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The base number of 'loxoscaphoid' *Asplenium* species and its implication for cytoevolution in Aspleniaceae

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• *Background and Aims* 'Loxoscaphoid' *Asplenium* species are morphologically a remarkably distinct group of Aspleniaceae. Except for two preliminary chromosome counts of *Asplenium theciferum*, the cytology of this group of species has, however, been largely unstudied.
• *Methods* Chromosome counts were obtained by acetocarmine squash preparations of one mitotic cell and several meiotic cells. Relative DNA content of gametophytic and sporophytic cells was determined by flow cytometry. The phylogenetic placement of *A. loxoscaphoides*, *A. rutifolium s.l.* and *A. theciferum s.l.* was investigated through an analysis of *rbcL* sequences.
• *Key Results* The dysploid base number is reported to be $x = 35$ in *Asplenium centrafricanum, A. loxoscaphoides, A. sertularioides* and *A. theciferum*. Analysis of *rbcL* sequences confirms that 'loxoscaphoids' nest robustly within *Asplenium*. Several high ploidy levels exceeding the tetraploid level were found in *A. theciferum s.l.* and *A. rutifolium s.l.* All taxa proved to be sexual.
• *Conclusions* Four base numbers are known at present for Aspleniaceae: $x = 39$, 38, 36 and 35. The dysploid base number $x = 35$ found in the 'loxoscaphoid' *Asplenium* spp. sheds a novel light on the cytoevolution of the whole family. We postulate a recurrent descending dysploid evolution within Aspleniaceae, leading to speciation at the (sub)generic and species/group level.

Key words: Dysploidy, cytology, cytoevolution, Aspleniaceae, *Asplenium centrafricanum*, *Asplenium loxoscaphoides*, *Asplenium rutifolium*, *Asplenium sertularioides*, *Asplenium theciferum*, polyploidization, chromosome base number, aneuploidy.

INTRODUCTION

The cosmopolitan Aspleniaceae are one of the largest and most species-rich families of leptosporangiate ferns and contain approx. 800 terrestrial, epilithic or epiphytic ferns (Kramer and Viane, 1990). Since 1950, many studies have focused on the cytology, taxonomy, biosystematics and phylogenetics of certain groups of Aspleniaceae (e.g. Manton, 1950, 1959; Wagner, 1952, 1954; Lovis and Lovis, 1955; Lovis, 1964, 1973, 1977; Tardieu-Blot, 1956*a*, *b*, 1957; Meyer, 1957, 1958, 1959, 1960, 1961; Bir, 1960, 1962, 1963; Vida, 1963; Sledge, 1965; Morton and Lellinger, 1966; Sleep, 1966, 1983; Braithwaite, 1972, 1986; Löve and Löve, 1973; Reichstein *et al.*, 1973; Holttum, 1974; Iwatsuki, 1975; Brownsey, 1976*a*, *b*; Bouharmont, 1977; Lovis *et al.*, 1977; Viane and Van Cotthem, 1977, 1979; Reichstein, 1981, 1984; Salvo *et al.*, 1982; Ching and Wu, 1984, 1985; Bir *et al.*, 1985; Werth *et al.*, 1985*a*, *b*; Mitui *et al.*, 1989; Wu, 1989*a*, *b*; Murakami and Moran, 1993; Murakami, 1995; Cheng and Murakami, 1998; Murakami *et al.*, 1998, 1999; Vogel *et al.*, 1996, 1998*a*–*c*, 1999*a*, *b*; Gastony and Johnson, 2001; Herrero *et al.*, 2001; Pinter *et al.*, 2002; Van den heede *et al.*, 2002, 2003, 2004; Van den heede and Viane, 2002; Trewick *et al.*, 2002; Sylvestre and Windisch, 2003; Van den heede, 2003; Viane and Reichstein, 2003; Schneider *et al.*, 2004; Chaerle, 2005; Perrie and Brownsey, 2005*a*, *b*; Schneider *et al*, 2005) resulting in the present generic delimitation of the family into two genera: *Asplenium* with >700 species and *Hymenasplenium* with >30 species. According to micromorphological studies (Viane and Van Cotthem, 1977, 1979; Viane, 1992) and recent molecular data (Murakami *et al.*, 1999; Gastony and Johnson, 2001; Pinter *et al.*, 2002; Trewick *et al.*, 2002; Van den heede *et al.*, 2003; Schneider *et al.*, 2004; Smith *et al.*, 2006), the generic segregates *Camptosorus*, *Ceterach*, *Ceterachopsis*, *Diellia*, *Loxoscaphe*, *Phyllitis*, *Pleurosorus* and *Thamnopteris* clearly nest within *Asplenium s.l.*, but with regard to the recognition of natural infrageneric groups (subgenera or sections), *Asplenium* is still poorly understood (Kramer and Viane, 1990). Besides molecular techniques and breeding experiments, cytological studies still have a major importance when studying the phylogenetics of this genus that is well known for its reticulate evolution resulting from hybridization and polyploidization (Wagner, 1953; Lovis, 1973, 1977).

Knowledge of fern cytology and chromosome base numbers has rapidly increased since the publication of Irene Manton's book *Problems of cytology and evolution in the Pteridophyta* in 1950. In this work, the first accurate chromosome counts for 11 European and four Macaronesian *Asplenium* species were published, all of which pointed towards a base number of $x = 36$ (Manton, 1950). Four years later Manton and Sledge (1954) presented the first chromosome numbers for a tropical *Hymenasplenium* species, counting $n = 80$ and $2n =$ approx.

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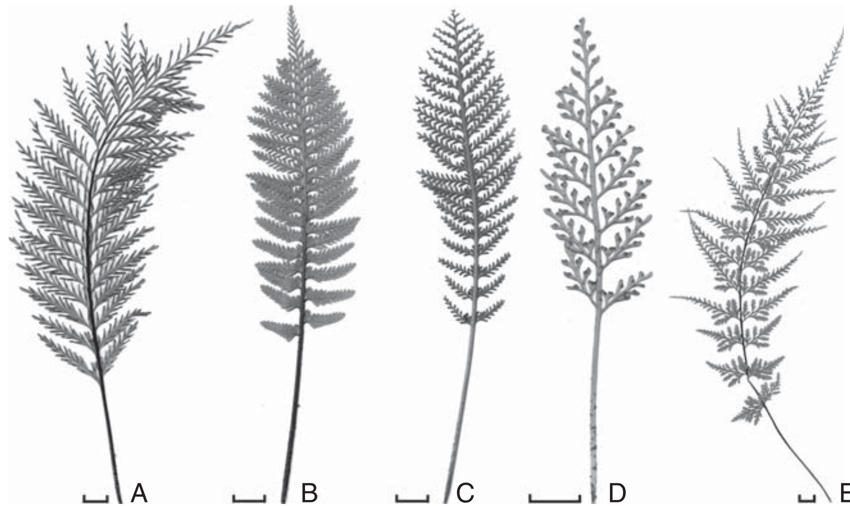

FIG. 1. 'Loxoscaphoid' *Asplenium*: abaxial view of fronds: (A) *Asplenium centrafricanum* (4*x*, Uganda); (B) *A. loxoscaphoides* (4*x*, Kenya); (C) *A. rutifolium* (8*x*, Zimbabwe); (D) *A. theciferum* (8*x*, Venezuela); (E) *A. sertularioides* (4*x*, Uganda). Scale bars = 20 mm.

158 in *H. unilaterale s.l.* from Sri Lanka. Many comparable chromosome numbers for *H. unilaterale* and related species were reported later for India, Japan and China (cited in Cheng and Murakami, 1998). Until 1989 the basic chromosome numbers for *Hymenasplenium* were considered to be $x = 40$ and $x = 36$. However, in 1989 Mitui *et al.* (1989) showed that the basic chromosome number in eight Japanese *Hymenasplenium* species was $x = 39$, with one exception of $x = 38$ in *H. subnormale.* The count of $x = 40$ for *H. unilaterale* has not been confirmed in later studies, whereas the base number $x = 36$ for *Asplenium* is supported by practically all cytological research on *Asplenium* since 1950.

The five *Asplenium* species examined were from Ethiopia, Kenya, Réunion, South Africa, Tanzania, Uganda, Venezuela and Zimbabwe: *Asplenium centrafricanum* Pic. Serm., *A. loxoscaphoides* Baker, *A. rutifolium* Kunze, *A. sertularioides* Baker and *A. theciferum* (Kunth) Mett. All species are characterized by short and submarginal sori, with the indusium fused with the lamina at its lateral ends to form a pouch-like structure, and a subcoriaceous and more or less reduced lamina (Fig. 1). These five species are mainly distributed in the Afro-montane regions, except for *A. theciferum* which also occurs in mountainous regions of Central and South America. Because the group includes *A. theciferum*, the type of *Loxoscaphe* T. Moore, the term 'loxoscaphoid' is used for these five taxa. *Loxoscaphe* in a purely descriptive sense is characterized by the presence of short and cup-shaped sori with the appearance of a *Davallia* sorus. The type, *A. theciferum*, was firstly described in the genus *Davallia* (as *Davallia thecifera* Kunth), and the presence of cup-shaped or 'davallioid' sori, has historically been interpreted as a primitive character for *Asplenium* and an intermediate state between *Asplenium* and *Davallia* (Holttum, 1966). We prefer to use the term 'loxoscaphoid' for all taxa related to *A. theciferum*, instead of other more confusing or less well-defined circumscriptions such as 'caenopteroid', derived from *Caenopteris* (Bergius, 1786) and lectotypified (Copeland, 1947) by *A. rutifolium* or 'dareoid' from *Darea* (de Jussieu, 1789). Both are based on superficial characters such as finely dissected fronds, reaching their extreme when each ultimate segment encloses only a single vein, bearing a single sorus with the lateral sides of the indusium sometimes fused to the lamina and occasionally forming a pouch-like structure (Copeland, 1947).

Asplenium theciferum was examined cytologically by Manton and Sledge (1954) and Gomez-Pignataro (1971), but these authors did not report exact or documented chromosome numbers. *Asplenium centrafricanum*, *A. loxoscaphoides, A. rutifolium* and *A. sertularioides* have never been studied cytologically.

The objective of this study was to clarify the cytological nature of the five 'loxoscaphoids' mentioned above. The chromosome numbers were identified through chromosome counts of meiotic spore mother cells and one mitotic sporangial stalk cell. Correlation of the chromosome counts with flow cytometric analysis revealed the ploidy. The reproductive nature of the species examined was investigated by growing gametophytes from spores and analysing their ploidy using flow cytometry. Three 'loxoscaphoids' were included in the present *rbcL* analysis: *A. loxoscaphoides*, *A. rutifolium* and *A. theciferum.*

MATERIALS AND METHODS

Origin of plants used in this study

Sporophytes. All material for the meiotic and mitotic chromosome counts and flow cytometry was collected during field trips by Bellefroid and Viane to Ethiopia (1998), Kenya (2006), Réunion (1999), South Africa (1997 and 2007), Tanzania (1998), Uganda (2007), Venezuela (2005) and Zimbabwe (2005). Some samples were collected from plants in the field, but others were collected later from living plants cultivated in the Ghent Botanical Garden. The field localities, specimen identifications and voucher numbers are listed in Appendix 1. The localities, specimen identifications and voucher numbers of material used for the molecular analysis are listed in Appendix 2. An overview is listed in Table 1. Herbarium vouchers of all specimens examined are deposited in the Ghent University Herbarium (GENT).

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TABLE 1. *Chromosome number, ploidy, locality and voucher of cytologically and molecularly examined specimens*

Species	Chromosome number (counted)	Ploidy level (flow cytometry)	Molecular analysis (X)	Voucher	Origin
A. centrafricanum	$n = 70^{II}$	4x	–	EB392	Uganda
A. centrafricanum	$n = 70^{II}$	4x	–	EB393	Uganda
A. centrafricanum	–	4x	–	EB396	Uganda
A. centrafricanum	–	4x	–	RV11236	Uganda
A. centrafricanum	–	4x	–	RV11237	Uganda
A. centrafricanum	–	4x	–	RV11246	Uganda
A. loxoscaphoides	$n = 70^{II}$	4x	–	EB324	Kenya
A. loxoscaphoides	–	4x	X	EB325	Kenya
A. loxoscaphoides	$n = 70^{II}$	4x	–	EB337	Kenya
A. loxoscaphoides	–	4x	X	EB339	Kenya
A. loxoscaphoides	–	4x	X	RV7548	Tanzania
A. loxoscaphoides	$n = 70^{II}$	4x	X	RV7549	Tanzania
A. rutifolium	–	4x	X	RV6377C	South Africa
A. rutifolium	$n =$ approx. 70^{II}	4x	–	RV11549	South Africa
A. rutifolium	–	4x	–	RV11561	South Africa
A. rutifolium	–	8x	–	RV8300	Réunion
A. rutifolium	–	8x	X	RV8301	Réunion
A. rutifolium	–	8x	–	RV8302	Réunion
A. rutifolium	–	8x	X	RV8730	Zimbabwe
A. rutifolium	–	8x	X	EB240	Zimbabwe
A. rutifolium	–	8x	–	EB256	Zimbabwe
A. sertularioides	–	4x	–	EB348	Uganda
A. sertularioides	$n = 70^{II}$	4x	–	EB353	Uganda
A. sertularioides	$n = 70^{II}$	4x	–	EB358	Uganda
A. sertularioides	$n = 70^{II}$	4x	–	EB360	Uganda
A. sertularioides	$n = 70^{II}$	4x	–	EB373	Uganda
A. sertularioides	–	4x	–	EB383	Uganda
A. sertularioides	$n = 70^{II}$	4x	–	EB408	Uganda
A. sertularioides	$2n = 140$	4x	–	EB408	Uganda
A. sertularioides	–	4x	–	RV11131	Uganda
A. sertularioides	–	4x	–	RV11156	Uganda
A. theciferum	$n = 70^{II}$	4x	–	EB306	Kenya
A. theciferum	$n = 70^{II}$	4x	X	EB308	Kenya
A. theciferum	$n = 70^{II}$	4x	–	EB342	Uganda
A. theciferum	$n = 70^{II}$	4x	–	EB388	Uganda
A. theciferum	$n = 70^{II}$	4x	–	RV7219	Ethiopia
A. theciferum	–	4x	X	RV7541	Tanzania
A. theciferum	–	8x	–	EB404	Uganda
A. theciferum	–	8x	–	EB407	Uganda
A. theciferum	–	8x	X	RV10140	Venezuela
A. theciferum	–	8x	–	RV10141	Venezuela
A. theciferum	–	8x	X	RV10336	Venezuela
A. theciferum	$n =$ approx. 210^{II}	12x	X	EB238	Zimbabwe
A. theciferum	–	12x	X	EB244	Zimbabwe
A. theciferum	–	12x	–	EB248	Zimbabwe
A. theciferum	–	12x	–	EB395	Uganda
A. theciferum	$n =$ approx. 210^{II}	12x	–	EB401	Uganda
A. theciferum	–	12x	–	RV11234	Uganda
A. theciferum	–	12x	–	RV11488	South Africa
A. theciferum	–	12x	–	RV11494	South Africa
A. theciferum	–	12x	–	RV11500	South Africa

Gametophytes. Spores were collected from plants cultivated in the Ghent Botanical Garden and were sown on agar-solidified medium containing a nutrient solution recommended by Dyer (1979). The cultures were maintained under natural light, without direct sunlight, at room temperature. When the gametophytes were approx. 5 months old, prior to the formation of antheridia and archegonia, they were harvested and thoroughly washed with distilled water.

Cytology

For chromosome counts of meiotic cells, immature sporangia were collected in the field or in the greenhouses of Ghent Botanical Garden (Table 1). The material was treated with 8-hydroxyquinolinine for 3 h at room temperature and then fixed in freshly prepared, ice cold 3 : 1 absolute ethanol : glacial acetic acid. The material was stored at −20 °C until studied. The meiotic spore mother cells were observed at metaphase I.

For the chromosome counts of mitotic cells, sori with young sporangia were treated in a similar way. The mitotic stalk cells of the young sporangia were then studied at metaphase. Several efforts were made to use gametophytic cells for mitotic chromosome counts, but all attempts failed.

Acetocarmine squash preparations were made as described by Heitz (1925) and Manton (1950). Photographs were taken with

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an Olympus BH2 phase-contrast microscope equipped with a Canon EOS 10D digital camera. Permanent preparations were made by dehydrating the cover slip and slide in graded mixtures of acetic acid and absolute ethanol, followed by mounting in Euparal (3C 239; Chroma-Gesellschaft, Köngen, Germany). All permanent preparations are kept in the Research Group of Pteridology of the Department of Biology at Ghent University. For the production of analytical diagrams, enlarged prints were used for interpretation and drawing at a magnification of ×1500. Drawings of the best cells were digitized using Corel Draw 12.

Flow cytometry

To quantify the relative DNA content of the specimens examined, flow cytometric analyses were conducted on both gametophytic and sporophytic material. For each species, the sporophytic specimens with accurate chromosome counts were used as standards to deduce the ploidy of the remaining sporophytes by correlating their relative DNA content. Similarly, the counted sporophytic specimens of each species were used as standards to deduce the ploidy of the corresponding gametophytes. The combination of flow cytometric analyses and chomosome counts allowed the ploidy of a large number of specimens to be determined from different populations (Table 1).

For flow cytometric analysis of sporophytes fresh petiole samples were used. Whole gametophytes were also prepared for analysis. The samples were individually chopped using a sharp razor blade in a glass Petri dish containing nucleus isolation buffer [2·1 % (w/v) citric acid monohydrate, 0·5 % (w/v) Tween 20, distilled water] and filtered through a 50-µm nylon mesh. The nuclei suspension was supplemented with DAPI reagent [6·5 % $NaH_2PO_4.2H_2O$, 40 µL DAPI stock solution (5 mg DAPI/mL distilled water), distilled water], amount 1 : 4 nucleus isolation buffer : DAPI reagent. The samples were analysed using a flow cytometer (Partec PA-1; Partec, GmbH, Munster, Germany), following the supplier's instructions. To calculate the relative nuclear DNA content, *Agave stricta* ($2x$, $2n = 60$, 2C = 7·8 pg; Zonneveld *et al.*, 2005) was used as an internal standard in most cases. *Agave sisalana* ($5x$, $2n = 150$, 2C = 20·2pg; Zonneveld *et al.*, 2005) was used when the relative nuclear DNA content of the gametophyte coincided with that of *Agave stricta*.

Molecular analysis

Thirty-eight taxa of Aspleniaceae from various locations (Appendix 2) were sampled. Two *Hymenasplenium* spp. were used as the out-group. Total genomic DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN), following the manufacturer's instructions or using the CTAB DNA extraction protocol (Doyle and Doyle, 1987). A partial region of the *rbcL* was amplified with primer 5′-ATGTCAC CACAAACAGAGACTAAAGC-3′ and 5′-GCAGCAGCTAG TTCCGGGCTCCA-3′ (Hasebe *et al.*, 1994). PCR reagents were prepared according to the instructions of i-TagTM DNA polymerase PCR kit by iNtRON Biotechnology, Inc. PCR amplifications were carried out on a PTC-100TM thermocycler (MJ Research, Inc.). Cycling parameters were fitted accordingly to the PCR kit manufacturer's recommendations for *rbcL*: initial denaturation of 120 s at 94 °C, followed by 35 cycles of 20 s denaturation at 94 °C, 20 s annealing at 57 °C, 105 s extension at 72 °C and a final extension of 5 min at 72 °C. PCR products were cleaned using MEGAquick-spinTM kit by iNtRON Biotechnology, Inc. Both strands of each gene were sequenced by Macrogen, Inc., South Korea, and assembled using Sequence Manipulation Suite (Stothard, 2000); *rbcL* sequences of 1209 bp were aligned using ClustalX (Thompson *et al.*, 1997). The best evolution model for maximum likelihood was tested using modeltest 3·06 (Posada and Crandall, 2001). Maximum likelihood analysis was performed using GARLI v0·942 (Zwickl, 2006*a*, *b*). The consensus tree of 1000 bootstrap replicates was obtained using PAUP 4·0 (Swofford, 2002).

RESULTS

Chromosome counts and flow cytometry

For various reasons, observing meiotic stages in the species examined was difficult. Loxoscaphoid sori are small and enclose relatively few sporangia, resulting in a limited number of potentially meiotic cells. All stages of sporangial development coexist in a single sorus, indicating that the ripening of sporangia and spores extends over a long period of time. Therefore the chance of finding sporangia with meiotic spore mother cells is rather small. Moreover, meiosis seems to take place early in sporangial development when the sporangia are still extremely small, and the spores rapidly develop an exospore wall within the immature sporangia. It is almost impossible to produce cytological preparations with meiotic stages, without the presence of spores. The relatively rigid exospore walls hinder proper squashing, essential for obtaining good chromosome spreads. Moreover, the chromosomes of *A. centrafricanum* and of *A. sertularioides*, in particular, have relatively long arms, frequently overlapping each other during metaphase I.

Observation of dividing mitotic cells in metaphase was even more problematic than observing dividing meiotic cells. The first reason is merely due to the large number of chromosomes ($2n = 140$) in mitotic sporophytic cells, which makes good and satisfactory chromosome spreading difficult. Only for *A. sertularioides* was an accurate mitotic chromosome count obtained. To overcome the problem of the large number of chromosomes, an attempt was made to count chromosomes in gametophytic mitotic cells, since flow cytometric analysis confirmed that gametophytes possess only half the DNA content of their corresponding sporophyte. Unfortunately these attempts failed, partly due to the low mitotic activity in the gametophytes.

In all cytological preparations, a mixture of metacentric, submetacentric and acrocentric chromosomes and a broad range of variation in chromosome size were observed. For specimens of *A. rutifolium* and *A. theciferum* with a ploidy exceeding $4x$, meiosis and sporogenesis were occasionally irregular. Some meiotic spore mother cells showed irregular pairing of chromosomes, with the formation of univalents, bivalents, clumped bivalents or multivalents and chomosome threads and/or bridges. As a result, anaphase I was often

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extremely disturbed, showing numerous lagging chromosomes. However, this phenomenon was not constant for all spore mother cells in a given sporangium. In a later stage of meiosis, irregular lobing or budding and splitting of the haploid daughter nuclei leading to abortion of spores in pairs, was sporadically observed. Further research is needed to examine the nature of the clumped bivalents in metaphase I, in order to determine whether these represent true multivalents. The hypothetical ability of these taxa of high ploidy to form multivalents during metaphase I would indicate their possible autopolyploid origins, although allopolyploidy cannot be ruled out at this stage. The majority of sporangia contained 16 spore mother cells and subsequently 64 spores. Occasionally, sporangia with only 32 spores, which were not always significantly larger or morphologically different from spores produced in normal sporangia, were observed.

Because various irregularities were observed during meiosis and sporogenesis in *A. rutifolium* and *A. theciferum*, the reproductive nature of all five examined species was examined by growing gametophytes from spores and analysing their ploidy using flow cytometry. The gametophytes of all examined species analysed, across different ploidies had only half the relative DNA content found in the corresponding sporophytes from which the spores were collected. This implies that normal meiosis took place prior to sporogenesis. As a consequence we assume these taxa to have a sexual reproduction, since apogamously reproducing taxa would form gametophytes with the same DNA content as the corresponding sporophytes. However, the possibility of the occasional formation of viable diplospores that could possibly germinate to form gametophytes with the same DNA content as the corresponding sporophyte cannot be ruled out, especially given the observation of occasionally 32-spored sporangia.

Asplenium sertularioides. Meiotic metaphase I cells of *A. sertularioides* had $n = 70$ bivalents (Fig. 2A, B). The mitotic metaphase cell of *A. sertularioides* (Fig. 2C, D) had $2n = 140$. All counted specimens proved to be tetraploid ($4x$) with a chromosome number based on $x = 35$ (Table 1). The counted specimens were used as standards to calculate the ploidy of the remaining specimens by flow cytometry. All collected specimens possesed the same ploidy (Table 1).

Asplenium centrafricanum. Meiotic metaphase I cells of *A. centrafricanum* had $n = 70$ bivalents (Fig. 3A, B). Counted specimens all proved to be tetraploid ($4x$) with a chromosome number based on $x = 35$ (Table 1). The counted specimens were used as standards to calculate the ploidy of the other specimens by flow cytometry. All collected specimens possessed the same ploidy (Table 1).

Asplenium loxoscaphoides. Meiotic metaphase I cells of *A. loxoscaphoides* had $n = 70$ bivalents (Fig. 3C, D). Counted specimens all proved to be tetraploid ($4x$) with a chromosome number based on $x = 35$ (Table 1). Counted specimens were used as standards to calculate the ploidy of the other specimens by flow cytometry. All collected specimens had the same ploidy (Table 1).

Asplenium theciferum. Specimens from Kenya and Ethiopia and some from Uganda had $n = 70$ bivalents at metaphase I (Fig. 3E, F). Specimens from Zimbabwe and some from Uganda showed $n =$ approx. 210 bivalents at metaphase I (Table 1). Both cytotypes have a chromosome number based on $x = 35$. Counted specimens were used as standards to determine the ploidy of other specimens by flow cytometry. Thus tetraploids ($4x$) were discovered in Ethiopia, Kenya, Tanzania and Uganda, octoploids ($8x$) in Uganda and Venezuela and dodecaploids ($12x$) in South Africa, Uganda and Zimbabwe (Table 1).

Asplenium rutifolium. *Asplenium rutifolium* proved to be extremely difficult to examine cytologically, and no exact chromosome counts were obtained. Based on an approximate counting (one specimen from South Africa with $n =$ approx. 70 bivalents) and correlation with flow cytometric analyses, putative tetraploids ($4x$) for South Africa and putative octoploids ($8x$) for Réunion and Zimbabwe are reported (Table 1).

Molecular analysis

In the *rbcL* analysis (Fig. 4), three 'loxoscaphoids', *A. loxoscaphoides*, *A. rutifolium s.l.* and *A. theciferum s.l.*, were included. In contrast to other recent molecular analyses of 'loxoscaphoid' *Asplenium* taxa (Murakami, 1995; Gastony and Johnson, 2001; Schneider *et al.*, 2004) only 'loxoscaphoid' samples of known ploidy were added, and these were collected over a wide geographical range (Appendix 2).

The phylogenetic tree, constructed on the basis of *rbcL* sequences using maximum likelihood reveals a 'loxoscaphoid' clade [*A. loxoscaphoides* (EB325, EB339, RV7548 and RV7549), *A. rutifolium* (EB240, RV6377C, RV8301 and RV8730) and *A. theciferum* (EB238, EB244, EB308, RV7541, RV10140 and RV10336)], robustly nested within *Asplenium*. The nearest sister groups to this clade are the '*A. sandersonii–A. daucifolium*' group, the '*A. thunbergii–A. tenerum*' group and the '*A. phyllitidis–A. nidus*' group.

The placement of *A. rutifolium* with *A. theciferum* and *A. loxoscaphoides* is consistent with their morphological similarity. Within *A. theciferum*, tetraploid (EB308 and RV7541) and dodecaploid (EB238 and EB244) specimens from Africa and octoploids (RV10140 and RV10336) from Venezuela strongly group together. The tetraploid (RV6377C) and octoploid (EB240, RV8301, RV8730) *A. rutifolium* specimens also strongly group together.

DISCUSSION

Recurrent descending dysploid evolution in Aspleniaceae

In this paper, new chromosome numbers, based on $x = 35$, are presented for *A. centrafricanum*, *A. loxoscaphoides* and *A. sertularioides*, and the provisional counts for *A. theciferum* of Manton and Sledge (1954) (specimen from Kenya, $n = 70–72$) and Gomez-Pignataro (1971) (specimen from Costa Rica, $n = 70$) confirmed. Hence, the present findings corroborate the establishment of a 'new' base number ($x = 35$ within *Asplenium*. In addition, the molecular phylogenetic tree (Fig. 4) shows that the 'loxoscaphoid' clade (*A. loxoscaphoides*, *A. rutifolium* and *A. theciferum*) with the base number $x = 35$ robustly nests within *Asplenium*. This phylogenetic placement is in concordance with previous

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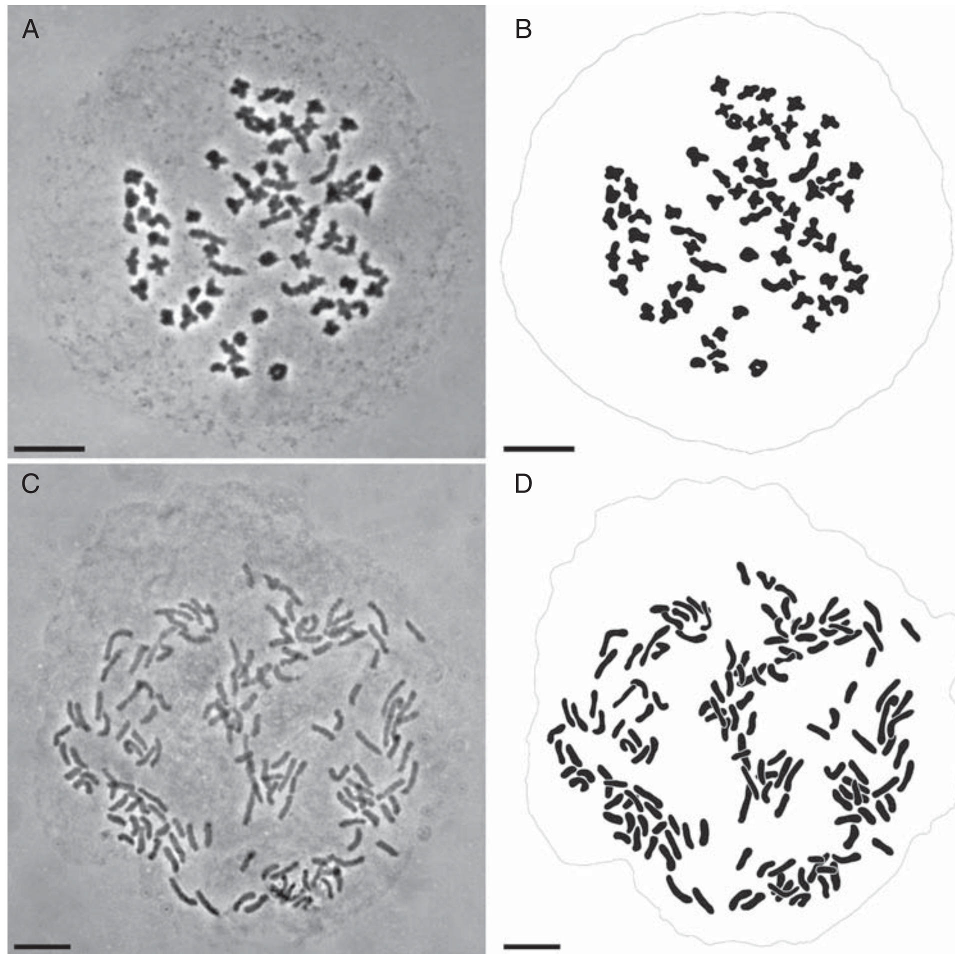

FIG. 2. Cytological preparations of *Asplenium sertularioides* (EB408): (A, B) meiosis with $n = 70$ bivalents; (C, D) mitosis with $2n = 140$ chromosomes; (A, C) photographs; (B, D) analytical drawings. Scale bars = 10 μm.

micromorphological (Viane and Van Cotthem, 1977; Viane, 1992), taxonomic (Kramer and Viane, 1990) and molecular analyses (Murakami, 1995; Gastony and Johnson, 2001; Schneider *et al.*, 2004). Although it was not possible to obtain an exact chromosome count for *A. rutifolium*, its macro- and micromorphology and phylogenetic placement in the molecular analysis (Fig. 4) indicate that this species is closely related to *A. loxoscaphoides* and *A. theciferum*. Therefore, we also postulate a chromosome number based on $x = 35$ for *A. rutifolium*. Biogeographical, morphological (data not shown) and cytological data suggest that *A. centrafricanum* and *A. sertularioides* are closely related to *A. loxoscaphoides*, *A. rutifolium* and *A. theciferum*, and belong to the 'loxoscaphoid' clade, which thus contains at least these five species. The cytologically examined taxa of the three nearest groups ('*A. sandersonii*–*A. daucifolium*' group, '*A. thunbergii*–*A. tenerum*' group and '*A. phyllitidis*–*A. nidus*' group) to the 'loxoscaphoid' clade are tetraploids and have chromosome numbers based on $x = 36$ [see Manton and Sledge (1954) for *A. tenerum* ($2n = 144$); Manton (1959) for *A. sandersonii* ($2n = 144$); Bir (1960) and Abraham *et al.* (1962) for *A. nidus* ($2n = 144$) and *A. phyllitidis* ($2n = 144$)]. Consequently we postulate that the base number $x = 35$ is a dysploid derivate from an ancestor with base number $x = 36$.

This hypothesis is supported by the presence of heterogeneous karyotypes with acrocentric and submetacentric chromosomes and a great range of variation in chromosome size (Stebbins, 1971; Schubert, 2007). The terms 'dysploidy' and 'aneuploidy' are often used interchangeably, but denote two different processes with different consequences. The term 'dysploidy' is used here in the sense of Stace and James (1996) to indicate the process whereby the euchromatin of a genome is rearranged by inversions and translocations onto a greater or lesser number of centromeres. 'Aneuploidy', on the other hand, defines the gain or loss of whole chromosomes. Chromosome number reduction through dysploidy or so-called chromosome 'fusion', combined with polyploidy has been suggested as an important evolutionary process in several genera of non-flowering plants (Lovis, 1977; Brownsey, 1983; Pichi Sermolli, 1987; Windham and Yatskievych, 2003) and flowering plants (Rye and James, 1992; Knox and Kowal, 1993; Stace *et al.*, 1993, 1997; Stace and James, 1996; Schneeweiss *et al.*, 2004; Lysak *et al.*, 2006; Shan *et al.*, 2006).

Due to morphological, anatomical, geographical, cytological and molecular affinities between the taxa examined, we

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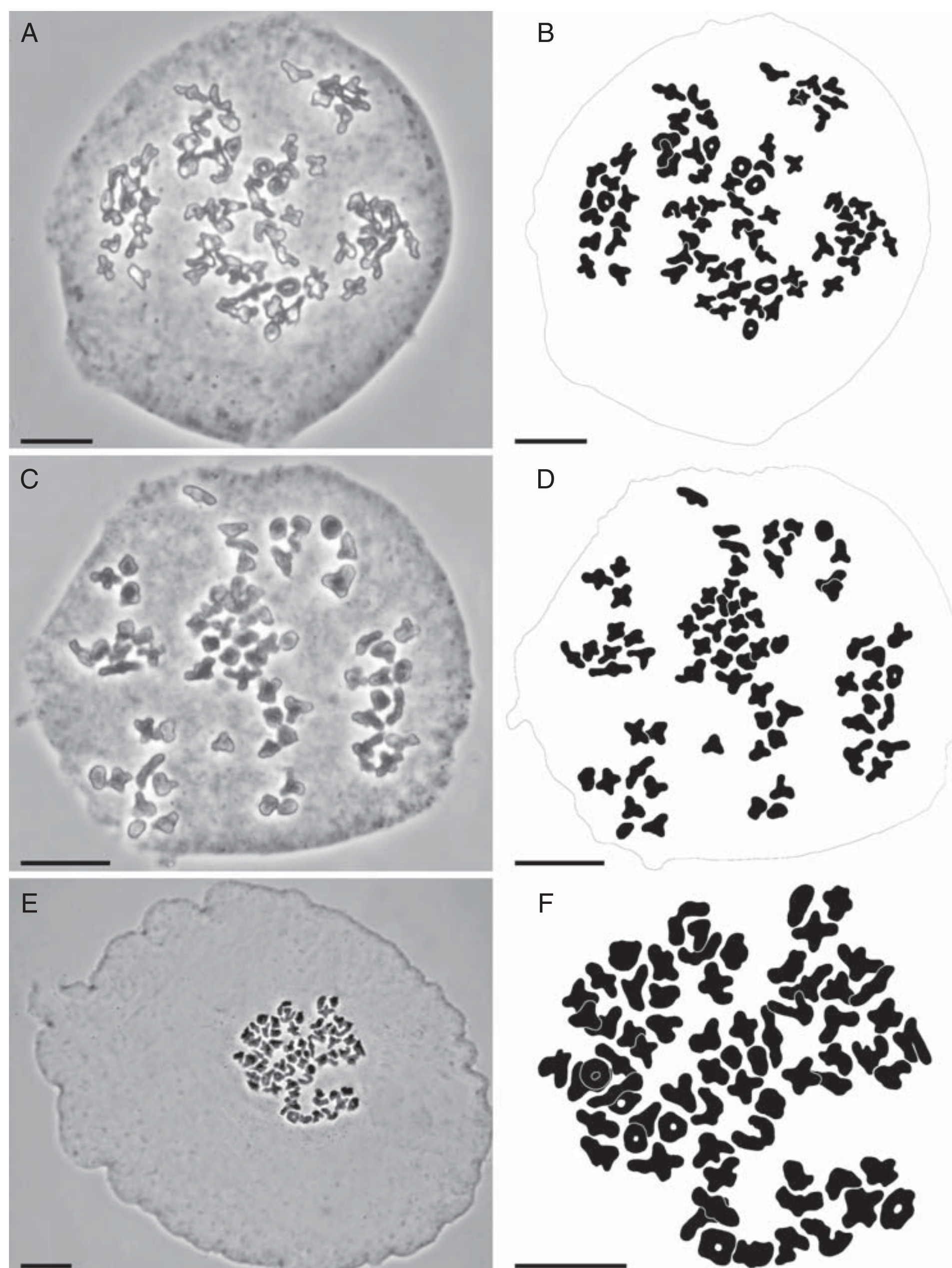

FIG. 3. Cytological preparations of *Asplenium centrafricanum*, *A. loxoscaphoides* and *A. theciferum*: (A, B) *Asplenium centrafricanum* (EB392), meiosis with $n = 70$ bivalents; (C, D) *Asplenium loxoscaphoides* (RV7549), meiosis with $n = 70$ bivalents; (E, F) *Asplenium theciferum* (RV7219), meiosis with $n = 70$ bivalents (an enlargement of the central part of cell is shown in F); (A, C, E) photographs; (B, D, F) analytical drawings. Scale bars = 10 μm.

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may presume a single common ancestor for the 'loxoscaphoid' taxa treated in this study. As no diploid species with chromosome numbers based on $x = 35$ have been detected to date, we cannot state with certainty whether this dysploid reduction occurred at the diploid level ($n = 36$ to $n = 35$) or at the tetraploid level ($n = 72$ to $n = 70$), nor can we be certain whether this was a single or recurrent evolutionary event.

A first explanation would be chromosomal reduction at the tetraploid level. This hypothesis would be supported by the fact that the lowest ploidy of the 'loxoscaphoids' and their sister groups is tetraploid or higher (this study; Perrie and Brownsey, 2005*b*). A major discovery of the past few decades is the extent and speed of genome reorganization in polyploids (Soltis *et al.*, 2003). Recent studies demonstrate that polyploidy involves more than the passive fusion of two or more genomes, instead it encompasses a whole spectrum of physiological and molecular adjustments (Soltis and Soltis, 1993, 1999, 2000; Scheid *et al.*, 1996; Leitch and Bennett, 1997, 2004; Wendel, 2000; Soltis *et al.*, 2003, 2004; Adams and Wendel, 2005; Schubert, 2007). Levy and Feldman (2004) demonstrated that extensive genomic rearrangements often arise with the onset of allopolyploidization. Allopolyploidization has been shown to be a driving force in plant evolution. Furthermore, rearranged chromosomes contribute to reproductive isolation and to speciation, as a result of reduced

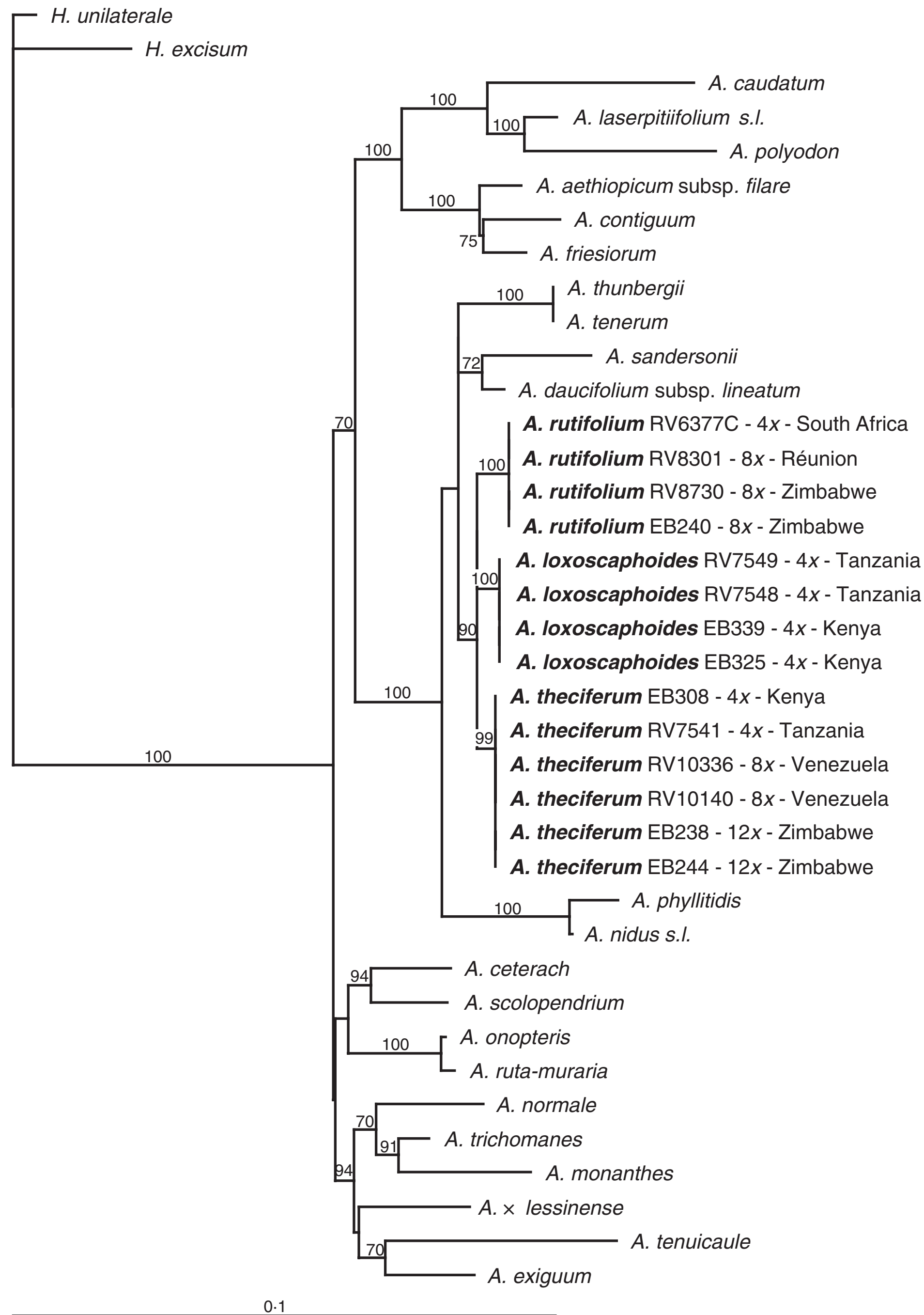

FIG. 4. Consensus *rbcL*-tree for 38 members of Aspleniaceae, including samples of three 'loxoscaphoid' *Asplenium* taxa, constructed using maximum likelihood, with bootstrap values >70 % from a 1000-replicate analysis.

fertility of heterozygous carriers and/or reduced gene flow caused by suppressed recombination (Werth and Windham, 1991; Lynch and Force, 2000; Rieseberg, 2001; Taylor *et al.*, 2001). Consequently, there is a realistic possibility of a dysploid chromosome number reduction during the formation of an allopolyploid *Asplenium* species (Fig. 5C). This would involve the reduction of the expected tetraploid karyotype with two chromosome-pairs ($n = 72^{\mathrm{II}}$ to $n = 70^{\mathrm{II}}$) in the course of the allopolyploidization event, through chromosomal reorganization. Again we cannot state whether this was a single or recurrent evolutionary event, even though many hybridization events in Aspleniaceae have been shown to be recurrent (Lovis, 1973; Werth *et al.*, 1985*a*, *b*; Prelli *et al.*, 1998; Vogel *et al.*, 1998*a*, *c*, 1999*a*, *b*; Rumsey *et al.*, 2004). Subsequent diploidization, genome downsizing, hybridization, geographic isolation and reproductive isolation might then have led to different closely related tetraploid taxa with a chromosome number based on $x = 35$. In this case there would have never been a diploid *Asplenium* species with

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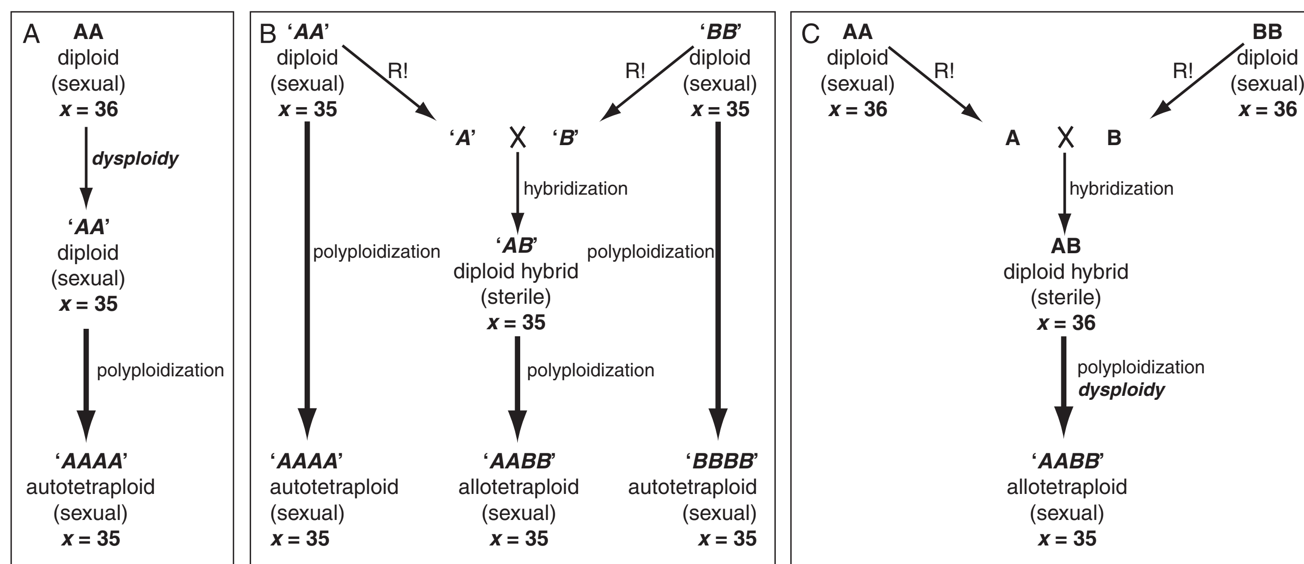

FIG. 5. Possible pathways in the formation of dysploid tetraploid karyotypes with base number $x = 35$. Regular bold characters indicate regular karyotypes based on $x = 36$. Italic bold characters between quotation marks indicate derived dysploid karyotypes based on $x = 35$; R! indicates meiosis. (A) Formation of an autotetraploid with base number $x = 35$ via autopolyploidization of a 'dysploid diploid' with $x = 35$, formed by dysploid chromosome number reduction in a diploid with $x = 36$. (B) Formation of either auto- or allotetraploids, with $x = 35$, after either polyploidization,or hybridization and polyploidization in or between two closely related 'dysploid diploids' with $x = 35$. (C) Formation of a 'dysploid allotetraploid' with base number $x = 35$ through hybridization of two sexual diploids with $x = 36$, and subsequent polyploidization and dysploid chromosome number reduction.

$x = 35$. In our phylogenetic tree, the three 'loxoscaphoid' *Aspleniums* form a polytomy, possibly representing a sudden radiation in speciation following an allopolyploidization event.

It should be noted that if a chromosome number reduction at the polyploid level is assumed, it is also necessary to take into account the possibility of an aneuploid chromosome number reduction. Theoretically the loss of whole chromosomes at the polyploid level is not necessarily lethal. However aneuploid chromosome number reduction in plants has never been irrefutably proven and recent studies have revealed much more complex interactions than the simple loss of whole chromosomes (Lovis, 1977; Brownsey, 1983; Pichi Sermolli, 1987; Rye and James, 1992; Knox and Kowal, 1993; Stace *et al.*, 1993; Stace and James, 1996; Stace *et al.*, 1997; Windham and Yatskievych, 2003; Schneeweiss *et al.*, 2004; Lysak *et al.*, 2006; Shan *et al.*, 2006).

A second hypothesis, chromosomal reduction at the diploid level, would involve the elimination of only one centromere/minichromosome, followed by polyploidization (Fig. 5A). The relative differences in total genome size (data not shown) between the taxa examined might indicate the possibility of a recurrent process of chromosome number reduction and polyploidization from a single common ancestor (Leitch and Bennett, 2004). After differential processes of genome rearrangements and downsizing following the polyploidization, different species might have originated (Leitch and Bennett, 2004). Recurrent processes of descending dysploidy at the diploid level may have led to different diploids with $x = 35$, which may have given rise to both auto- and allopolyploids after hybridization, polyploidization, genome restructuring and downsizing, as well as geographical and subsequent reproductive isolation (Fig. 5B). However, at present no diploid species with $x = 35$ are known.

Aspleniaceae have long been considered to be extraordinarily uniform in cytological terms, with a base number of $x = 36$. Indeed, the majority of data on chromosome numbers reveal 36 bivalents or euploid derivates, suggesting $x = 36$ as the plesiomorphic state for Aspleniaceae. In *Hymenasplenium*, the only other genus currently recognized besides *Asplenium*, most representatives have cytotypes based on $x = 39$ (Mitui *et al.*, 1989; Cheng and Murakami, 1998), although Cheng and Murakami (1998) found exceptions: *H. costarisorum* appears to have two cytotypes (sexual diploids and tetraploids) based on $x = 36$ and *H. subnormale* two cytotypes (sexual diploids and tetraploids) based on $x = 38$. Following the assumption that $x = 36$ is the plesiomorphic state for Aspleniaceae and therefore also in *Hymenasplenium*, *H. costarisorum* would have maintained the primitive chromosome number for this genus. This, however, is not supported by the molecular data of Murakami (1995), as the *rbcL* tree indicates that this species is closely related to *H. obscurum* with a base number $x = 39$. Therefore, Cheng and Murakami (1998) stated that the $x = 36$ of *H. costarisorum* cannot be a plesiomorphic state in *Hymenasplenium*, but possibly a result of convergent or reversal evolution from $x = 39$ to 36. Apart from *H. costarisorum* ($x = 36$) and *H. subnormale* ($x = 38$) all *Hymenasplenium* species investigated have cytotypes based on $x = 39$. Molecular phylogenetic analyses using *rbcL* sequences have shown *Hymenasplenium* to be the most basally diverged monophyletic genus in Aspleniaceae, only distantly related to any of the remaining species of the family (Murakami, 1995; Gastony and Johnson, 2001; Schneider *et al.*, 2004). As a consequence, we interpret the $x = 36$ of *H. costarisorum* as a convergent dysploid evolution in *Hymenasplenium* from $x = 39$ to 36. *Hymenasplenium subnormale* with $n = 38$ and $n = 76$, is closely related to

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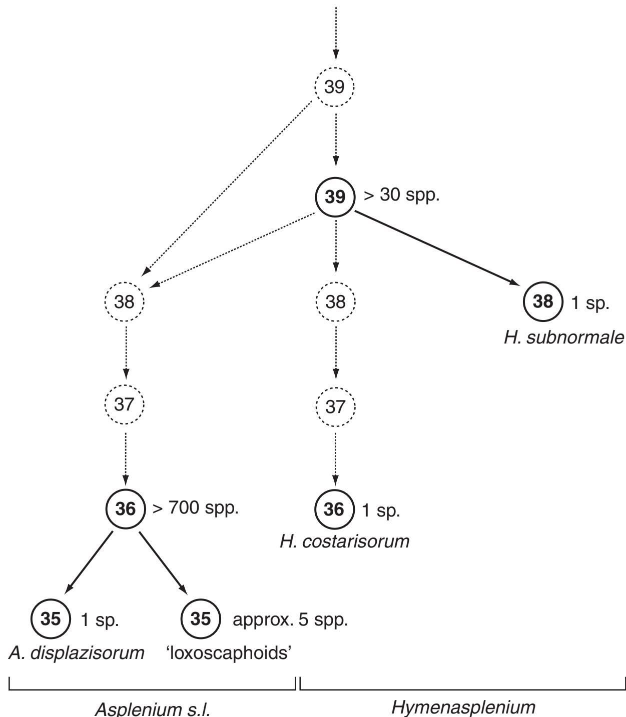

FIG. 6. Putative phyletic scheme of cytoevolution within Aspleniaceae, based on cytological and *rbcL* data. Continuous lines and bold numbers in unbroken circles indicate most probable lines of evolution based on established basic chromosome numbers. Dotted lines and numbers in dotted circles indicate putative lines of evolution based on postulated transitional chromosome numbers.

H. cataractarum, *H. apogamum* and *H. hondoense* (Murakami, 1995), all based on $x = 39$. This indicates that dysploid karyotypes evolved at least twice independently in *Hymenasplenium*.

The establishment of the very successful base number $x = 36$ of *Asplenium* was another independent dysploid karyotype evolution within the family. Albeit the base number $x = 36$ is clearly dominant and most likely plesiomorphic for *Asplenium*, it is not as conservative and uniform as previously assumed. The tendency of descending dysploid cytoevolution of the family persists in the genus *Asplenium* with the evolution of the 'loxoscaphoids' with chromosome numbers based on $x = 35$. Additionally, Manton (1959) reported a chromosome number of $n = 70^{II}$ (tetraploid, with unusually large chromosomes) for *A. diplazisorum* from Ghana. There are no ecological, morphological or geographical indications that *A. diplazisorum* is related to the 'loxoscaphoid' *Asplenium* spp., and thus the dysploid reduction from $x = 36$ to 35 may have evolved at least twice independently within *Asplenium*.

With the confirmation of $x = 35$, currently four different base numbers that are well established within Aspleniaceae are known: $x = 39$ (*Hymenasplenium*: approx. 30 spp.), $x = 38$ (*Hymenasplenium subnormale*), $x = 36$ (*Asplenium*: approx. 700 spp., *Hymenasplenium costarisorum*) and $x = 35$ [*Asplenium diplazisorum* (to be confirmed), and 'loxoscaphoids': approx. five spp.]. These base numbers are plotted on a phyletic scheme of Aspleniaceae in Fig. 6, illustrating the postulated recurrent descending dysploid evolution of the family.

Although many intriguing questions remain, it appears highly plausible that 'loxoscaphoid' *Asplenium* species evolved through dysploid chromosome number reduction from an ancestral karyotype with $x = 36$. We also propose that dysploid chromosome number reduction occurred at least five times in the (cyto)evolution of Aspleniaceae, leading to speciation at both the (sub)generic and species (group) level.

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APPENDIX 1

List of taxa and samples used for meiotic and mitotic chromosome counts and flow cytometry. Species names and vouchers are in bold.

Asplenium centrafricanum *Bellefroid* **EB392**: Uganda, Western Prov., montane rainforest along road from Kabale to Kisoro, epiphytic, approx. 2302 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB393**: Uganda, Western Prov., montane rainforest along road from Kabale to Kisoro, epiphytic, approx. 2302 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB396**: Uganda, Western Prov., montane rainforest along road from Kabale to Kisoro, epiphytic, approx. 2302 m a.s.l., Ploidy: 4*x*; *Viane* **RV11236**: Uganda, Kanungu Distr., Bwindi Impenetrable National Park, *Podocarpus* forest with bamboo along track from Ruhija to Ikumba, epiphytic, approx. 2430 m a.s.l., Ploidy: 4*x*; *Viane* **RV11237**: Uganda, Kanungu Distr., Bwindi Impenetrable National Park, *Podocarpus* forest with bamboo along track from Ruhija to Ikumba, epiphytic, approx. 2430 m a.s.l., Ploidy: 4*x*; *Viane* **RV11246**: Uganda, Kabale Distr., Bwindi Impenetrable National Park, *Podocarpus* forest with bamboo along track from Ruhija to Ikumba, approx. 2260 m a.s.l., Ploidy: 4*x*. ***A. loxoscaphoides*** *Bellefroid* **EB324**: Kenya, Coast Prov., Taita region, approx. 90 km south from Voi, Kasigau, path from Rukanga along water pipeline to water catchment area for Rukanga, lowland shrub forest, terrestrial in rocky soil, approx. 886 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB325**: Kenya, Mt Kasigau, route from Rukanga along water pipeline to top, epilithic, approx. 886 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB337**: Kenya, Coast Prov., Taita region, approx. 90 km south from Voi, Kasigau, at the water catchment area for Jora. edge of montane rainforest, epilitic on rocky outcrop, approx. 1150 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB339**: Kenya, Mt Kasigau, route from Rukanga along water pipeline for Kiteghe, at water intake, epilithic, approx. 1174 m a.s.l., Ploidy: 4*x*; *Viane* **RV7548**: Tanzania, S. of Arusha National Park (Mt Meru), forest with *Impatiens hochstetteri* along tack to Mamela Gate, approx. 1780 m a.s.l., Ploidy: 4*x*; *Viane* **RV7549**: Tanzania, South of Arusha National Park (Mt Meru), forest with *Impatiens hochstetteri* along tack to Mamela Gate, approx. 1780 m a.s.l., Ploidy: 4*x*. ***A. rutifolium*** *Bellefroid* **EB240**: Zimbabwe, Eastern Prov., Bvumba Mountains, Bunga Forest, epilithic, approx. 1622 m a.s.l., Ploidy: 8*x*; *Bellefroid* **EB256**: Zimbabwe, Eastern Prov., Chimanimani Mountains National Park, along Baileys Folly trail, epiphytic, approx. 1572 m a.s.l., Ploidy: 8*x*; *Viane* **RV6377C**: South Africa, N. Transvaal, below Mariepskop, 40 km N of Graskop, approx. 1250 m a.s.l., Ploidy: 4*x*; *Viane* **RV8730**: Zimbabwe, Forest patch along rivulet along private 4-wheel track to Pungwe falls (Nyazengu Priv. Nat. Res.), approx. 2100 m a.s.l., Ploidy: 8*x*; *Viane* **RV11549**: South Africa, Western Cape, 26·5 km NE of Plettenberg, montane forest in Bloukranspass, approx. 600 m NW of bridge on Bloukransriver, approx. 150 m a.s.l., Ploidy: 4*x*; *Viane* **RV11561**: South Africa, Western Cape, approx. 7·5 km E of George, 3·4 km N of Wilderness, forest along George-Knyssna Rd, E bank of Silver River, approx. 120 m a.s.l., Ploidy: 4*x*; *Viane* **RV8300**: Reunion, NE of Cilaos, forest along GRR1, path to Caverne Dufour, above parking 'Le Bloc', approx. 1420 m a.s.l., Ploidy: 8*x*; *Viane* **RV8301**: Reunion, NE of Cilaos, forest along GRR1, path to Caverne Dufour, above parking 'Le Bloc', approx. 1420 m a.s.l., Ploidy: 8*x*; *Viane* **RV8302**: Reunion, NE of Cilaos, forest along GRR1, path to Caverne Dufour, above parking 'Le Bloc', approx. 1420 m a.s.l., Ploidy: 8*x*. ***A. sertularioides*** *Bellefroid* **EB348**: Uganda, Western Prov., Rwenzori Mountains National Park, Central Circuit Trail between Nyabitaba Hut and John Matte Hut, terrestrial along path in op patch between bamboo forest and *Hagenia abyssinica* montane cloud forest, approx. 2926 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB353**: Uganda, Western Prov., Rwenzori Mountains National Park, Central Circuit Trail between Nyabitaba Hut and John Matte Hut, terrestrial along path trough *Hagenia abyssinica* montane cloud forest, approx. 2926 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB358**: Uganda, Western Prov., Rwenzori Mountains National Park, Central Circuit Trail between Nyabitaba Hut and John Matte Hut, epilithic on rocks along Nyamleju River, Ericaceous zone, approx. 3234 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB360**: Uganda, Western Prov., Rwenzori Mountains National Park, Central Circuit Trail in vicinity of Guy Yeoman Hut, terrestrial, Ericaceous zone, approx. 3483 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB373**: Uganda, Western Prov., Rwenzori Mountains National Park, Central Circuit Trail in vicinity of Guy Yeoman Hut, terrestrial, Ericaceous zone, approx. 3483 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB383**: Uganda, Western Prov., Rwenzori Mountains National Park, Central Circuit Trail between Nyabitaba Hut an Guy Yeoman Hut, terrestrial, *Podocarpus* zone, approx. 2650 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB408**: Uganda, Kisoro District, Mgahinga Gorilla National park, Sabinyo Gorge, epiphytic, approx. 2464 m a.s.l., Ploidy: 4*x*; *Viane* **RV11131**: Uganda, Kasese Distr., eastern slopes of Rwenzori Mountains National Park, *Hagenia* zone between Nyabitaba Hut and John Matte Hut, epiphytic on *Hagenia*, approx. 2835 m a.s.l., Ploidy: 4*x*; *Viane* **RV11156**: Uganda, Kasese Distr., eastern slopes of Rwenzori Mountains National Park, *Erica arborea*–*Hypericum*–*Lobelia* vegetation above Guy Yeoman Hut, approx. 3450 m a.s.l., Ploidy: 4*x*. ***A. theciferum*** *Bellefroid* **EB238**: Zimbabwe, Eastern Prov., Bvumba Mountains, Bunga Forest, epiphytic, approx. 1610 m a.s.l., Ploidy: 12*x*; *Bellefroid* **EB244**: Zimbabwe, Eastern Prov., Bvumba Mountains, Bunga Forest, epilithic, approx. 1634 m a.s.l., Ploidy: 12*x*; *Bellefroid* **EB248**: Zimbabwe, Eastern Prov., Chimanimani Mountains National Park, near the waterfall left from the Mountain Hut, epilithic, approx. 1589 m a.s.l., Ploidy: 12*x*; *Bellefroid* **EB306**: Kenya, Coast Prov., Taita Hills, approx. 40 km west from Voi, Ngangao Forest, near the top with pine plantations, epiphytic on pine, approx. 1920 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB308**: Kenya, Coast Prov., Taita Hills, approx. 40 km west from Voi, Ngangao Forest, near the rocky top, approx. 1900 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB342**: Uganda, Kasese District, Rwenzori Mountains National Park, Central Circuit Trail in vicinity of Nyabitaba Hut, epiphytic in *Podocarpus* forest, approx. 2665 m a.s.l., Ploidy: 4*x*; *Bellefroid* **EB388**: Uganda, Kasese District, Rwenzori Mountains National Park, Central Circuit Trail between Guy Yeoman Hut and Nyabitaba Hut, epiphytic in *Podocarpus* forest, approx. 2700 m a.s.l., Ploidy: 4*x*;

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Bellefroid **EB395**: Uganda, Western Prov., montane rainforest along road from Kabale to Kisoro, epiphytic, approx. 2302 m a.s.l., Ploidy: 12*x*; *Bellefroid* **EB401**: Uganda, Kisoro District, montane rainforest along road from Kabale to Kisoro, epiphytic, approx. 2302 m a.s.l., Ploidy: 12*x*; *Bellefroid* **EB404**: Uganda, Kisoro District, Mgahinga Gorilla National park, Sabinyo Gorge, epiphytic, approx. 2464 m a.s.l., Ploidy: 8*x*; *Bellefroid* **EB407**: Uganda, Kisoro District, Mgahinga Gorilla National park, Sabinyo Gorge, epiphytic, approx. 2464 m a.s.l., Ploidy: 8*x*; *Viane* **RV7219**: Ethiopia, southern slopes of Mt Batu, degraded forest, along track, approx. 2320 m a.s.l., Ploidy: 4*x*; *Viane* **RV7541**: Tanzania, South of Arusha National Park (Mt Meru), forest with *Impatiens hochstetteri* along tack to Mamela Gate, approx. 1780 m a.s.l., Ploidy: 4*x*; *Viane* **RV10140**: Venezuela, Sierra de la Culata, roadside forest margin at La Vergara, approx. 2400 m a.s.l., Ploidy: 8*x*; *Viane* **RV10141**: Venezuela, Sierra de la Culata, roadside forest margin at La Vergara, approx. 2400 m a.s.l., Ploidy: 8*x*; *Viane* **RV10336**: Venezuela, Aragua, N slopes of Pico Codazzi, jeep track, forest with *Marattia* and *Didymochlaena*, approx. 1860 m a.s.l., Ploidy: 8*x*; *Viane* **RV11234**: Uganda, Kanungu Distr., Bwindi Impenetrable National Park, along track from Butogota to Ruhija, approx. 2325 m a.s.l., Ploidy: 12*x*; *Viane* **RV11488**: South Africa, E. Transvaal (Mpumalunga), approx. 3·5 km NNE of Graskop, montane forest on escarpment N of Driekopskloof, approx. 1480 m a.s.l., Ploidy: 12*x*; *Viane* **RV11494**: South Africa, E. Transvaal (Mpumalunga), approx. 3·5 km NNE of Graskop, montane forest on escarpment N of Driekopskloof, approx. 1480 m a.s.l., Ploidy: 12*x*; *Viane* **RV11500**: South Africa, KwaZulu Natal, Ngoma forest approx. 62·5 km E of Vryheid and 25·4 km NW of Nongoma, SSE exposed slopes, approx. 1040–1060 m a.s.l., Ploidy: 12*x*

APPENDIX 2

List of taxa and samples used for the molecular analysis. Species names vouchers and GenBank accession numbers are in bold.

Asplenium aethiopicum* (Burm.f.) Becherer subsp. *filare* (Forsk.) A.F.Braithw.** *Viane* **RV7416**: Kenya, Mt Elgon Nat. Park, *Hagenia-Erica* arborea zone, rocky outcrop along track, approx. 3290 m a.s.l., **GU586821**. ***A. caudatum* G.Forst.** *Siti Khadijah Rambe* **KH161**: Peninsular Malaysia, Bukit Larut, terrestrial, approx. 600 m a.s.l., **GU586818**. ***A. ceterach* L.** *Harris Chandra Pande* **107295**: India, Tangling Village, Pawari, Peo, Kinnaur, **GU586814**. ***A. laserpitiifolium* Lam. *s.l. *Viane* **RV9714**: Myanmar, Kachin State, Nam Sar Bung, sandy riverbed, approx. 450 m a.s.l., **GU586816**. ***A. nidus* L. *s.l.*** *Siti Khadjah Rambe* **KH162**: Peninsular Malaysia, Bukit Larut, epiphytic, approx. 1070 m a.s.l., **GU586813**. ***A. contiguum* Kaulf.** *Siti Khadijah Rambe* **KH196**: Indonesia, Nusa Tenggara Barat–Mataram, Mt Rinjani, terrestrial, approx. 2376 m a.s.l., **GU586819**. ***A. daucifolium* L. subsp. *lineatum* (Sw.) C.V.Morton** *Viane* **RV8220**: Réunion, Forêt de Bélouve, road to Gite, *Cryptomeria* plantation, approx. 1548 m a.s.l., **GU586808**. ***A. exiguum* Bedd.** *Viane* **RV9341**: China, Yunnan, Dêqên County, South of Dêqên, side valley of River Mekong, approx. 4 km North of Yanmen, approx. 2100 m a.s.l., **GU586826**. ***A. friesiorum* C.C**h. *Viane* **RV7715**: Tanzania, SE of Mt Kilimanjaro, rainforest between Mandara Hut and Mandara Gate, approx. 2360 m a.s.l., **GU586820**. ***A. loxoscaphoides*** *Bellefroid* **EB325**: Kenya, Mt Kasigau, route from Rukanga along water pipeline to top, epilithic, approx. 886 m a.s.l., **GU586802**; *Bellefroid* **EB339**: Kenya, Mt Kasigau, route from Rukanga along water pipeline for Kiteghe, at water intake, epilithic, approx. 1174 m a.s.l., **GU586801**; *Viane* **RV7548**: Tanzania, S. of Arusha National Park (Mt Meru), forest with *Impatiens hochstetteri* along tack to Mamela Gate, approx. 1780 m a.s.l., **GU586800**; *Viane* **RV7549**: Tanzania, S. of Arusha National Park, Mt Meru, forest with *Impatiens hochstetteri* along tack to Mamela Gate, approx. 1780 m a.s.l., **GU586803**. ***A. monanthes* L.** *Viane* **RV7368**: Kenya, Aberdare Nat. Park, Chania Falls, Hagenia-Hypericum zone, approx. 2950 m a.s.l., **GU586823**. ***A. normale* D.Don.** *Viane* **RV9705**: Myanmar, Kachin State, along track in forest West of Nan Hti village (East of Putao), approx. 710 m a.s.l., **GU586824**. ***A. onopteris* L.** *Viane* **RV8094**: Spain, La Palma, SE of Llano Negro, above Casas de las Palmeras, Pinar, approx. 1270 m a.s.l., **GU586792**. ***A. phyllitidis* D.Don.** *Siti Khadijah Rambe* **KH172**: Indonesia, Nusa Tenggara Barat–Mataram, Mt Rinjani, epiphytic, approx. 510 m a.s.l., **GU586812**. ***A. polyodon* G.Forst.** *Viane* **RV8545**: Réunion, NW exposed slope of 'Bras de la Plaine', Le Pont d'Yves, sentier de la Petite Ravine, approx. 615 m a.s.l., **GU586817**. ***A. ruta-muraria* L.** *Viane* **RV9553**: Italy, Mt Lessini, between Mte. Terrazzo and Psso Ristele, limestone rocks along '202', limestone rocks under *Pinus mugo*, approx. 1715 m a.s.l., **GU586793**. ***A. rutifolium*** *Viane* **RV6377C**: South Africa, N. Transvaal, below Mariepskop, 40 km N of Graskop, approx. 1250 m a.s.l., **GU586804**; *Viane* **RV8301**: Réunion, NE of Cilaos, forest along GRR1, path to Caverne Dufour, above parking 'Le Bloc', approx. 1420 m a.s.l., **GU586807**; *Bellefroid* **EB240**: Zimbabwe, Eastern Prov., Bvumba Mountains, Bunga Forest, epilithic, approx. 1622 m a.s.l., **GU586806**; *Viane* **RV8730**: Zimbabwe, Forest patch along rivulet along private 4-wheel track to Pungwe falls (Nyazengu Priv. Nat. Res.), approx. 2100 m a.s.l., **GU586805**. ***A. sandersonii* Hook.** *Viane* **RV7228**: Ethiopia, S slopes of Mt Batu, degraded forest, along track, approx. 1610 m a.s.l., **GU586811**. ***A. scolopendrium* L.** *Viane* **RVsn**: Belgium, Namur, Bauche, East of Yvoir, **GU586815**. ***A. tenerum* G.Forst.** *Siti Khadijah Rambe* **KH174**: Indonesia, Nusa Tenggara Barat–Mataram, Mt Rinjani, epiphytic, approx. 550 m a.s.l., **GU586810**. ***A. tenuicaule* Hayata** *Viane* **RV9991**: Russian Federation, Altay Rep., N exposed limestone-marble rocks in valley on left bank of Katun, approx. 580 m a.s.l., **GU586825**. ***A. theciferum*** *Bellefroid* **EB308**: Kenya, Coast Prov., Taita Hills, approx. 40 km west from Voi, Ngangao Forest, near the rocky top, approx. 1900 m a.s.l., **GU586796**; *Viane* **RV7541**: Tanzania, South of Arusha National Park, Mt Meru, forest with *Impatiens hochstetteri* along tack to Mamela Gate, approx. 1780 m a.s.l., **GU586799**; **Viane RV10140**: Venezuela, Sierra de la Culata, roadside forest margin at La Vergara, approx. 2400 m a.s.l., **GU586798**; *Viane* **RV10336**: Venezuela,

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Aragua, N slopes of Pico Codazzi, jeep track, forest with *Marattia* and *Didymochlaena*, approx. 1860 m a.s.l., **GU586794**; *Bellefroid* **EB238**: Zimbabwe, Eastern Prov., Bvumba Mountains, Bunga Forest, epiphytic, approx. 1610 m a.s.l., **GU586797**; *Bellefroid* **EB244**: Zimbabwe, Eastern Prov., Bvumba Mountains, Bunga Forest, epilithic, approx. 1634 m a.s.l., **GU586795**. ***A. thunbergii*** **Kunze** *Siti Khadijah Rambe* **KH176**: Indonesia, Nusa Tenggara Barat–Mataram, Mt Rinjani, epiphytic, approx. 550 m a.s.l., **GU586809**. ***A. trichomanes*** **L.** *Harris Chandra Pande* **106448**: India, Niti Village, Joshimath Pawari, Chamali, Uttaranchal, **GU586822**. ***A.* × *lessinense*** **Vida & Reichst.** *Viane* **RV9547**: Italy, Mt Lessini, Colle della Gazza, SE of Rifugio CA. Battisti, approx. 1195 m a.s.l., **GU586827**. ***H. excisum*** **(C.Presl) Tagawa & K.Iwats.** *Siti Khadijah Rambe* **KH17**: Indonesia, Jambi privince, Kerinci-Seblat National Park, Mt Kerinci, approx. 1675 m a.s.l., **GU586828**. ***H. unilaterale*** **(Lam.) Hayata** *Siti Khadijah Rambe* **KH165**: Malaysia, Pahang state, Tioman Island, Mt Kajang, epilithic, approx. 200 m a.s.l., **GU586829**.

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