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Polyploidy in *Asplenium*

Schneider et al.

Neo- and Paleopolyploidy contribute to the species diversity of *Asplenium* – the most species-rich genus of ferns

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Abstract

Polyploidy is widely considered as a major process in the evolution of plants but the accumulation of polyploid species diversity is still controversial. Some recent studies proposed increased extinction risk in neopolyploids compared with their diploid ancestors. The high proportion of polyploid ferns is expected to be formed mainly by neopolyploids, whereas paleopolyploid species are predicted to be clustered in clades founded by whole genome duplications. Here, we test this prediction by exploring the evolution of polyploidy in the derived fern family Aspleniaceae. The family has a global distribution and shows the highest frequency of polyploid taxa among all ferns. To test the hypothesis, we obtained a comprehensive phylogeny using chloroplast DNA sequences of 883 specimens representing 292 species. All published chromosome counts were mapped onto this phylogenetic framework in order to explore the evolution of polyploids. We recovered evidence for several whole genome duplications in the history of Aspleniaceae. Phylogenetic relationships of polyploids exceeding the tetraploid level suggest that tetraploid *Asplenium* species may have replaced their diploid ancestors as the main evolutionary players in some clades of this family.

Key words: chromosome number, diversification, extinction risk, genome evolution, macroevolution, neopolyploidy, paleopolyploidy.

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1 Introduction

Polyploidy has been widely recognized as a major process in the accumulation of plant diversity (e.g., Otto, 2007; Leitch & Leitch, 2013; Madlung, 2013; Wendel, 2015; Kellogg, 2016; Soltis et al., 2016). Up to 15% of angiosperm and 31% of fern speciation events have been inferred to involve polyploidy (Wood et al., 2009), and whole genome duplications are recognized as key events in the history of seed plants (Fawcett et al., 2009; Soltis & Soltis, 2009; Jiao et al., 2011; Leitch & Leitch, 2013). However, some widely accepted hypotheses emphasized the limitations of polyploids, either by interpreting them as evolutionary dead-ends (Stebbins, 1950) or by recognizing a reduced diversification rate in recently found polyploid taxa compared with their diploid relatives (Mayrose et al., 2011; Arrigo & Baker, 2012). The latter hypothesis – the extinction-risk hypothesis – was challenged by emphasizing the need to use exhaustively sampled datasets to test macroevolutionary hypotheses about polyploidy (Soltis et al., 2014; but see Mayrose et al., 2015). The high proportion of polyploidy in ferns (Wood et al., 2009) makes them especially suitable to explore macroevolutionary processes shaping the accumulation of polyploid taxa (Wood et al., 2009). Taking into account the limited evidence supporting ancient whole genome duplications in ferns (Barker, 2013; Clark et al., 2016), the majority of polyploid ferns are considered to be neopolyploids – having closely related extant diploid ancestors – so that the accumulation of polyploids is the result of a successful strategy of niche space-filling through recurrent formation of neopolyploid fern taxa (see Werth & Windham, 1990; Wood et al., 2009; Madlung, 2013; Marchant et al., 2016; Parisod & Broennimann, 2016). Accumulation of neopolyploids may reflect selective advantages carried by polyploid taxa showing new genetic combinations (Otto, 2007; Abbott et al., 2013; Hollister, 2014) or reproductive advantages displayed by polyploids in general (Soltis et al., 2007; Parisod et al., 2010). In light of the existing evidence, the evolution of polyploidy in ferns may provide a well-suited system to test

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the hypothesis of a high extinction-risk limiting the establishment of paleopolyploid lineages lacking of closely related diploid ancestors.

The interpretation of polyploidy in ferns needs to consider a range of observations supporting the hypothesis that genome evolution of ferns is distinct from that of seed plants, with polyploidy as the predicted predominant mechanism of genome size expansion (Barker, 2013; Leitch & Leitch, 2013; Wolf et al., 2015). With an average of n = 60.5 chromosomes (Clark et al., 2016), ferns show a much higher chromosome number than angiosperms, which have an average of n = 15.99 (Klekowski & Baker, 1966). Chromosome number and genome size measured as C-DNA values are strongly correlated in ferns but not in angiosperms (Bainard et al., 2011; Barker, 2013; Leitch & Leitch, 2013; Clark et al., 2016). Although mitigation of inbreeding has been considered as a major factor in the accumulation of polyploidy in ferns (Klekowski, 1973; Werth & Windham, 1990), studies on the reproductive systems of ferns have revealed that the majority of ferns reproduce preferentially as outbreeders (Haufler & Soltis, 1986). Furthermore, protein assays have suggested rapid gene silencing in polyploid ferns, but provided little evidence for genome size reduction (Barker, 2013; Leitch & Leitch, 2013). Evidence so far supports the hypothesis that polyploid fern genomes are probably diploidized despite retaining polyploid chromosome numbers (Haufler, 1987; Clark et al., 2016). This argument appears to be consistent with the relative scarcity of documented dysploid events in the phylogeny of fern lineages (Lovis, 1977; Bellefroid et al., 2010; Hennequin et al., 2010; Wang et al., 2010; Wolf et al., 2015), and the recent report of static genome size for more than 180 million years in royal ferns (Bomfleur et al., 2014; Schneider et al., 2015).

Here, we explore the accumulation of polyploidy in the evolutionary history of the derived fern family Aspleniaceae, known colloquially as spleenworts. This globally distributed

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fern family is dominated by the genus *Asplenium* L.*,* which is one of the most species-rich fern genera with approximately 700 species (Smith et al., 2006). These mostly epiphytic or saxicolous ferns occur in all tropical and temperate regions and are only absent from habitats subjected to severe water deficiency. Among derived ferns, spleenworts show the highest proportion of polyploids and the highest proportion of ploidy levels of octoploid or higher (Schneider, unpublished data). Furthermore, there is evidence for the recurrent formation of polyploid taxa either as a result of frequent reticulate evolution as, for example, in the Appalachian spleenworts (Werth et al., 1985), the New Zealand *Asplenium* complex (Shepherd et al., 2008b), the *A. monanthes* complex (Dyer et al., 2012, 2013), and the *A. normale* complex (Chang et al., 2013), or due to the reproductive advantages of the polyploids (e.g., Vogel et al., 1999; Suter et al., 2000; Hunt et al., 2011). The genus is also known for a stunning example of cryptic speciation in the mainly epiphytic *A. nidus* complex (Yatabe et al., 2001, 2009). The diversification of this clade provides further support for the hypothesis of polyploidy as an evolutionary strategy in spleenworts (Werth & Windham, 1990) because existing chromosome counts suggest that this complex is exclusively composed of polyploids (Dong, 2011).

In the present study, we test four predictions that are based on the working hypothesis that polyploids experience a higher extinction risk compared with their diploid ancestors, and establishment of paleopolyploid fern lineages is rare.

1. Polyploidy is expected to be fairly equally distributed throughout the evolutionary tree of *Asplenium*; this prediction follows assumptions that the recurrent formation of polyploids is random and that polyploids are short-lived as a result of enhanced extinction risks.

2. The majority of polyploids share their plastid DNA with diploid relatives under the predicted preponderance of neopolyploidy; alternatively, there is plastid DNA sequence variation unique to

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clades comprising exclusively polyploid species if polyploids have existed for a long time and speciated without reduction of their chromosome number.

3. Species richness and polyploidy are expected to be positively correlated; this prediction follows from the hypothesis that polyploid taxa are recurrently formed and accumulate together with their diploid ancestors.

4. The frequency of polyploids is predicted to be positively correlated with long-term stability of areas and strong niche competition, which lead to fragmentation and differentiation; this expectation follows from the hypothesis of high extinction risks of polyploids compared with their diploid relatives.

2 Material and Methods

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2.1 Reconstructing the phylogenetic framework

A phylogeny was reconstructed by taking advantage of a large range of studies focusing either on the global phylogeny of Aspleniaceae (Murakami et al., 1999; Schneider et al., 2004, 2005; Leroux et al., 2011) or particular species and species complexes (e.g., Murakami, 1995; Schulze et al., 2001; Pinter et al., 2002; Perrie & Brownsey, 2005; Li & Lu, 2006; Shepherd et al., 2008a, 2008b; Bellefroid et al., 2010; Perrie et al., 2010; Dyer et al., 2012, 2013; Schneider et al., 2012; Chang et al., 2013; Ohlsen et al., 2014; Loriga et al., 2017). The majority of these studies used the DNA sequences of *rbcL*, the chloroplast coded large subunit of the RUBISCO enzyme. We downloaded all *rbcL* sequences of this family available from GenBank (www.ncbi/nlm.nih.gov) in March 2014. In addition, we generated new sequences by using previously described protocols to extract DNA and amplify the selected gene using polymerase chain reactions and automated sequencing (e.g., Murakami et al., 1999; Ohlsen & Field, 2013). Newly obtained DNA sequences

were assembled, carefully edited using standard software, and submitted to GenBank (Table S1). New sequences contributed more than one-third of the taxonomic coverage of this study. All available sequences were assembled into a single alignment using Mesquite 2.75 (Maddison & Maddison, 2012). The alignment was adjusted manually and the *rbcL* sequence of the whole plastid genome of *Adiantum capillus-veneris* L. (AY178864; Wolf et al., 2003) was used to adjust the reading frame. Both nucleotide and protein alignments were used to assess reading errors in sequences obtained from GenBank. *Hemidictyum marginatum* (L.) C. Presl. was selected as outgroup based on accepted phylogenetic hypotheses (e.g., Schuettpelz & Pryer, 2007; PPG1, 2016). The phylogeny was reconstructed using maximum likelihood, as implemented in RAxML 8 (Stamatakis, 2014), and Bayesian inference of phylogeny, as implemented in MrBayes 3.2 (Ronquist et al., 2012). Models of sequence evolution were determined using jModelTest 2 (Darriba et al., 2012). The output of the Bayesian analyses was explored using TRACER version 1.6 (Rambaut & Drummond, 2013), and the trees of all analyses were visualized using FigTree version 1.4 (Rambaut, 2012). The initial dataset included sequences from 913 specimens, of which 549 were downloaded from GenBank and 364 were newly generated. In total, we excluded 21 sequences from those obtained from GenBank because of detected problems (e.g., species misidentifications and short sequences) or when sequences were from F1 hybrids. The final analyses were carried out with sequences from 883 specimens representing approximately 292 species (for further information, see Table S1).

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2.2 Taxonomic database and geographical distribution

In the absence of a modern revision of *Asplenium*, we compiled a checklist of currently recognized species of Aspleniaceae based on recent floristic treatments such as those for China

(Lin & Viane, 2013), Mexico (Mickel & Smith, 2004), and the Afromadagascan region (Roux, 2009). This list comprised 720 currently accepted species (Table S2). Information about chromosome numbers and geographical distribution were obtained from the abovementioned sources, online databases such as Tropicos (www.tropicos.org), and specialized reports (Tindale & Roy, 2002). Based on documented occurrences, these species were assigned to six biogeographic regions – Neotropics (i.e., South America, Central America, and Caribbean), Afromadagascar (i.e., Africa and Madagascar), North America, Europe, Asian + Malesia, and Australasia + Pacific – and two climatic regions. These two regions were defined as tropical and non-tropical based on the occurrences inside or outside the tropical region demarcated by the Tropics of Cancer and Capricorn.

2.3 Exploring the distribution of polyploidy

The distribution of polyploidy in the dataset was explored by using both phylogeny-independent and phylogeny-dependent statistics. Phylogeny-independent analyses were carried out using standard statistics software, whereas phylogenetic comparative analyses were carried out using phylogenetic-independent contrasts (Felsenstein, 1985) as implemented in the PDAP:PDTREE 1.16 module (Midford et al., 2011) that is executed by Mesquite. These analyses were calculated considering the reconstructed phylogenetic hypothesis. We compared the phylogenetic distribution of four parameters (overall species-richness, species-richness in tropical and non-tropical climate zones, proportion of polyploids, and proportion of polyploids with $x > 4$) among the 10 main clades identified. In total, chromosome counts were available for 54% of the included taxa and 72% of the total of 236 spleenwort species with known chromosome counts were included. It is also important to note that, for some species, multiple ploidy levels were reported. Some currently accepted species contain more than one karyotype, such as diploids and

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tetraploids. Each karyotype was treated as an independent species in statistical analyses requiring species number estimates. For example, we recognized the diploid *A. caucasicum* (Fraser-Jenk. & Lovis) Viane (=*A. septentrionale* (L.) subsp. *caucasicum* Fraser-Jenk. & Lovis) as a separate species from the tetraploid *A. septentrionale* (L.) Hoffm. (=*A. septentrionale* subsp. *septentrionale*). In other cases, such as *A. abscissum* Willd., the taxonomy is not available to address the issue so elegantly. Three different counts were reported for this species, namely $2n$ = 72, $2n$ = 144, and $2n$ = 288 (Mickel & Smith, 2004), and thus we assumed for purposes of our analysis that these counts correspond to at least three species sharing a highly similar morphology. Throughout the paper, the number of chromosomes per haploid gametic chromosome set (n) is considered as 35, 36, 39, or 40, that is, four different base chromosome numbers (x) for the family (Smith et al., 2006). Polyploidy levels were estimated based on these base chromosome numbers (x). The variation of base chromosome numbers x = 35, 36, 39, 40) was considered as the result of dysploidy with the most common base chromosome number x = 36 considered as the plesiomorphic character state. Throughout the manuscript, allo- and autopolyploids are not distinguished because their differentiation is difficult to apply across the sampling. Some polyploids may appear as autopolyploids because of the lack of taxonomic revision work enabling the recognition of distinct diploid species with highly similar morphology contributing to allotetraploids (Doyle & Sherman-Broyles, 2016). The formation of polyploids involving closely related species may be considered to result in the establishment of segmental allopolyploids which are than considered intermediates in a continuous spectrum ranging from autopolyploidy to allopolyploidy (Shinohara et al., 2010). Given the quality of the taxonomic treatments and the extent of biosystematic studies available, it appears premature to include the genus *Asplenium* in meta-analyses aiming to estimate relative abundance of auto- and

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allopolyploids as aimed in Barker et al. (2016). Furthermore, species numbers of polyploids are arguably inflated because of recurrent origins of karyological and morphological highly similar species with shared parental diploid taxa (Werth et al., 1985).

To study the evolution of chromosome numbers, we used the methods available in Mesquite for ancestral character state reconstruction (squared-change parsimony for continuous characters) and the software package ChromEvol 2.0, which was specifically designed to reconstruct the evolution of chromosome numbers (Glick & Mayrose, 2014). The first approach enables the independent reconstruction of the evolution of base chromosome number and polyploidy, whereas ChromEvol was used to explore models that considered integration of polyploidy and chromosome number evolution. Phylogenetic uncertainty was taken into account by calculating the phylogenetic contrasts for 100 trees that were randomly selected from the plateau phase of the Bayesian analysis. Evolutionary distinctiveness of polyploids was measured by identifying the proportion of species with unique plastid DNA compared with the proportion of species sharing the same plastid DNA. Furthermore, we identified the proportion of plastid DNA shared between diploids only, polyploids only, and both diploids and polyploids. Changes of the base chromosome number (e.g., replacement of $x = 36$ by $x = 35$) were considered as dysploid events because we have no evidence to support an interpretation of aneuploidy, which would assume a correlation of chromosome number change and reduction of genome size. In all analyses, we considered reports of $2n = 72$ or in some cases $2n = 70$, 78, or 80 as the diploid state throughout the spleenworts phylogeny. This interpretation is consistent with the assumption of a higher frequency of polyploidization events compared with reduction of the chromosome number through chromosome fusion. This assumption is consistent with the reconstructions of the chromosome evolution in derived ferns (Schneider, unpublished data).

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3 Results

3.1 Phylogenetic framework

The recovered phylogenetic hypothesis resembles previously published results, even though the number of sampled species has been increased by more than 50% compared with previous analyses. Based on our checklist of 720 species, our sampling represents 41% of the total species diversity of spleenworts. Taking into account the estimated number of species per geographical region, the Neotropical region is underrepresented in the phylogenetic sampling, with 26% contribution to the sampled species diversity versus 33% contribution to the global checklist. The temperate regions of Europe, North America, and Australasia-Pacific are overrepresented, with 11% contribution to the species sampled compared with 6% according to the global checklist, 4% versus 2%, and 25% versus 13%, respectively (Table 1). The phylogeny recovered 10 main clades (Figs. 1, S1) that were having deep nodes with high posterior confidence values ($P \geq 0.95$). These 10 clades – *Aegeum* (AE), *Asplenium* s.s. (AS), *Auritum* (AU), *Bullatum* (BU), *Camptosorus* (CA), *Hymenasplenium* (HY), *Neottopteris* (NE), *Phyllitis* (PH), *Pleurosorus* (PL), and *Tarachia* (TA) – differ considerably in species richness and geographic range (Table 1). Two of them (AU and HY) have relatively long branches and form the sister clades to a core clade with very short basal branches (Fig. 1). Some main clades such as the *Auritum* clade (AU in Fig. 1) are further subdivided into two subclades, and the most species-rich main clade, the *Neottopteris* clade (NE), comprises seven subclades. The *Aegeum* clade (AE) is the smallest clade and is likely sister to the *Asplenium* s.s. clade (AS). The *Phyllitis* (PH) and *Pleurosorus* clades (PL) are found to be sister, but the posterior confidence value is $P < 0.95$.

3.2 Biogeographic distribution of Aspleniaceae diversity

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Some 82% of *Asplenium* species occur exclusively in tropical regions whereas approximately 17% of the species are restricted to non-tropical climatic zones (Table 1). Several clades occur predominately in non-tropical zones: *Aegeum* (AE in Fig. 1), *Camptosorus* (CA), *Phyllitis* (PH), and *Pleurosorus* (PL). The *Bullatum* clade (BU in Fig. 1) occurs mainly in warm temperate to tropical climates of SE Asia. These clades are all rather species-poor, with the exception of the *Camptosorus* clade (CA in Figs. 1, S1), the only species-rich clade that shows a preference for non-tropical climatic zones (63%). The *Camptosorus* clade contributes 48% of the European *Asplenium* species flora and 55% of the North American *Asplenium* species (Table 1). Four clades, including the species-rich *Neottopteris* and *Tarachia* clades (NE and TA, respectively, Fig. 1), occur mainly in the tropical climate zone. The tropical clades show distinct preferences in their geographic distribution, with the *Asplenium* s.s. and *Auritum* clades (AS and AU, respectively, Fig. 1), contributing 46% of the total Neotropical spleenwort species diversity. The *Neottopteris* and *Tarachia* clades (NE and TA, respectively, Fig. 1) occur primarily in the Paleotropics, contributing 33% and 20%, respectively, to the Afromadagascar spleenwort species diversity, as well as 49% and 26% to the Australasian-Pacific spleenwort species diversity. Both clades have a strong presence (23%) in the Asian-Malesian region (Table 1), where they are only second to *Camptosorus* (CA) with 28%. The *Hymenasplenium* clade (HY in Fig. 1) shows a similar division into a derived Paleotropical clade and a Neotropical basal grade (Fig. 1). The *Camptosorus* clade comprises several species complexes with a tropical preference.

3.3 Distribution of polyploidy in Aspleniaceae

Nearly half (49%) of the sampled species are tetraploid, exceeding the diploids, which constitute only 25%. Higher ploidy levels contribute the remaining 26% of cytotaxonomic diversity (Table 2). The frequency of diploids is lower in tropical regions (19%) compared with non-tropical

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regions (35%), but both climate zones show a similar proportion of tetraploids (52% and 49%, respectively), due to the higher contribution of high-level polyploids in the tropics (29% vs. 16%). Differences are also found among geographic regions. The highest proportion of diploids are in Europe (47%) and North America (35%), whereas the highest proportion of taxa with ploidy levels above 4x are found in the Neotropics (48%) and in the Australasia + Pacific region (37%). All regions show rather similar proportions of tetraploids, ranging from 43% in the Neotropics to 59% in Afromadagascar. The most distinct pattern is found in the distribution of ploidy across the phylogeny. Diploids constitute half or more of the diversity in the *Aegeum* (50%), *Asplenium* s.s. (61%), *Camptosorus* (49%), *Hymenasplenium* (62%), and *Pleurosorus* clades (67%). No diploids are documented in the *Auritum*, *Bullatum*, or *Tarachia* clades (Table 2), and diploids contribute less than 2% to the diversity of the *Neottopteris* clade.

ChromEvol analyses supported a model explaining the evolution of chromosome number mainly as a result of whole genome duplications and rare dysploidy events. The analyses supported n = 72 (4x) as the ancestral chromosome number for the three clades with no diploids and the one clade with only a single diploid (black ovals, Fig. 1), whereas clades with a considerable proportion of diploids were found to have an ancestral chromosome number of n = 36 (2x). The *Tarachia* clade showed the highest proportion (55%) of taxa higher than 4x. The proportion of tetraploids ranged from 31% in the *Asplenium* s.s. (AS, Fig. 1) clade to 69% in the *Neottopteris* clade. Approximately 26% of unique plastid DNA is restricted to diploid taxa, 23% is shared between diploids and polyploids, and 51% of the plastid DNA is found exclusively in polyploids (Table 3).

4 Discussion

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We found no evidence supporting the hypothesis that polyploid spleenwort species are mainly neopolyploids. Instead, our results suggest that paleopolyploid clades contribute to the accumulation of spleenwort species diversity. Contrary to our prediction 1, polyploidy was not evenly distributed across the phylogeny of spleenworts, with some clades showing little or no evidence for diploids $n = 36$ ($2x$). The existence of clades with an ancestral chromosome number of $n = 72$ ($4x$) is supported by inference of chromosome number evolution using maximum parsimony and the likelihood model used in ChromEvol. Four main clades, *Auritum*, *Bullatum*, *Neottopteris*, and *Tarachia*, arose from tetraploid ancestors ($n = 72$), suggesting the occurrence of at least three whole genome duplication events (WGD) coinciding with the foundation of new lineages of spleenworts (black ovals, Fig. 1). As well as WGDs associated with main clades, additional WGDs are found in the *Camptosorus* clade (two WGD) and the *Phyllitis* clade (one WGD). Instead of a single WGD, the *Neottopteris* clade may have evolved through several of these events as indicated by the occurrence of rare diploids. The ancestors of these putative paleopolyploid clades are characterized by a duplication of the genome resulting in tetraploid chromosome numbers. The newly formed taxa behave as diploids despite the duplication of the chromosome numbers. This hypothesis is consistent with the observation that the proportional distribution of tetraploids versus higher polyploids in these putative paleotetraploid lineages resembles the proportional distribution of diploids versus polyploids in lineages including taxa with $2n = 72$ (Table 2, Fig. 1).

The recognition of paleopolyploid lineages is possibly affected by incomplete taxon sampling, with less than half (approximately 40%) of the global spleenwort species diversity included in the phylogenetic hypothesis (Table 1) and with chromosome numbers available for only about 36% of the spleenwort species. In fact, only 23% of the investigated species had both

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rbcL sequence information and chromosome counts. Thus, the absence/rarity of diploids in four main clades may result from low sampling density. Support for this possibility is provided by the observation of a small fraction of diploids in the *Neottopteris* clade. This clade contains a single confirmed diploid, *Asplenium surrogatum* P. S. Green (Tindale & Roy, 2002), which is nested in a well-studied South Pacific *Asplenium* clade (Fig. S1). The phylogenetic position of this diploid taxon within a polyploid clade requires either the proposal of reverse polyploidy (Perrie & Brownsey, 2005) or alternatively the recurrent formation of paleopolyploidy. The other putative diploid, *A. griffithianum* Hook., nested in the *Neottopteris* clade. The reported diploid level requires further confirmation because some authors report this taxon as polyploid in part of its range (Lin & Viane, 2013). In contrast, the identification of other paleopolyploid clades may not be affected by sampling density and probability of overlooked diploid counts. All species of the *Bullatum* clade (BU in Fig. 1) were repeatedly reported as polyploids with *A. bullatum* Wall. having $2n = 144$ and $2n = 288$, *A. ritoense* Hayata having $2n = 144$, and *A. wrightii* D. C. Eaton ex Hook. having $2n = 144$ and $2n = 288$ (Table 2).

No convincing case of reverse polyploidy has been documented for asplenioid ferns (Perrie & Brownsey, 2005). In this context, it is worth noting that only one case of dysploidy has been well documented in spleenworts so far, namely for the tetraploid *Loxoscaphe* complex (Bellefroid et al., 2010). This complex is nested in the *Neottopteris* clade and shows the replacement of $x = 36$ by $x = 35$. The base chromosome number also varies in the *Hymenasplenium* clade. This clade comprises a core subclade composed exclusively of paleotropical species. These taxa have $x = 39$ or $x = 40$ (Murakami, 1995). However, very little is known about the chromosome numbers of the Neotropical species belonging to this clade. At least some Neotropical species of *Hymenasplenium* have counts based on $x = 36$ or $x = 39$ and

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the Neotropical species form a grade at the base of the *Hymenasplenium* clade (see Cheng & Murakami, 1998; Regalado & Prada, 2011). Thus, $x = 36$ was recovered as the putative ancestral character state of Aspleniaceae, but the lack of robustness of the deeper relationships in the *Hymenasplenium* clade does not allow rejection of the alternative hypothesis that $x = 39$ is the ancestral base chromosome number of the family. Finally, a further instance of dysploidy may occur in the *A. serra* clade (SER, Fig. 1); the African species, *A. friesiorum* C. Chr., was recorded as $2n$ = ca. 140 (Manton, 1959), but this count needs further investigation because related species such as *A. serra* Langsd. & Fisch. were reported as $2n = 144$ (Jarrett et al., 1968) and $2n$ = ca. 288 (Walker, 1985).

Our results support the hypothesis of recurrent establishment of polyploid taxa as long as the tetraploid state is not considered as the ancestral character state in spleenworts. Indeed, basal nodes were reconstructed to be polyploid and not diploid when an equal rate transition between $n = 36$ and $n = 72$ was assumed. This result may be caused either by non-sampling of diploid representatives and/or the occurrence of paleopolyploid lineages with conserved $n = 72$. Considering the four putative paleopolyploid main clades as functional diploids, as suggested by the ChromEvol analyses, polyploids are increasingly restricted to terminal nodes with the exception of a few clades that lacked diploids in our sampling, such as the *Asplenium cordatum* complex (COR, Fig. 1). This complex of Afromadagascan species is nested in the *Camptosorus* clade, and our sampling did not include diploid representatives of this complex such as *A. capense* (Kunze) Bir. Fraser-Jenk. & Lovis (Bir et al., 1985). This is one of the few examples in which species with known chromosome counts were not represented in the reconstructed phylogeny using DNA sequence data. However, some of these polyploid lineages comprise species reported only as tetraploids to octoploids such as the *A. radicans* complex, which is

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nested in the *Camptosorus* clade (Fig. S1) or the *A. aureum* complex in the *Ceterach* subclade of *Phyllitis* (Fig. S1). These complexes are considered to represent paleopolyploids in the ChromEvol analyses. The majority of the remaining polyploid species shares plastid DNA with diploid relatives and such species are interpreted as neopolyploids. Of course, these results are based on the assumption of $2n$ = 72 as the ancestral chromosome number in the sporophytic generation because the alternative of $2n$ = 144 as the ancestral chromosome number requires repeated reduction of the chromosome number from $2n$ = 144 to $2n$ = 72 through chromosome fusion and deletion.

To identify recently established neopolyploids, we adopted the criterion that neopolyploids share their plastid DNA variation with the maternal diploid ancestor (Vogel et al., 1998). Without considering the above-discussed interpretation of some clades as paleopolyploid lineages, the proportion of neopolyploids is approximately 23% (Table 3), but increases to 55% with acceptance of four paleopolyploid lineages. With this correction, the proportion of neopolyploids is lowest in the *Asplenium* (27%) and *Camptosorus* clades (32%), whereas the four clades interpreted as paleopolyploids show percentages of neopolyploids above or close to 55%, namely *Auritum* (67%), *Bullatum* (67%), *Neottopteris* (48%), and *Tarachia* (67%). The highest frequencies of neopolyploids are found in the species-poor *Aegeum* (100%) and *Hymenasplenium* clades (71%). In the latter, the majority of neopolyploids are recorded in China and Japan. The proportion of neopolyploids is consistent with prediction 2, as long as the paleotetraploid lineages with $2n$ = 144 are identified. However, future research may imply a more continuous criterion using genetic distances between polyploids and their diploid relatives.

These data need to be interpreted with care because the small sample size of some clades makes the statistics prone to sampling bias. As discussed earlier, the observed pattern may be

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affected by the better known cytology of temperate spleenworts compared with their tropical relatives (Table 1). The phylogeny is likely also affected by the lack of information on the ploidy levels of collected species, and thus some sequences may be incorrectly treated as unique to diploid or polyploid taxa. Furthermore, our study is based solely on maternally inherited plastid DNA and thus the contribution of both the parents was not inferred in this study. This lack of evidence prevents us from inferring the proportion of allotetraploids among the neopolyploids. Furthermore, some taxa recovered as not contributing to the formation of allopolyploids may actually contribute as the paternal parent. Finally, the argument used to define neopolyploidy may be judged as conservative. Thus, we explored the pattern by taking into account the criterion that neopolyploidy was rejected for those polyploids with a sister species with the same ploidy level. The paleopolyploidy interpretation found further support by the observation of allooctoploid taxa formed between species with $2n = 144$ in New Zealand (Perrie & Brownsey, 2005; Shepherd et al., 2008b), indicating these tetraploids are outcrossing. In comparison, neopolyploids such as *A. quadrivalens* (D. E. Mey.) Landolt (Suter et al., 2000) and *A. ruta-muraria* L. (Schneller, 1996) reproduce preferably by obligate inbreeding, preventing the formation of allopolyploids among neopolyploid taxa.

As discussed above, the interpretation of polyploidy based on $x = 36$ for all clades or on $x = 72$ for some main clades had a considerable impact of the interpretation of polyploids as mainly neopolyploids or some paleopolyploids. We did not find a correlation between species richness and polyploidy in phylogeny-dependent statistics as long as $x = 36$ was considered to be the base chromosome number of all clades (SPR-POL, Table 4). Thus, our analyses do not support a correlation of the accumulation of diploids and neopolyploids (prediction 3). However, species richness and polyploidy are correlated if four main clades are interpreted as

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paleotetraploids with $2n = 144$ (Table 4). Interpreting the paleotetraploid lineages as functional diploids allowed identification of the proportion of neopolyploid taxa also within these four clades. Thus, the recovered significant correlation provided further evidence for paleopolyploidy in spleenworts (Table 4).

Our data do not provide unequivocal support for a higher proportion of polyploidy in tropical climatic zones compared with non-tropical climatic zones (prediction 4). Assuming climatic niche conservatism, Pleistocene climatic fluctuations may have had different effects on spleenworts growing in tropical climatic regions and temperate climatic regions, with the latter having more dramatic range shifts, and thus enhancing extinction risk. The higher “extinction-risk” hypothesis (Mayrose et al., 2011) implies an expected higher rate of polyploids in tropical regions (more stable regions) compared with non-tropical regions (less stable regions). Some evidence supporting this expectation was found by a marginally significant correlation of tropical occurrence and polyploids with ploidy levels exceeding tetraploidy (Table 4). However, these analyses may need to take into account the frequency of neo- and paleopolyploidy as well as the total number of polyploid taxa. The obtained results may suggest a correlation between climatic stability and the establishment of paleopolyploid lineages, which requires further investigation. For example, taxonomic sampling may have biased the statistics because of the much better coverage of temperate spleenworts. No correlation of species richness and the occurrence in tropical regions was found among the 10 main clades (Table 4), but these data may have been biased by the more exhaustive data available for temperate climatic zones, for example, Europe, New Zealand, and North America, and the often limited amount of data available for tropical regions such as northern South America and Malesia.

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The documented evidence might also biased by the influence of polyploidy on species recognition. In well-studied regions like Europe, the density of chromosome counts and related data allows us to segregate species distinguished by karyotype despite overlapping morphology. For example, the mainly north-temperate *Asplenium trichomanes* complex comprises at least four diploid and six tetraploid species (Schneider et al., unpublished data). However, comparatively little is known for the majority of tropical species. For these taxa, we relied mainly on species concepts based on morphology and thus may have underestimated species diversity. This argument is supported by the observations on three species complexes nested in the putative paleopolyploid *Neottopteris* (NA) clade. Cryptic speciation was documented in the tetraploid *A. nidus* complex (Yatabe et al., 2001, 2009) and karyological variation was found in morphologically highly variable species such as *A. affine* Sw. (Manton & Sledge, 1954; Sledge, 1962). The polyploid *A. polyodon* G. Forst. was found to be polyphyletic, which is consistent with recent studies on the nomenclature of these ferns (Salgado & Fraser-Jenkins, 2013). In turn, this discovery may support the notion that speciation processes may be different in these lineages with $x = 72$ compared to lineages conserving the ancestral $x = 36$. Future studies on these ferns need to incorporate karyological evidence as well as morphology and DNA sequence variation.

5 Conclusions

The results did not provide unambiguous support for the prediction of recurrent formation of neopolyploidy as the main process of polyploidy accumulation in spleenworts. Instead, evidence for paleopolyploidy was found for four of the 10 main clades of spleenworts. Thus, our results are in conflict with predictions 1 to 3 as long as paleopolyploid lineages were not taken into consideration. However, predictions 2 and 3 were supported with four main clades accepted as

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paleotetraploids. By recovering evidence for recurrent formation of paleopolyploid lineages, our results did not confirm the expectation of a high extinction risk of polyploids compared to the diploid relatives. Species richness and polyploidy were correlated only with the correction for paleopolyploidy. Finally, no evidence was found for a higher rate of polyploidy in areas less affected by Pleistocene climatic fluctuations, although the establishment of paleopolyploidy may be influenced by climatic stability.

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Figure Legend

Fig. **1.** Phylogenetic hypothesis shown as the Bayesian consensus phylogeny and plotted as circular tree representation. Each terminal branch corresponds to a single specimen. Main clades are indicated by two letter abbreviations: AE, *Aegeum*; AS, *Asplenium* s.s.; AU, *Auritum*; BU, *Bullatum*; CA, *Camptosorus*; HE, *Hemidictyum*; HY, *Hymenasplenium*; NE, *Neottopteris*; PH, *Phyllitis*; PL, *Pleurosorus*; TA, *Tarachia*. Branch color indicates the climatic preferences of clades: bright blue, Southern Hemisphere non-tropical; dark blue, Northern Hemisphere non-tropical; purple, tropical to warm temperate; red, tropical. Black ovals, whole genome duplications detected at the stem of main clades AU, BU, NE, and TA; orange stars, apomixis (=apogamy). Donuts visualize the proportion of species contributed (dark green) for each main clade compared to the diversity contributed by other clades (bright green). Pie charts visualize the proportion of ploidy per main clade with colors corresponding to ploidy levels as shown in the pie chart in the center of the figure.

Table 1 Summary of the taxonomic and geographic coverage of *Asplenium*

	NR	Total	AFM	NTR	NAM	EUR	ASM	AAP	nTRO	TRO
CH	720		25	33	2	6	34	13	17	82
SA	292	41	27	**26**	4	**11**	35	**25**	31	69
AE	2	0.7	0	0	0	**6**	0	0	100	0
AS	27	9	6	**30**	9	3	2	4	4	96
AU	13	4	1	16	0	0	1	0	0	100
BU	3	1	0	0	0	0	3	0	?	?
CA	74	25	25	28	**55**	**48**	28	14	63	34
HY	15	5	4	4	0	0	**11**	5	0	100
NE	82	28	**33**	6	0	0	23	**49**	26	74

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PH	12	4	5	3	**9**	**24**	4	0	100	0
PL	10	3	5	3	27	**18**	4	1	100	0
TA	54	18	20	10	0	0	23	**26**	0	100

Column NR gives the number of species as absolute number whereas all other columns give percentages (%). NR shows the number of species in the checklist (CH), in the assembled dataset (SA), and in the 10 main clades recognized in the recovered phylogeny: AE, *Aegeum*; AS, *Asplenium* s.s.; AU, *Auritum*; BU, *Bullatum*; CA, *Camptosorus*; HY, *Hymenasplenium*; NE, *Neottopteris*; PH, *Phyllitis*; PL, *Pleurosorus*; TA, *Tarachia*. The column “Total” gives the percentage of species sampled of those in the checklist for CH and SA, whereas the remaining values are given as the proportion of species per clade versus the number of species sampled (SA). The remaining columns show the percentage of species for the six geographical regions: AAP, Australasia plus Pacific; AFM, Afromadagascar; ASM, Asia plus Malesia; EUR, Europe including Azores, Madeira, and Macaronesia; NAM, North America (without Florida); NTR, Neotropics; and two climatic regions (NTRO, occurrence principally outside the tropical climate zone; TRO, occurrence principally inside the tropical climate zone). Bold print is used to mark percentages that are above expectation. The expectations are for the row SA given in the row CH and for all other rows in row SA. ?, these values can not be calculated for the members of this clade with our current knowledge on their ecological preferences.

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Table 2 Distribution of polyploidy in Aspleniaceae according to phylogeny and biogeography

	SCC	2*x*	4*x*	6*x*	8*x*	>8*x*	APO

SA	57	25	49	3	20	2	3.4
AE	**100**	**50**	**50**	0	0	0	0
AS	41	**61**	31	0	8	0	**11**
AU	38	0	**60**	0	**40**	0	0
BU	**100**	0	**60**	0	**40**	0	0
CA	**71**	**49**	44	3	2	2	**33**
HY	**87**	**62**	37	0	0	0	**33**
NE	35	2	**69**	2	18	**8**	**11**
PH	**100**	**35**	35	**27**	3	0	0
PL	**80**	**67**	33	0	0	0	0
TA	43	0	45	0	**42**	**13**	**11**
AFM	21	**29**	**59**	0	9	3	**7**
NTR	15	**31**	43	**4**	**20**	2	**36**
NAM	6	**35**	**58**	0	6	0	**21**
EUR	13	**47**	47	**2**	2	0	0
ASM	30	23	**56**	**3**	11	**6**	**28**

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AAP	14	17	46	**4**	**25**	**8**	**7**
tro	22	19	**52**	4	19	6	_
ntro	**82**	**35**	49	4	11	1	_

All values are given as percentages. SA is given as the percentage of species sampled versus species with known chromosome counts; the remaining rows show distribution of ploidy according to main clades recognized (AE, *Aegeum*; AS, *Asplenium* s.s.; AU, *Auritum*; BU, *Bullatum*; CA, *Camptosorus*; HY, *Hymenasplenium*; NE, *Neottopteris*; PH, *Phyllitis*; PL, *Pleurosorus*; TA, *Tarachia*) and the distribution according to biogeographic regions (AAP, Australasia plus Pacific; AFM, Afromadagascar; ASM, Asia plus Malesia; EUR, Europe including Azores, Madeira, and Macaronesia; NAM, North America (without Florida); NTR, Neotropics). Column SCC shows species with chromosome counts; subsequent columns report the fraction of diploids (2*x*), tetraploids (4*x*), hexaploids (6*x*), octoploids (8*x*), and higher ploidy levels (>8*x*). Row SA shows the proportion in all counts available, whereas the remaining rows show the proportion per clade. Column APO shows the proportion of apomixis reported versus all species with chromosome counts, respectively, per main clade. The rows “trop” and “ntrop” show the proportion of species with chromosome counts and ploidy levels occurring preferably in tropical climate zones (tro) or outside of the tropics (ntro). We note that the proportion of species with chromosome counts is much higher in non-tropical taxa versus tropical taxa. Boldface indicates percentages for clades that exceed the average expectation shown in row SA.

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Table 3 Species differentiation and ploidy measured by plastid DNA differentiation

	DiUn	DiCo	DiPo	PoUn	PoCo
AE	0	0	2	0	0
AS	2	4	3	2	0
AU	0	0	0	1	2
BU	0	0	0	1	2
CA	11	3	8	1	2
HY	2	0	5	0	0
NE	1	0	0	11	12
PH	2	0	2	0	1
PL	2	0	4	0	3
TA	0	0	0	5	10
%	19	7	24	20	31

Statistics take into account only species with both chromosome count and plastid DNA sequences available. Those *rbcL* sequences differing by less than 1% sequence difference were considered to be identical. DiCo, plastid DNA shared by at least two diploid species; DiPo, plastid DNA is shared by diploids and polyploids; DiUn, plastid DNA found in a single diploid species; PoCo, plastid DNA shared by several polyploid species; PoUN, plastid DNA found in a single polyploid species. Main clades recognized: AE, *Aegeum*; AS, *Asplenium* s.s.; AU, *Auritum*; BU, *Bullatum*; CA, *Camptosorus*; HY, *Hymenasplenium*; NE, *Neottopteris*; PH, *Phyllitis*; PL, *Pleurosorus*; TA, *Tarachia*.

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Table 4 Results of the phylogenetically independent contrast analyses addressing pairwise correlation of the following parameters in the evolution of spleenworts

	r(o)	p(o)	r(c)	p(c)
SPR-TRO	0.489 (0.326–0.602)	0.071 (0.025–0.163)	NA	NA
SPR-POL	0.678 (0.606–0.775)	0.012^{*} (0.002–0.122)	0.913 (0.913–0.945)	$5.5e\text{-}5^{***}$ ($5.5e^{-6}$–$1.1e^{-4}$)
SPR-POL > 4	0.423 (0.029–0.569)	0.084 (0.019–0.135)	0.473 (0.315–0.652)	0.084 (0.015–0.173)
TRO-POL	0.356 (0.047–0.618)	0.184 (0.021–0.446)	0.587 (0.508–0.702)	0.032^{*} (0.008–0.055)
TRO-POL > 4	0.741 (0.616–0.827)	0.008^{**} ($8.4e^{-4}$–0.022)	0.302 (0.149–0.450)	0.193 (0.082–0.331)
POL-POL > 4	0.558 (0.360–0.731)	0.068 (0.005–0.16)	0.664 (0.582–0.737)	0.015^{*} (0.005–0.03)

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Four main clades may be considered as paleotetraploids or may be considered as diploids in their phylogenetic contribution. To integrate this, the tests were also carried out with the adjusted

distribution of polyploidy. To distinguish the test statistics, r(o) and p(o) correspond to the original observations, r(c) and p(c) to the transformed observations. To incorporate phylogenetic uncertainty, the calculations were carried out for 100 phylogenetic hypotheses obtained from the results of the Bayesian analyses. *R*-values and *P*-values of the test statistics are given with mean value followed by minimum and maximum value in parenthesis. *$P < 0.05$ for mean *P*-value but not maximum *P*-value; **$P < 0.01$ for mean *P*-value but not maximum *P*-value; ***$P < 0.001$ for mean *P*-value as well as maximum *P*-value obtained. NA, not applicable; POL, number of polyploid taxa; POL > 4, number of taxa with ploidy level exceeding tetraploid such as hexaploid and octoploid; SPR, species richness; TRO, number of species occurring in the tropics.

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Supplementary Material

The following supplementary material is available online for this article at **http://onlinelibrary.wiley.com/doi/10.1111/jse.12271/suppinfo**:

Table S1. List of included specimens with species names, clade assignments as shown in Fig. S1, and GenBank accession numbers.

Table S2. Checklist of currently accepted species of Aspleniaceae including reported chromosome numbers, information about relationships, and distribution.

Fig. S1. Phylogenetic hypothesis summarized in Figure 1. This phylogeny is resolved to the level of species and species complexes (abbreviations given in Table S2) and shows branch support as

posterior confidence values. Black stars correspond to $P \geq 0.95$. Branch colors correspond to the inferred ancestral climate preferences: dark blue, northern temperate; light blue, southern temperate; purple, Southeast Asia transition from tropical to subtropical climates; red, tropics.

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