *A. trichomanes* subsp. *hastatum* S.Jessen, *A. trichomanes* subsp. *pachyrachis* (Christ) Lovis & Reichst. [=*A. csikii* Kuemmerle & Andras], *A. trichomanes* subsp. *quadrivalens* D.E.Mey. [=*A. quadrivalens* (D.E.Meyer) Landolt]. Only the last tetraploid taxon is not restricted in its occurrence to limestone rocks in Europe and Macaronesia (Lovis 1977; Tigerschiold 1981; Moran 1982; Reichstein 1984; Lovis and Reichstein 1985; Manton et al. 1986; Rasbach et al. 1990, 1991; Tutin et al. 1993; Jessen 1995; Hou and Wang 2000). The study takes advantage of research on the biosystematics and population genetics of these ferns in Europe (Bennert and Fischer 1993; Vogel et al. 1999; Suter et al. 2000; Ekrt and Stech 2008). In addition to those taxa occurring in Europe and Macaronesia, samples are included from other parts of the world: *A. trichomanes* subsp. *densum* (Brack.) W.H.Wagner [=*A. densum* Brack.] endemic to Hawaii and *A. trichomanes* subsp. *tripteropus* (Nakai) Á.Löve & D.Löve [=*A. tripteropus* Nakai] occurring in China, Korea, and Japan. The latter species has been reported to include both diploids and tetraploids (Wang 1989; Weng 1990), whereas no chromosome counts exist for the Hawaiian taxon. It is acknowledged that our sampling of the *Asplenium trichomanes* aggregate in China is far from comprehensive. Future studies will be needed to confirm the integration of several species that may also be part of the aggregate such as *A. humistratum* Ching ex H.S.Kung, *A. glanduliserrulatum* Ching ex S.H.Wu, and *A. microtum* Maxon. The Azorean tetraploid *A. azoricum* Lovis, Rasbach & Reichst. (Lovis et al. 1977; Rumsey et al. 2004) was excluded because the study of its relationships requires the inclusion of material of the *A. monanthes* aggregate (see Dyer et al. 2012).

Using this sampling, we aim to address the following questions: (1) have the diploid taxa accumulated sufficient DNA variation to discriminate them using plastid DNA alone, (2) do the tetraploids possess preferably the DNA of one diploid taxon, and (3) do those taxa with identical chromosome numbers differ in the level of accumulated genetic diversity.

Materials and methods

Taxon sampling for phylogenetic analyses

To avoid confusion, the classification of second edition of the Flora Europaea (Tutin et al. 1993) was adopted for all European taxa with the exception of the tetraploid *Asplenium trichomanes* subsp. *hastatum* (Jessen 1995). Non-European taxa were treated according to the most recent and relevant floras of these areas, e.g. China (Lin and Viane 2013) and Hawai'i (Palmer 2003). However, alternative species names were mentioned in parenthesis. To simplify the text, *Asplenium trichomanes* subsp. was reduced to *A. tri.* subsp. All identifications were carefully verified by studying both the morphology and evidence from isozyme analyses when available (Vogel pers. comm.). The isozyme analyses were carried out according to well-documented protocols (Vogel et al. 1998a, 1999; Suter et al. 2000; Hunt et al. 2009). Additive banding patterns were interpreted as evidence for polyploidy (Wendel and Weeden 1989). The sampling included five specimens of *A. anceps*, one specimen of *A. tri.* subsp. *coriaceifolium* (=*A. azomanes*), two specimens of *A. tri.* subsp. *densum* (=*A. densum*), four specimens of *A. tri.* subsp. *hastatum*, 17 specimens of *A. tri.* subsp. *inexpectans* (=*A. inexpectans*), nine specimens of *A. tri.* subsp. *pachyrachis* (=*A. csikii*), 12 specimens of *A. tri.* subsp. *quadrivalens* (=*A. quadrivalens*), 12 specimens of *A. tri.* subsp. *trichomanes* (=*A. trichomanes* s.s.), and 11 specimens of *A. tripteropus* (=*A. tri.* subsp. *tripteropus*) (Table 1). All vouchers are deposited at BM with the exception of the Chinese specimens that are deposited at DUKE (Schuettpelz), HITBC (Chang), PE (Zhang), and SZG (Liu). One sample per unique haplotype was made available via the website of Plant Systematics and Evolution (see Online Resource 1).

Sequence generating and phylogenetic analyses

Whole genomic DNA was extracted from material collected and stored in silica gel using a modified CTAB protocol (Doyle and Doyle 1987). PCR and DNA sequencing protocols follow standard protocols described previously including the primers used to amplify *psb*A-*trn*H IGS (Ebihara et al. 2010), *rps*4-*trn*S IGS (Schneider et al. 2005), and *trn*L-*trn*F region (Trewick et al. 2002). DNA sequences were assembled using Lasergene Core Suite11 (DNASTAR, Madison, USA) and aligned manually using Mesquite 2.75 (Maddison and Maddison 2011). The alignment of each region was carefully checked for sequence inversions and ambiguous regions. None were found; however, a few gaps were identified visually. The number and identity of haplotypes was determined using ALTER with gaps treated as unknown nucleotide (Glez-Pena et al. 2010). Alternative approaches, such as indel coding, were considered but were dismissed. The alignment contained only a few indels suitable to indel coding, and the reconstructed phylogeny was fully resolved by considering only substitution events. Subsequent analyses used data sets with a single representative for each haplotype. Evidence for the absence of topological conflicts among the three gene regions was explored by visual comparison of the consensus trees obtained in maximum parsimony analyses of each individual region. These analyses were carried out with 1000 bootstrap replicates, heuristic searches with 10 randomly assembled starting trees as implemented in PAUP 4.0 (Swofford 2002). Besides the maximum parsimony analyses, each plastid region was also analysed using maximum likelihood analyses in PhyML 3.0 (Guindon and Gascuel 2003; Guindon et al. 2010) with simultaneous model and topology searches. The results of the maximum likelihood bootstrap analyses found topologies including bootstrap values consistent with the results recovered in maximum parsimony analyses.

Statistical parsimony networks (SPN) were employed to explore the relationships within the in-group. They were generated for each plastid DNA region independently and for the combined data set using TCS (Clement et al. 2000). Each haplotype was included by a single accession, and the network was constructed by employing the 95% confidence limit. Initially, networks were also constructed for each of the three markers, whereas the final analyses were carried out using the combined data set. Visual comparison of the networks obtained for each region recovered them as consistent to each other and to the network obtained with the combined data set. Phylogenetic relationships were inferred using maximum likelihood analyses as implemented in PhyML 3.0 (Guindon and Gascuel 2003; Guindon et al. 2010). *Asplenium formosum* Willd., *A. normale* D. Don, and *A. viride* Huds were included as out-group taxa based on the reported relationship among the black-stemmed rock spleenworts (Schneider et al. 2004, 2005, 2013; Dyer et al. 2012; Chang et al. 2013; Schneider et al. 2017). jModeltest 2 was used to infer the best-fit model to the observed distribution of sequence variation (Darriba et al. 2012). The model was implemented in PhyML, but without specifying the parameter values. These values were inferred simultaneously with the optimal tree search. Confidence levels were explored by calculating nonparametric bootstrap values (BS) with 1000 bootstrap replicates as implemented in PhyML 3.0.

Discrimination of the diploid taxa was inferred by counting the number of hypothetical haplotypes separating the observed haplotypes in SPN. Besides, we inferred the ratio of intra-species genetic diversity versus inter-species genetic differentiation using uncorrected *p*-distances, with the maximum intra-species distance versus the minimum distance between the inferred species and its closest relatives. Ratios < 0.5 were regarded as support to species discovery.

Table 1 Information on specimens and observed haplotypes

Taxon	Specimen	Origin	Collector	*psbA-trnH*	*rps4-trnS*	*trnL-trnF*	combined
A. anceps	ANC-12-6	Madeira	Rumsey	ANCP	ANCR	ANCT	ANC
	ANC-13-A1	Madeira	Rumsey	ANCP	ANCR	ANCT	ANC
	ANC-13-B1	Madeira	Rumsey	ANCP	ANCR	ANCT	ANC
	ANC-20	Canary Islands	Rumsey	ANCP	ANCR	ANCT	ANC
	ANC-23-12	Canary Islands	Rumsey	ANCP	ANCR	ANCT	ANC
A. tri. subsp. *coriaceifolium*	COR-11-B-4	Spain	Vogel	INEP	INET3	INER1	INE3/1
A. tri. subsp. *densum*	TT-127-1	Hawai'i	Hemp	DENP	TRIR	TRIT	TRI
	TT-127-2	Hawai'i	Hemp	DENP	TRIR	TRIT	TRI
A. tri. subsp. *hastatum*	HAS-20-D	Germany	Vogel	INEP	INET1	INER3	INE1/3
	HAS-20-E	Germany	Vogel	INEP	INET1	INER3	INE1/3
	HAS-27-B	Austria	Vogel	INEP	INET1	INER3	INE1/3
	HAS-29-B	Austria	Vogel	INEP	INET1	INER3	INE1/3
A. tri. subsp. *inexpectans*	AZO-40-C	Croatia	Vogel	INEP	INET3	INER1	INE3/1
	AZO-42	Croatia	Vogel	INEP	INET3	INER1	INE3/1
	CRO-14	Croatia	Vogel	INEP	INET1	INER1	INE1/1
	D4	Greece	Vogel	INEP	INET4	INER1	INE4/1
	I-25-X-A	Austria	Vogel	INEP	INET1	INER3	INE1/3
	I-25-X-B	Austria	Vogel	INEP	INET1	INER4	INE1/4
	I-45-C1	France	Vogel	INEP	INET3	INER2	INE3/2
	I-46	France	Vogel	INEP	INET3	INER2	INE3/2
	I-50-C1	France	Vogel	INEP	INET3	INER2	INE3/2
	I-74	Balearic Islands	Vogel	INEP	INET1	INER1	INE1/1
	I-76	France	Vogel	INEP	INET1	INER1	INE1/1
	I-86-B-1	Spain	Vogel	INEP	INET1	INER1	INE1/1
	I-91-C	Croatia	Vogel	INEP	INET3	INER1	INE3/1
	I-99-A-3	Austria	Vogel	INEP	INET4	INER1	INE4/1
	I-120-A	Austria	Vogel	INEP	INET1	INER3	INE1/3
	I-126-E-3	Slovenia	Vogel	INEP	INET1	INER3	INE1/3
	I-130-A	Austria	Vogel	INEP	INET1	INER3	INE1/3
A. tri. subsp. *pachyrachis*	PAC-135	Spain	Vogel	INEP	INET1	INER4	INE1/4
	PAC-146-A	Hungary	Vogel	INEP	INET1	INER4	INE1/4
	PAC-146-C	Hungary	Vogel	INEP	INET1	INER4	INE1/4
	PAC-171	Sicily	Vogel	INEP	INET5	INER1	INE5/1
	PAC-172-F	France	Vogel	INEP	INET3	INER1	INE3/1
	PAC-181-B	Belgium	Krippel	INEP	INET1	INER4	INE1/4
	PAC-188	Luxemburg	Vogel	INEP	INET3	INER1	INE3/1
	PAC-191-B	Sardinia	Vogel	INEP	INET3	INER1	INE3/1
	PAC-195-3	Switzerland	Vogel	INEP	INET1	INER4	INE1/4
A. tri. subsp. *quadrivalens*	GEORG-Q-7	Georgia	Bystriakova	INEP	INET1	INER4	INE1/4
	Gueryeba 1	Altai Mts.	Gueryeba	INEP	INET1	INER4	INE1/4
	I-112	Morocco	Rumsey	INEP	INET1	INER4	INE1/4
	Q-CRE-1B	Crete	Vogel	INEP	INET1	INER4	INE1/4
	Q-298	Morocco	Vogel	INEP	INET1	INER4	INE1/4
	TT-50-A	Sweden	Vogel	INEP	INET1	INER4	INE1/4
	TT-71	UK	Vogel	INEP	INET1	INER1	INE1/1
	TT-77	Ukraine	Vogel	INEP	INET1	INER4	INE1/4
	TT-78	Yemen	Gibby	INEP	INET1	INER4	INE1/4
	TT-123	Bosnia	Vogel	INEP	INET3	INER1	INE3/1
	TT-129-6	USA-PA	Acock	INEP	INET1	INER4	INE1/4
	TRI-TUN-3	Tunisia	Vogell	INEP	INET2	INER1	INE2/1

Table 1 (continued)

Taxon	Specimen	Origin	Collector	*psbA-trnH*	*rps4-trnS*	*trnL-trnF*	combined
A. tri. subsp. *trichomanes*	Sayers 3675	India	Sayers	–	TRIR	–	TRI
	Sch 1054	Taiwan	Schuettpelz	TRIP	TRIR	TRIT	TRI
	Sch 1063	Taiwan	Schuettpelz	TRIP	TRIR	TRIT	TRI
	Sch 1097	Taiwan	Schuettpelz	TRIP	TRIR	TRIT	TRI
	Sch 1120	Taiwan	Schuettpelz	TRIP	TRIR	TRIT	TRI
	Sch 1132	Taiwan	Schuettpelz	TRIP	TRIR	TRIT	TRI
	Yanfen1	China, Yunnan	Chang	–	TRIR	–	TRI
	Q-315-6	Turkey	Vogel	TRIP	TRIR	TRIT	TRI
	TT-72-A	Norway	Vogel	TRIP	TRIR	TRIT	TRI
	TT-80	Germany	Vogel	TRIP	TRIR	TRIT	TRI
	TT-126	Canada-BC	Bjoerk	TRIP	TRIR	TRIT	TRI
	TT-128-3	UK	Acock	TRIP	TRIR	TRIT	TRI
A. tripteropus	HS-G-2012	China, Guizhou	Zhang	TRTP	TPTR1	TPTT	TPT1
	C1PT	China, Yunnan	Chang	–	TPTR1	–	TPT1
	C2PT	China, Yunnan	Chang	–	TPTR1	–	TPT1
	C3PT	China, Yunnan	Chang		TPTR1		TPT1
	C4PT	China, Yunnan	Chang		TPTR1		TPT1
	C5PT	China, Sichuan	Chang	–	TPTR1	–	TPT1
	C6PT	China, Sichuan	Chang	–	TPTR1	–	TPT1
	C10PT	China, Sichuan	Chang	–	TPTR1	–	TPT1
	C11PT	China, Sichuan	Chang		TPTR2	–	TPT2
	LIU1	China, Yunnan	Liu	TRTP	TPTR1	TPTT	TPT1
	LIU2	Ching, Yunnan	Liu	TRTP	TPTR1	TPTT	TPT1

Columns 1–8 provide information (from left to right): taxon name employed based on morphological identification (sometimes taken into account unpublished isozyme evidence by J. Vogel); sample number (mostly population numbers used by J. Vogel or collector number); geographic origin; collector; haplotypes identified for each of the three regions and the combined data set. Haplotype abbreviations are based on species name of putative diploid taxon (three letters), the first letter of the plastid region in the case of individual regions, and number of haplotypes observed in each region. Haplotypes abbreviations of the combined data include the taxon name (three letters) and a number composed by the contribution of the haplotypes inferred in *trn*L-*trn*F and *rps*4-*trn*S

Results

Haplotype characteristics

In total, 13 haplotypes were recovered in the combined data set (Table 1). Nine of these haplotypes were found to be unique to a single taxon without the distinction of diploids and tetraploids (Table 2). Haplotype diversity is unequally distributed among taxa (Tables 2, 3). The two diploids, *Asplenium anceps* and *A. tri.* subsp. *trichomanes,* comprised a single haplotype each, whereas two and six haplotypes were found in *A. tripteropus* and *A. tri.* subsp. *inexpectans,* respectively. The three tetraploids with at least four specimens studied comprised either one in *A. tri.* subsp. *hastatum*, three in *A. tri.* subsp. *pachyrachis*, or four haplotypes in *A. tri.* subsp. *quadrivalens*. Two haplotypes (*INE*2/1, *INE*5/1) were unique to tetraploids. All other haplotypes found in the European tetraploids were also present in *A. tri.* subsp. *inexpectans* (Tables 1, 2). The Hawaiian *A. tri.* subsp. *densum* possess a unique haplotype that is separated by the haplotype unique to *A. tri.* subsp. *trichomanes* by a single mutation (Tables 1, 2).

Several haplotypes were separated from the next closest haplotype by more than two hypothetical haplotypes in SPNs (Fig. 1). The haplotype *ANC*—unique to *Asplenium anceps*—was separated from haplotype *TPT*1—unique to *A. tripteropus*—by five hypothetical haplotypes. The haplotype *TRI*—found exclusively in *A. tri.* subsp. *trichomanes*—was separated from *TPT*1 by six hypothetical haplotypes. The haplotype *INE*1/1—found in *A. tri.* subsp. *inexpectans* and *A. tri.* subsp. *quadrivalens*—was separated from *TRI* by 12 hypothetical haplotypes and *TPT*1 by seven hypothetical haplotypes. Each of these haplotypes was found to be distinct in each of the three regions in the study. In contrast, haplotype *DEN*—unique to *A. tri.* subsp. *densum*—was found to be separated from *TRI* by a single substitution in *psb*A-*trn*H, but not distinct in *trn*L-*trn*F and *rps*4-*trn*S. Haplotypes closely related to *INE*1/1 were found to be the result of substitutions or single-base-pair insertions in *trn*L-*trn*F or *rps*4-*trn*S. Each of the *INE* haplotypes was linked

Table 2 Distribution of haplotypes recovered

	ANC	DEN	TRI	TPT	INE	COR*	HAS*	PAC*	QUA*	TH	HUA	HUD
ANC	5	–	–	–	–	–	–	–	–	5	U	U
DEN	–	2	–	–	–	–	–	–	–	2	U	U
INE1/1	–	–	–	–	4	–	–	–	1	5	N	U
INE2/1	–	–	–	–		–	–	–	1	1	U	N/A
INE3/1	–	–	–	–	3	1	–	3	1	8	N	U
INE3/2	–	–	–	–	3	–	–	–	–	3	U	U
INE4/1	–	–	–	–	2	–	–	–	–	2	U	U
INE5/1	–	–	–	–		–	–	1	–	1	U	N/A
INE1/3	–	–	–	–	4	–	4	–	–	8	N	U
INE1/4	–	–	–	–	1	–	–	5	9	15	N	U
TPT1	–	–	–	10		–	–	–	–	10	U	U
TPT2	–	–	–	1		–	–	–	–	1	U	U
TRI	–	–	12	–		–	–	–	–	12	U	U
TT	5	2	12	11	17	1	4	9	12	73	–	–
TUA	U	U	U	U	N	N	N	N	N	–	–	–
TUD	U	U	U	U	U	N	N	N	N	–	–	–

Columns 2–9 show the distribution of haplotypes (left column) versus taxa (top row) for all 72 specimens studied. The occurrence is given as number of specimens with the haplotype per taxon. Column *TH* shows the absolute number of specimens with this haplotype; column *HUA* (=haplotype uniqueness in all taxa) and *HUD* (=haplotype uniqueness in diploid taxa) indicate the taxonomic uniqueness of haplotypes considering all taxa or only diploid taxa. *Asplenium tripteropus* is treated as diploid in this analyses. Abbreviations of haplotypes as in Table 1; taxon abbreviations: *ANC Asplenium anceps*, *DEN A. trichomanes* subsp. *densum*, *TRI A. trichomanes* subsp. *trichomanes*, *TPT A. tripteropus*, *INE A. trichomanes* subsp. *inexpectans*, *COR A. trichomanes* subsp. *coriaceifolium*, *HAS A. trichomanes* subsp. *hastatum*, *PAC A. trichomanes* subsp. *pachyrachis*, *QUA A. trichomanes* subsp. *quadrivalens*. Clade abbreviations: *TRClade* clade including haplotypes found in ANC, DEN, TRI, TPT; *INClade* clade including haplotypes found in INE, COR, HAS, PAC, QUA; general abbreviations: *U* unique, *N* not unique, *N/A* not applicable. Exclusive tetraploid taxa are marked with stars (*)

Table 3 Taxonomic summary of the observed haplotype diversity

	NS	CTS	NH	CHS	NUH	CUHS
ANC	5	6.8	1	7.7	1	7.7
DEN	2	2.7	1	7.7	1	7.7
TRI	12	16.4	1	7.7	1	7.7
TPT	11	15.1	2	15.4	2	15.4
TRClade	30	41.1	5	38.4	5	38.4
INE	17	23.3	6	46.1	2	15.4
COR*	1	1.4	1	7.7	0	0
HAS*	4	5.5	1	7.7	0	0
PAC*	9	12.3	3	23.1	1	7.7
QUA*	12	16.4	4	30.8	1	7.7
INClade	43	58.9	8	61.5	8	61.8

Columns left to right: taxon or clade (abbreviations as in Table 2); *NS* number of specimens studies; *CTS* contribution of taxon to the total number of specimens sampled (in %); *NH* number of observed haplotypes, *CHS* contribution to the observed haplotype number (in %); *NUH* number of unique haplotypes; *CUHS* contribution to diversity by unique haplotypes (in %); clade membership according to phylogenetic analyses

* ploidy level according to references

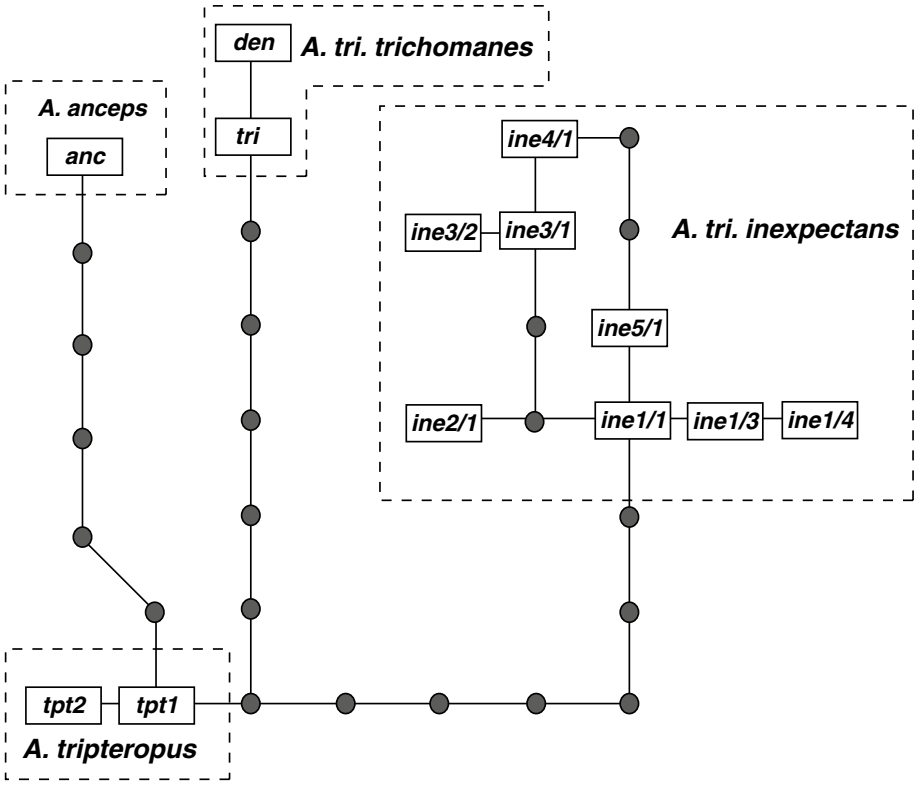

Fig. 1 Statistical parsimony network obtained for the combined data set using TCS with 95% confidence interval. Each line corresponds to a single change. Grey circles correspond to hypothetical haplotypes; squares indicate observed haplotypes. Haplotypes are named according to Table 1. Boxes with dashed lines indicate discrimination of species using a five hypothetical haplotypes separation as the cut-off. Taxa names are assigned according to the occurrence of diploids and priority rules. *Asplenium trichomanes* subsp. *densum* (haplotype *DEN*) is treated as belonging to *A. trichomanes* subsp. *trichomanes*

to its closest haplotype by either a single or maximum three mutations.

Phylogenetic relationships among different haplotypes

Phylogenetic analyses and SPNs (Figs. 1, 2) recovered 13 haplotypes in two major clades. Eight formed one clade (BS = 63%), whereas the remaining five haplotypes formed its sister clade (BS = 96%). The clade with five haplotypes included three morphologically distinguished taxa: *Asplenium anceps* with a single haplotype *ANC*; *A. tripteropus* with two haplotypes *TPT*1 & *TPT*2; and *A. tri.* subsp. *trichomanes* with a single haplotype *TRI*. The clade also included the Hawaiian endemic *A. tri.* subsp. *densum* separated by a single mutation in *psb*A-*trn*H from the closest related haplotype *TRI*. In SPNs, *A. tripteropus* was separated from *A. anceps* by five and *A. tri*. subsp. *trichomanes* by six hypothetical haplotypes. Phylogenetic analyses (Fig. 2) recovered *A. tripteropus* as sister to a clade comprising *A. tri.* subsp. *densum* and *A. tri*. subsp. *trichomanes* (BS < 75%) with *A. anceps* sister to this clade.

The clade formed by eight haplotypes—*INE*1/1 and derivates—consisted of specimens identified either as the diploid *Asplenium trichomanes* subsp. *inexpectans* or the tetraploids *A. tri*. subsp. *coriaceifolium*, *A. tri*. subsp. *hastatum*, *A. tri*. subsp. *pachyrachis*, and *A. tri*. subsp. *quadrivalens*. Haplotype *INE*1/1 was found to be the putative ancestral haplotype of this clade (Figs. 1, 2). This haplotype was found in specimens belonging to *A. tri*. subsp. *inexpectans* and *A. tri*. subsp. *quadrivalens* (Table 1).

Discussion

Delimitation of taxa using plastid DNA

Plastid DNA delimitates four separate operational taxonomic units (OTUs) considering three criteria: (1) the uniqueness of the genetic variation to a single OTU (Table 3); (2) the distinctiveness of the genetic variation of two OTUs by the occurrence of two or more hypothetical haplotypes; and (3) the existence of a "barcoding gap". This gap was inferred by calculating the ratio between the maximum distance within an OTU and the minimum distance between the two most closely related OTUs. The gap was considered to be present with the intra-/inter-values (I/I) < 0.5. Based on the application of these criteria, the following OTUs were recovered: OTU1 with I/I < 0.007 included *Asplenium anceps*; OTU2 with I/I = 0.11 included *A. tri*. subsp. *trichomanes* and *A. tri*. subsp. *densum*; OTU3 with I/I = 0.12 included *A. tripteropus*; OTU4 with I/I < 0.27 included *A. tri*. subsp. *inexpectans* and all tetraploids with European occurrences (see Tables 1, 3). Two of the OTUs correspond to a single taxon: OTU1 = *A. anceps* and OTU3 = *A. tripteropus*. OTU2 comprised *A. tri*. subsp. *densum* and *A. tri*. subsp. *trichomanes.*

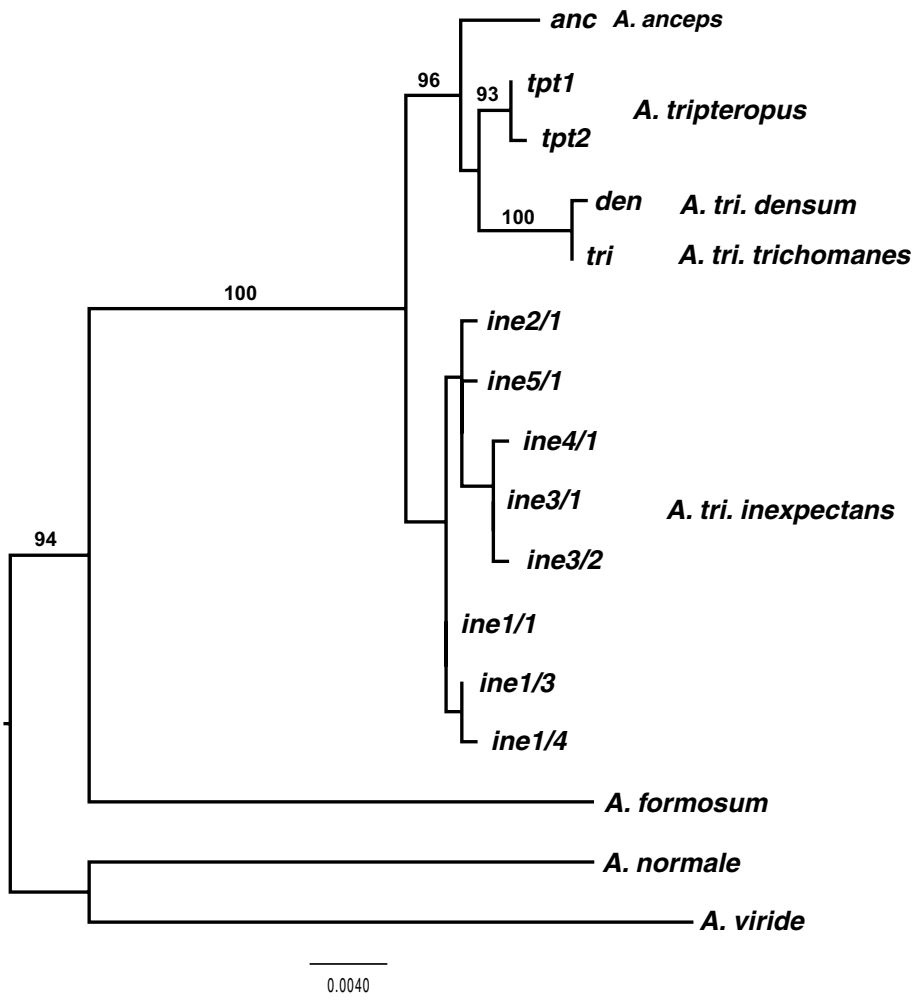

Fig. 2 Phylogram obtained from maximum likelihood analyses of the combined data set. Three species—*Asplenium formosum*, *A. normale*, and *A. viride*—were included as outgroup taxa representing closely related clades (see Schneider et al. 2004, 2005, 2013). Bootstrap values ≥ 75% are shown above branches. Abbreviation of haplotypes follows Table 1. Taxon names follow the classification given in Table 1. Taxa names were assigned based on the distribution of haplotypes in diploids (or putative diploids in the case of *A. tri.* subsp. *densum*)

The little genetic differentiation of the Hawaiian endemics suggests a rather recent separation of this island endemic, but this needs to be confirmed with an expanded sampling of *A. tri.* subsp. *trichomanes*. The ploidy level of the Hawaiian taxon is currently unknown, and its status requires further investigation. OTU4 comprised the diploid *A. tri.* subsp. *inexpectans* and four tetraploids occurring in Europe: *A. tri.* subsp. *coriaceifolium*, *A. tri.* subsp. *hastatum*, *A. tri.* subsp. *pachyrachis*, and *A. tri.* subsp. *quadrivalens*. In summary, diploid taxa occurring in Europe or Macaronesia can be reliably distinguished from each other based on their unique plastid DNA variation by applying either distance methods (e.g. DNA barcoding gap) or using a character-based approach (Jean-Molina et al. 2015), whereas European tetraploids cannot be reliably distinguished based on plastid DNA variation only (see also Tables 1, 3).

The tetraploids in Europe are not distinct from the diploid *Asplenium trichomanes* subsp. *inexpectans,* but well distinct from the diploid *A. tri.* subsp. *trichomanes*. Since the plastid DNA in ferns is maternally inherited (Gastony and Yatskievych 1992; Vogel et al. 1998b), this pattern suggests *A. tri.* subsp. *inexpectans* is either the single parent of these tetraploids (= autotetraploids) or the maternal parent of a hybrid formed with another species of the complex (allotetraploids). The later hypothesis assumes an asymmetrical hybridization. Considering the tetraploids in isolation, some patterns are inferred. The three taxa sampled with at least four specimens—*A. tri.* subsp. *hastatum*, *A. tri.* subsp. *pachyrachis*, and *A. tri.* subsp. *quadrivalens*—show distinct patterns of genetic variation. *Asplenium tri.* subsp. *hastatum* comprises a single haplotype (*INE*1/3), which is not found in any other tetraploid, but does occur in the diploid *A. tri.* subsp. *inexpectans.* The other two tetraploids—*A. tri.* subsp. *pachyrachis* and *A. tri.* subsp. *quadrivalens*—show similar levels of genetic variation with three and four haplotypes, respectively (Table 1). In the latter, nine out of 12 samples showed the haplotype *INE*1/4. This haplotype is also common in *A. tri.* subsp. *pachyrachis,* but rare in *A. tri.* subsp. *inexpectans* (Table 1, 2, 3). The patterns require to be confirmed by expanding the number of sampled individuals and populations.

Accumulation of plastid DNA variation

The studied taxa show different levels of sequence variation in their plastid DNA (Table 3). Two out of the three European diploid taxa—*Asplenium anceps* and *A. tri.* subsp. *trichomanes*—possess a single haplotype, whereas the third one—*A. tri.* subsp. *inexpectans*—contains six haplotypes. This difference is unlikely to be due to the result of sampling size, but arguably reflect the independent evolutionary histories of these taxa including variation of the population structure. The four European tetraploid subspecies—*A. tri.* subsp. *coriaceifolium*, *A. tri.* subsp. *hastatum*, *A. tri.* subsp. *pachyrachis*, and *A. tri.* subsp. *quadrivalens*—display different levels of genetic variation ranging from one to four haplotypes (Tables 1, 2, 3).

The two island endemics—*Asplenium anceps* and *A. tri.* subsp. *densum*—share the occurrence of a single haplotype, whereas the other two diploids occurring in the European continent—*A. tri.* subsp. *inexpectans* and *A. tri.* subsp. *trichomanes*—differ substantially in their genetic diversity. However, this difference appears to be independent from the spatial range because *A. tri.* subsp. *trichomanes* has a nearly global distribution, whereas *A. tri.* subsp. *inexpectans* occurs only in Europe. Thus, the haplotype diversity is higher in the less widespread distributed taxon. This hypothesis is consistent with the rather low diversity found in the SE Asian *A. tripteropus*. Among tetraploids, the globally distributed *A. tri.* subsp. *quadrivalens* shows similar variation as *A. tri.* subsp. *pachyrachis* despite the range of the latter is restricted to limestone areas of Europe. In addition, *A. tri.* subsp. *quadrivalens* differs from *A. tri.* subsp. *pachyrachis* not only in the nearly global versus European range, but also in the substrate vagueness of *A. tri.* subsp. *quadrivalens* versus the restriction to limestone of *A. tri.* subsp. *pachyrachis*. Thus, neither in diploids nor in tetraploids is there a correlation between accumulation of genetic diversity and size of the distribution range.

Previous studies have reported evidence that suggests there is a correlation between the accumulation of genetic diversity and evolution of breeding systems in fern species (Haufler and Soltis 1986; Murakami et al. 1997; Suter et al. 2000; Haufler 2002; Ranker and Geiger 2008; Wubs et al. 2010; Haufler et al. 2016). The diploid limestone taxon *Asplenium trichomanes* subsp. *inexpectans* displays genetic variation in its plastid genomes resembling the genetic variation reported for two other European diploid limestone ferns—*A. fontanum* (Hunt et al. 2009) and *A. viride* (James et al. 2008). In contrast, the widespread *A. tri.* subsp. *trichomanes* possesses a single haplotype. The failure to assemble diversity may be either related to a very recent population size fluctuation or by a reproductive system which limits the accumulation of genetic variation. A probable explanation is provided by the scenario that *A. tri.* subsp. *trichomanes* reproduces preferentially by intragametophytic selfing (Vogel, pers. comm.). Shifts in the reproductive biology from outbreeding to obligate intragametophytic selfing have been documented in various fern lineages. The obtained results are consistent with the theoretical predictions of the haplotype distribution in populations with a recent shift to obligate intragametophytic selfing, e.g. fixation of haplotypes. This reproductive preference would allow the taxon to colonize new areas via single-spore range expansion, which is consistent with its distribution covering the temperate regions of both the northern and southern hemispheres. In turn, intragametophytic selfing causes the fixation of genotypes. This preferential intragametophytic selfing may also explain why the plastid DNA of allotetraploids involving *A. tri.* subsp. *trichomanes* as one parent is consistent with that of the chloroplast in the other parent, as exemplified by *A. adulterinum* which shares the cpDNA of *A. viride* and not of its other parent *A. tri.* subsp. *trichomanes* (James et al. 2008; Schneider et al. 2013). Unpublished allozyme data support this assumption and are also evidence for preferred outcrossing in *A. tri.* subsp. *inexpectans* (Vogel, pers. comm.).

A different explanation is needed to understand the reason for the differences in genetic variation which was recovered from the European tetraploids studied. The population structure for two of these tetraploid subspecies—*Asplenium trichomanes* subsp. *pachyrachis* and *A. tri.* subsp. *quadrivalens*—has been studied (Vogel et al. 1999; Suter et al. 2000). These studies indicate that these taxa reproduce mainly by intragametophytic selfing. For this reason, the accumulation of plastid DNA in these two taxa is likely due to the result of multiple origins of tetraploids instead of sexual recombination. Unpublished nuclear genome evidence supports the hypotheses that each of these tetraploid taxa originated from multiple hybridization events involving the same parental species, but not the same parental populations. Recurrent formation of polyploid taxa is a common process in plants (Doyle et al. 1990; Soltis and Soltis 1991) and has been discussed for European polyploids belonging to the genus *Asplenium* (Vogel et al. 1998a, 1999; Suter et al. 2000; Trewick et al. 2002). Combining plastid DNA with other evidence may make it possible to trace independent origins of these tetraploids. For example, the morphologically distinct *A. tri.* subsp. *hastatum* was found to contain a single haplotype that was not shared with any other tetraploid. This observation requires further confirmation by increasing the sampling size of other populations with the morphology of *A. tri.* subsp. *hastatum*. With all the current available evidence, this taxon is considered to have a single area of origin. In contrast, the morphologically and ecologically distinct *A. tri.* subsp. *pachyrachis* and *A. tri.* subsp. *quadrivalens* are considered to have multiple origins. These results when confirmed will be critical to clarify the status of these taxa.

Taxonomy of the *Asplenium trichomanes* complex

This study confirmed the distinction of four diploid species. *Asplenium anceps* and *A. tripteropus* have in common the occurrence of three wings at the petiole and demonstrate other similarities such as the shape of the pinnae. These two species are distinct from each other in the absence/presence of leaf buds (Lovis et al. 1977). However, these two species are distinct from their relatives by the presence of a third wing located at the abaxial side of the rachis. All other species inferred possess only two wings on the adaxial side of the petiole. The other two diploids—*A. tri.* subsp. *trichomanes* and *A. tri.* subsp. *inexpectans*—are distinct from each other in the shape of the pinnae, especially the terminal pinnae and other morphological features (Tigerschiold 1981; Reichstein 1984; Tutin et al. 1993; Saez 2000; Ekrt and Stech 2008). In conclusion, the reported plastid DNA supports the argument that the complex includes at least four diploid species. *Asplenium tri.* subsp. *trichomanes* and *A. tri.* subsp. *inexpectans* should be recognized as different species instead of different subspecies. All required combinations have been introduced including *A. inexpectans* (Loves) Landolt (2010). However, there is a problem with regard to the typification of *A. trichomanes.* Bobrov (1984) selected the specimen Herb. Linn. No. 1250. 12 (LINN) as the lectotype. Characters as pinnae shape suggest that this specimen correspond what is currently identified as *A. tri.* subsp. *quadrivalens*. It is suggested that the problem would be avoided with the alternative proposal of Burser XX: 14 as the lectotype by Viane (in Jonsell and Jarvis 1994), but its publication post-dates Bobrov's lectotypification.

Our results suggested *Asplenium trichomanes* subsp. *densum* to be a local variant of *A. tri.* subsp. *trichomanes.* Thus, this taxon may be best treated as a subspecies as long as no further evidence is reported supporting its segregation (see also Palmer 2003). Future studies may need to test the proposal that this taxon is of anagenetic origin in Hawai'i. Further attention needs to be given to the taxonomy of *A. tripteropus* because this taxon contains a diploid and a tetraploid karyotype.

The treatment of the tetraploid subspecies of *Asplenium trichomanes* is a challenge because they represent a poorly understood assemblage of morphological and/or cytological distinct units. Most of these units may have multiple origins, but share the same or rather similar ancestries. In addition, there is evidence that suggests intragametophytic selfing as the common reproductive strategy (Vogel et al. 1999; Suter et al. 2000). The observed plastid DNA variation is consistent with the hypothesis of multiple origins of tetraploids, which has consequences for the treatment at the species level of *A. tri.* subsp. *quadrivalens* and *A. tri.* subsp. *pachyrachis*. These taxa may not form monophyla, but further work is required to confirm this. In contrast, a single haplotype was found in the tetraploid *A. tri.* subsp. *hastatum* which may indicate a single origin of this morphological distinct limestone tetraploid.

Given the limited understanding of the evolutionary history and the genetic distinctness of the tetraploid forms, it seems premature to introduce a new classification despite the obvious limitations of their treatment as subspecies as promoted in the Flora Europaea (Tutin et al. 1993). Confirmation of the status of the tetraploids is required using not only plastid DNA sequences, but also sequences of low-copy nuclear genes (Schneider et al. 2013). In contrast to the tetraploids, the status of the diploids is now rather clear and *Asplenium inexpectans* need to be accepted as a distinct species from *A. trichomanes* aggregate. At this point, it is important to note that no further conclusions can be made on Asian taxa because they were either not sampled or the sampling was rather limited in the case of *A. tripteropus.*

Conclusions

The results provide evidence that DNA barcodes can be successfully employed to identify specimens of closely related fern species as long as the studied taxa are diploids. The inclusion of polyploids representing either allo- or autopolyploids introduces levels of complexity that cannot be solved using plastid DNA barcodes alone. A further important observation is the discovery of distinct differences and the amount of plastid DNA variation that exists among diploid taxa. These differences in the observed variation may have profound impact on the required number of specimens per species in a DNA barcoding database. The results of this study carry not only implications for DNA barcoding studies on ferns, but also for other groups of land plants that tend to accumulate species diversity via the formation of polyploids instead of cladogenesis resulting in the formation of diploid species (Fazekas et al. 2009).

Taxonomic treatment

As discussed above, combinations exist for all European taxa recognized in this study at both species and subspecies level with the exception of *Asplenium trichomanes* subsp. *hastatum* (Christ) S.Jess. which is based on the basionym *A. trichomanes* var. *hastatum* Christ. The existence of the Neotropical *A. hastatum* Klotzsch ex Kunze prevents the transfer of the basionym to the species level. We therefore introduce here *Asplenium jessenii* in recognition of the important contribution of Stefan Jessen to the taxonomy of this species aggregate.

Asplenium jessenii H.M.Liu & H.Schneid., **sp. nov.** ≡ *Asplenium trichomanes* var. *hastatum* Christ, Beitr. Kryotpgamenfl. Schweiz 1(2): 92. 1900.≡ *Asplenium*

trichomanes subsp. *hastatum* (Christ) S.Jess., Ber. Bayer. Bot. Ges. 65: 111. 1995.

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Authors' contributions HML, JCV, and HS designed the study and drafted the manuscript and worked out the taxonomy. SR generated most of the new sequence data, whereas JCV provided most of the material and background information, especially the information on the unpublished allonym data. All authors approved the final manuscript.

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Compliance with ethical standards

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

Information on Electronic Supplementary Material

Online Resource 1. Haplotypes recovered based on three non-coding plastid genome regions with ambiguous regions deleted. Sequence: trnL-trnF, rps4-trnS, psbA-trnH. Total length: 1537 base pairs. Haplotype classification as in Fig. 1 and Table 2. For convenience of users, the supplement is provided as a nexus file.

References

Arca M, Hinsinger DD, Cruaud C, Tillier A, Bousquet J, Frascaria-Lacoste N (2012) Deciduous trees and the application of universal DNA barcodes: a case study on the circumpolar *Fraxinus*. PLoS ONE 7:e34089. https://doi.org/10.1371/journal.pone.0034089

Barrington DS, Haufler CH, Werth CR (1989) Hybridization, reticulation, and species concepts in the ferns. Amer Fern J 79:55–64

Bell D, Long DG, Forrest AD, Hollingswirth M, Blom HH, Hollingsworth PM (2012) DNA barcoding of European *Herbertus* (Marchantiopsida, Herbertaceae) and the discovery and description of new species. Molec Ecol Resources 12:36–47

Bennert HW, Fischer G (1993) Biosystematics and evolution of the *Asplenium trichomanes* complex. Webbia 48:743–760

Bobrov AE (1984) Conspectus Filicatum Asiae Mediae et Kazakhstaniae. Novosti Sistematiki Vysshikh I Nizshikh Rasteni 21:5–21

Borisjuk N, Chu P, Gutierrez R, Zhang H, Acosta K, Friesen N, See KS, Garcia C, Appenroth KJ, Lam E (2015) Assessment, validation and deployment strategy of a two-barcode protocol for facile genotyping of duckweed species. Pl Biol 17:42–49. https://doi.org/10.1111/plb.12229

Braukmann TWA, Kuzmina ML, Sills J, Zakharov EV, Hebery PDN (2017) Testing the efficacy of DNA barcodes for identifying the vascular plants of Canada. PLoS ONE 12:e0169515. https://doi.org/10.1073/journal.pone.0169515

Caetano Wyler S, Naciri Y (2016) Evolutionary histories determine DNA barcoding success in vascular plants: seven case studies using intraspecific broad sampling of closely related species. BMC Evol Biol 16:103. https://doi.org/10.1186/s12862-016-0678-0

CBOL Plant Working Group (2009) A DNA barcode for land plants. Proc Natl Acad Sci USA 106:12794–12797. https://doi.org/10.1073/pnas.0905845106

Chang YF, Li J, Lu SG, Schneider H (2013) Species diversity and reticulate evolution in the *Asplenium normale* complex (Aspleniaceae) in China and adjacent areas. Taxon 62:673–687

Chen CW, Huang YM, Kuo LY, Nguyen QD, Luu HT, Callado JR, Farrar DR, Chiou WL (2013) *trnL*-Fis a powerful marker for DNA identification of field vittarioid gametophytes (Pteridaceae). Ann Bot (Oxford) 111:663–673. https://doi.org/10.1093/aob/mct004

Clement WL, Donoghue MJ (2012) Barcoding success as a function of phylogenetic relatedness in *Viburnum*, a clade of woody angiosperms. BMC Evol Biol 12:73. https://doi.org/10.1186/1471-2148.12-73

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. Molec Ecol 9:1657–1660. https://doi.org/10.1046/j.1365-294x.2000.01020

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Meth 9:772. https://doi.org/10.1038/nmeth.2109

de Groot GA, During HJ, Maas JW, Schneider H, Vogel JC, Erkens RHJ (2011) Use of *rbc*L and *trn*L-F as a two-locus DNA barcode for identification of NW-European ferns: an ecological perspective. PLoS ONE 6:e16371. https://doi.org/10.1371/journal.pone.0016371

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure from small quantities of fresh leaf tissue. Phytochem Bull Boc Soc Amer 19:11–15

Doyle JJ, Doyle JL, Brown AH, Gace JP (1990) Multiple origins of polyploids in the *Glycine tabacina* complex inferred from chloroplast DNA polymorphisms. Proc Natl Acad Sci USA 87:714–717

Dyer RJ, Savolainen V, Schneider H (2012) Apomixis and reticulate evolution in the *Asplenium monanthes* fern complex. Ann Bot (Oxford) 110:1515–1529. https://doi.org/10.1093/aob/mcs202

Ebihara A, Nitta J, Ito M (2010) Molecular species identification with rich floristic sampling: DNA barcoding the pteridophyte flora of Japan. PLoS ONE 5:e15136. https://doi.org/10.1371/journal.pone.0015136

Ebihara A, Yamaoka A, Muzikami N, Sakoda A, Nitta JH, Imaichi R (2013) A survey of the fern gametophyte flora of Japan: frequent independent occurrences of noncordiform gametophytes. Amer J Bot 100:735–743. https://doi.org/10.3732/ajb.1200555

Ekrt L, Stech M (2008) A morphometric study and revision of the *Asplenium trichomanes* group in the Czech Republic. Preslia 80:325–347

Fazekas AJ, Kesanakurti PR, Burgess KS, Percy DM, Graham SW, Barrett SCH, Newmaster SG, Hajibabaei M, Husband BC (2009) Are plant species inherently harder to discriminate than animal species using DNA barcoding markers? Molec Ecol Resources 9(Suppl. 1):130–139. https://doi.org/10.1111/J.1755-0998.2009.02652

Fazekas AJ, Kuzmina ML, Newmaster SG, Hollingsworth PM (2012) DNA Barcoding methods for land plants. Meth Molec Biol 858:223–252

Garcia-Robledo C, Erickson DL, Staines CL, Erwin TL, Kress WJ (2013) Tropical plant-herbivore networks: reconstructing species interactions using DNA barcodes. PLoS ONE 8:e5296. https://doi.org/10.1371/journal.pone.0005296

Gastony GJ, Yatskievych G (1992) Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. Amer J Bot 79:716–722

Ghorbani A, Saeedi Y, de Boer HJ (2017) Unidentifiable by morphology: DNA barcoding of plant material in local market in Iran. PLoS ONE 12:e0175722. https://doi.org/10.1371/journal.pone.0175722

Glez-Pena D, Gomez-Blanco D, Reboiro-Jato Fdez-Riverola F, Posada D (2010) ALTER: program-oriented conversions of DNA and protein alignments. Nucl Acids Res 28(Suppl. 2):W14–W18. https://doi.org/10.1093/nae/gkq321

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704. https://doi.org/10.1080/10635150390235520

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321. https://doi.org/10.1093/sysbio/syq010

Hassel K, Segreto R, Ekrem T (2013) Restricted variation in plant barcoding markers limits identification in closely related bryophytes species. Molec Ecol Resources 13:1047–1057

Haufler CH (2002) Homosporoy 2002: An odyssey of progress in pteridophyte genetics and evolutionary biology. BioScience 52:1081–1093. https://doi.org/10.1641/0006-3568(2002)052[1081:HAOOPI]2.0.CO;2

Haufler CH, Soltis DE (1986) Genetic evidence suggests that homosporous ferns with high chromosome numbers are diploid. Proc Natl Acad Sci USA 83:4389–4393

Haufler CH, Pryer KM, Schuettpelz E, Sessa EB, Farrar DR, Moran R, Schneller JJ, Watkins JE, Windham MC (2016) Sex and the single gametophyte: revising the homosporous vascular plant life cycle in light of contemporary research. BioScience 666:928–937. https://doi.org/10.1093/biosci/biw108

Hollingsworth ML, Clark AA, Forest LL, Richardson J, Pennington RT, Long DG, Cowan R, Chase MW, Gaudeul M, Holligsworth PM (2009) Selecting barcoding loci for plants: evaluating of seven candidate loci with species-level sampling in three divergent groups of land plants. Molec Ecol Resources 9:439–457. https://doi.org/10.1111/j.1755-0998.2008/-2439

Hollingsworth P, Li DZ, van der Bank M, Twyford AD (2016) Telling plant species apart with DNA: from barcodes to genomes. Philos Trans Roy Soc B Bio Sci 371:1702. https://doi.org/10.1098/rstb.2015.0338

Hou X, Wang ZR (2000) A subspecific taxonomic study on *Asplenium trichomanes* L. from China. Acta Phytotax Sin 38:242–255

Hunt HV, Ansell SW, Russell SJ, Schneider H, Vogel JC (2009) Genetic diversity and phylogeography in two diploid ferns, *Asplenium fontanum* subsp. *fontanum* and *A. petrarchae* subsp. *bivalens*, in the western Mediterranean. Molec Ecol 23:4940–4954. https://doi.org/10.1111/j.1365-294X.2009.04402

James KE, Schneider H, Ansell SW, Evers M, Robba L, Uszynski G, Pedersen N, Newton A, Russell SJ, Vogel JC, Kilian A (2008) Diversity arrays technology (DArT) for pan-genomic evolutionary studies of non-model organisms. PLoS ONE 3:e1682. https://doi.org/10.1371/journal.pone.0001682

Jean-Molina R, Marrero-Rodriguez A, Reyes-Betancort JA, Santos-Guerra A, Naranjo-Suarez J, Caujape-Castells J (2015) Molecular Taxonomic identifications in the absence of a 'barcoding gap': a test with the endemic flora of the Canadian oceanic hotspot. Molec Ecol 15:42–56. https://doi.org/10.1111/1755-0998.12292

Jessen S (1995) *Asplenium trichomanes* L. subsp. *hastatum*, stat. nov.—eine neue Unterart des Braunstiel-Streifenfarnes in Europa und vier neue intraspezifische Hybriden (Aspleniaceae, Pteridophyta). Ber Bayer Bot Ges 65:107–132

Jonsell B, Jarvis CE (1994) Lectotypifications of Linnean names for Flora Nordica, Vol. 1 (Lycopodiaceae-Papaveraceae). Nordic J Bot 14:145–164. https://doi.org/10.1111/j.1756-1051.1994.tb00581

Kress WJ, Erickson DL (2007) A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. PLoS ONE 2:e508. https://doi.org/10.1371/journal.pone.0000508

Kress WJ, Erickson DL, Swenson NG, Thompson J, Uriarte M, Zimmermann JS (2010) The use of DNA barcodes to build a community phylogeny for tropical trees in a Puerto Rican forest dynamics plot. PLoS ONE 5:e15409. https://doi.org/10.1371/journal.pone.0000508

Landolt E (2010) Flora Indicativa: Oekologische Zeigerwerte und biologische Kennzeichen zur Flora der Schweiz und der Alpen. Verlag Paul Haupt, Bern

Li FW, Tan BC, Buchbender Y, Moran RC, Rouhan G, Wang CN, Quandt D (2009) Identifying a mysterious aquatic fern gametophyte. Pl Syst Evol 281:77–86. https://doi.org/10.1007/s00606-009-0188-2

Li DZ, Gao LM, Li HT, Wang H, Ge XJ, Liu J, Chen ZD, Zhou SL, Chen SL, Yang JB, Yu WB, Zeng CZ, Yan HF, Zhu YJ, Sun YS, Chen SY, Zhao L, Wang K, Yang T, Duan GW (2010) Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proc Natl Acad Sci USA 108:19641–19646. https://doi.org/10.1073/pnas.1104551108

Li FW, Kuo LY, Rothfels CJ, Ebihara A, Chiou WL, Windham MD, Pryer KM (2011) *rbcL* and *matK* earn two thumbs up as the core DNA barcode for ferns. PLoS ONE 6:e26597. https://doi.org/10.1371/journal.pone.0026597

Lin YX, Viane R (2013) Aspleniaceae. In: Wu ZY, Raven PH, Hong DY (eds) Flora of China (Lycopodiaceae through Polypodiaceae), vol. 2–3. Science Press, Beijing, pp 267–316

Liu Y, Ge TC, Ge XJ (2011) A care study of DNA barcoding in Chinese Grimmiaceae and a moss recorded in China for the first time. Taxon 60:185–193

Liu ZH, Zeng X, Yang D, Chu GY, Yuan ZR, Chen SL (2012) Applying DNA barcodes for identification of plant species in the family Araliaceae. Gene 499:76–80. https://doi.org/10.1016/j.gene.2012.02.016

Lovis JD (1964) The taxonomy of *Asplenium trichomanes* in Europe. Fern Gaz 9:147–160

Lovis JD (1977) Evolutionary patterns and processes in ferns. Advances Bot Res 4:229–415

Lovis JD, Reichstein T (1985) *Asplenium trichomanes* subsp. *pachyrachis* (Aspleniaceae) and a note on the typification of *A. trichomanes*. Willdenowia 15:187–205

Lovis JD, Rasbach HG, Rasbach K, Reichstein T (1977) *Asplenium azoricum* and other ferns of the A. *trichomanes* group from the Azores. Amer Fern J 67:81–93

Maddison WP, Maddison DR (2011) Mesquite: a modular system for evolutionary analyses. Vers. 2.75. Available at: http://mesquiteproject.org. Accessed 25 Mar 2017

Maia VH, Mata CSD, Franco LO, Cardoso MA, Cardoo SRS, Hemerly AS, Ferreira PCG (2012) DNA barcoding Bromeliaceae: achievements and pitfalls. PLoS ONE 7:e29877. https://doi.org/10.1371/journal.pone.0082977

Manton I, Lovis JD, Vida G, Gibby M (1986) Cytology of the fern flora of Madeira. Bull Brit Mus (Nat Hist) Bot 15:123–161

Moran RC (1982) The *Asplenium trichomanes* complex in the United States and adjacent Canada. Amer Fern J 72:5–11

Murakami N, Nishiyama T, Satoh H, Suzuku T (1997) Marked spatial genetic structure in three populations of a weedy fern, *Pteris multifida Poir.*, and re-estimation of its selfing rate. Pl Spec Biol 12:97–106

Nitta JH (2008) Exploring the utility of three plastic loci for barcoding filmy ferns (Hymenophyllaceae) of Moorea. Taxon 57:725–736
Nitta JH, Meyer JY, Taputuarai R, Davis CC (2017) Life cycle matters: DNA barcoding reveals contrasting community structure between fern sporophytes and gametophytes. Ecol Monogr 87:278–296. https://doi.org/10.1002/ecm.1246
Palmer DD (2003) Hawai'i's ferns and fern allies. University of Hawai'i Press, Honolulu
Pang X, Luo H, Sun C (2012) Assessing the potential of candidate DNA barcodes for identifying non-flowering seed plants. Pl Biol 14:839–844. https://doi.org/10.1111/j.1438-8677.2011.00554
Parmentier I, Duminil J, Kuzmina M, Philippe M, Thomas DW, Kenfack D, Chuyong GB, Cruaud C, Hardy O (2013) How effective are DNA barcodes in the identification of African rainforest trees? PLoS ONE 8:e54921. https://doi.org/10.1371/journal.pone.0054921
Piredda R, Simone MC, Attimonelli M, Bellarosa R, Schirone B (2011) Prospects of barcoding the Italian wild dendroflora: oaks reveal severe limitations to tracking species identity. Molec Ecol Resources 11:72–83. https://doi.org/10.1111/j.1755-0998.2010.02858
Pryer KM, Schuettpelz E, Huiet Grusz AL, Rothfels CJ, Avent T, Schwartz D, Windham MD (2010) DNA barcoding exposes a case of mistaken identity in the fern horticultural trade. Molec Ecol Resources 10:979–985
Ranker TA, Geiger JMO (2008) Population genetics. In: Ranker TA, Haufler CH (eds) Biology and evolution of ferns and lycophytes. Cambridge University Press, Cambridge, pp 107–133
Rasbach H, Rasbach K, Reichstein T, Bennert HW (1990) *Asplenium trichomanes* subsp. *coriaceifolium*, a new subspecies and two new intraspecific hybrids of the *A. trichomanes* complex (Aspleniaceae, Pteridophyta) I. Nomenclature and typification. Willdenowia 19:471–474
Rasbach H, Rasbach K, Reichstein T, Bennert HW (1991) *Asplenium trichomanes* subsp. *coriaceifolium*, a new subspecies and two new intraspecific hybrids of the *A. trichomanes* complex (Aspleniaceae, Pteridophyta). II. Description and illustration with an appendix on pairing behaviour of chromosomes in fern hybrids. Willdenowia 21:239–261
Reichstein T (1984) *Asplenium*. In: Kramer KU (ed) Gustav Hegi, Illustrierte Flora von Mitteleuropa. Band I Pteridophyta-Spermatophyta. Teil I. Pteridophyta, 3rd edn. Verlag Parey, Nerlon, pp 211–266
Rumsey F, Russell S, Schäfer H, Rasbach H (2004) Distribution, ecology and cytology of *Asplenium azoricum* Lovis, Rasbach & Reichstein (Aspleniaceae, Pteridophyta) and its hybrids. Amer Fern J 94:113–125. https://doi.org/10.1640/0002/8444
Saez L (2000) El complex d' *Asplenium trichomanes* L. (Aspleniaceae, Pteridophyta) al nord-east de la Peninsula Iberia. Orsis 15:27–43
Schneider H, Schuettpelz E (2006) Identifying fern gametophytes using DNA sequences. Molec Ecol Notes 6:989–991. https://doi.org/10.1111/j.1471-8286.2006.01424
Schneider H, Russell SJ, Cox CJ, Bakker F, Henderson S, Rumsey F, Barrett J, Gibby M, Vogel JC (2004) Chloroplast phylogeny of asplenioid ferns based on *rbc*L and *trn*L-F spacer sequences (Polypodiidae, Aspleniaceae) and its implications for biogeography. Syst Bot 29:260–274
Schneider H, Ranker TA, Russell SJ, Cranfill R, Geiger JMO, Aguraiuja R, Wood KR, Grundmann M, Kloberdanz K, Vogel JC (2005) Origin of the endemic fern genus *Diellia* coincides with the renewal of Hawaiian terrestrial life in the Miocene. Proc Roy Soc Lond B Biol Sci 272:455–460. https://doi.org/10.1098/rspb.2004.2965
Schneider H, Navarro-Gomez A, Russell SJ, Ansell S, Grundmann M, Vogel JC (2013) Exploring the utility of three nuclear regions to reconstruct reticulate evolution in the fern genus *Asplenium*. J Syst Evol 51:142–153. https://doi.org/10.1111/j.1759-6831.2012.00226.x
Schneider H, Liu HM, Chang YF, Ohlsen D, Perrie LR, Shepherd L, Kessler M, Karger DN, Hennequin S, Marquardt J, Russell S, Ansell S, Lu NT, Kamau P, Loriga J, Regalado L, Heinrichs J, Ebihara A, Smith AR, Gibt M (2017) Neo- and paleopolyploidy contribute to the species diversity of Asplenium—the most species-rich genus of ferns. J Syst Evol 55:353–364. https://doi.org/10.1111/jse.12271
Shepherd LD, Perrie LR, Brownsey PJ (2007) Fire and ice: volcanic and glacial impacts on the phylogeography of the New Zealand forest fern *Asplenium hookerianum*. Molec Ecol 21:4536–4549. https://doi.org/10.1111/j.1365-294X.2007.03451
Soltis DE, Soltis PS (1987) Polyploidy and breeding systems in homosporous pteridophyta: a reevaluation. Amer Naturalist 130:219–232
Soltis PS, Soltis DE (1991) Multiple origins of the allotetraploid *Tragopogon mirus* (Compositae): rDNA evidence. Syst Bot 16:487–493. https://doi.org/10.2307/2419333
Stech M, Veldman S, Larrain J, Munoz J, Quandt D, Hassel K, Kruijer H (2013) Molecular species delimitation in the *Racomitrium canescens* complex (Grimmiaceae) and implications for DNA barcoding of species complexes in mosses. PLoS ONE 8:e53134. https://doi.org/10.1371/journal.pone0053134
Suter M, Schneller JJ, Vogel JC (2000) Investigations into the genetic variation, population structure, and breeding systems of the fern *Asplenium trichomanes* subsp. *quadrivalens*. Int J Pl Sci 161:233–244. https://doi.org/10.1086/314258
Swofford DL (2002) PAUP*: Phylogenetic analysis using parsimony (* and other methods). version. 4.0b10, Sinauer, Sunderland
Tigerschiold E (1981) The *Asplenium trichomanes* complex in East Central Sweden. Nordic J Bot 1:12–16. https://doi.org/10.1111/j.1756-1051/1981.tb01027.x
Tindale MD, Roy SK (2002) A cytotaxonomic survey of the Pteridophyta of Australia. Austral Syst Bot 15:839–937. https://doi.org/10.1071/SB0003
Trewick SA, Morgan-Richards M, Russell SJ, Henderson S, Rumsey FJ, Pinter I, Barrett JA, Gibby M, Vogel JC (2002) Polyploidy, phylogeography and Pleistocene refugia of the rockfern *Asplenium ceterach*: evidence from chloroplast DNA. Molec Ecol 11:2003–2012. https://doi.org/10.1046/j.1365-294X.2002.01583
Tutin TG, Heywood VH, Burges NA, Valentine DH, Walter SM, Webb DA (1993) Flora Europaea, vol. 1, 2nd edn. Lycopodiaceae to Plantaginaceae. Cambridge University Press, Cambridge
Valentini A, Pompanon F, Taberlet P (2009) DNA barcoding for ecologists. Trends Ecol Evol 24:110–117. https://doi.org/10.1016/j.tree.2008.09.011
van Velzen R, Weitschek E, Felici G, Bakker FT (2012) DNA barcoding of recently diverged species: relative performance of matching methods. PLoS ONE 7:e30490. https://doi.org/10.1371/journal.pone.0030490
Vogel JC, Rumsey FJ, Schneller JJ, Russell SJ, Holmes JS, Barrett JA, Gibby M (1998a) The origin, status and distribution of *Asplenium presolanense* spec. nov. (Aspleniaceae, Pteridophyta). Bot Helvetica 109:269–288
Vogel JC, Russell SJ, Rumsey FJ, Barrett JA, Gibby M (1998b) Evidence for maternal transmission of chloroplast DNA in the genus *Asplenium* (Aspleniaceae, Pteridophyta). Pl Biol 111:247–249. https://doi.org/10.1111/j.1438-8677.1998.tb00704
Vogel JC, Rumsey FJ, Russell SJ, Cox CJ, Holmes JS, Bujnoch W, Stark C, Barrett JA, Gibby M (1999) Genetic structure, reproductive biology and ecology of isolated populations of *Asplenium csikii* (Aspleniaceae, Pteridophyta). Heredity 83:604–612. https://doi.org/10.1038/sj.hdy.6886120

Wang ZR (1989) A preliminary report on the cytology of some species of *Asplenium* from China. In: Shing KH, Kramer KU (eds) Proceedings of international symposium on systematic pteridology, Science and Technology Press, Beijing, pp 133–134

Wang L, Wu ZQ, Byrstiakova N, Ansell SW, Xiang QP, Heinrichs J, Schneider H, Zhang XC (2011) Phylogeography of the Sino-Himalayan fern *Lepisorus clathratus* on "The Roof of the World". PLoS ONE 6:e25896. https://doi.org/10.1371/journal.pone.0025896

Wang L, Schneider H, Wu ZQ, He LJ, Zhang XC, Xiang QP (2012) Indehiscent sporangia enable the accumulation of local fern diversity at the Qinghai-Tibetan Plateau. BMC Evol Biol 12:158. https://doi.org/10.1186/1471-2148-12-15

Wang FH, Lu JM, Wen J, Ebihara A, Li DZ (2016) Applying DNA barcodes to identify closely related species of ferns: a case study of the Chinese *Adiantum* (Pteridaceae). PLoS ONE 11:e0160611. https://doi.org/10.1371/journal.pone.0160611

Wendel JF, Weeden NF (1989) Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS (eds) Isozymes in plant biology. Chapman and Hall, London, pp 5–45

Weng RF (1990) Cytological observations of some Chinese ferns. Acta Phytotax Sin 28:27–33

Williams E, Theis Z, Hoess C (2016) Identifying a cryptic *Adiantum population* through DNA barcoding. Amer Fern J 106:135–142. https://doi.org/10.1640/0002-8444-106.2.135.1

Wubs ERJ, de Groot GA, During HJ, Grundmann M, Bremer P, Schneider H (2010) Mixed mating system in the fern *Asplenium scolopendrium*: implications for colonization potential. Ann Bot (Oxford) 106:583–590. https://doi.org/10.1093/aob/mcq157

Zhang W, Fan XH, Zhu SF, Zhao H, Fu LZ (2013) Species-specific identification from incompletely sampling: applying DNA barcodes to monitoring invasive *Solanum* plants. PLoS ONE 8:e55927. https://doi.org/10.1371/journal.oine.0055927