Exploring natural hybridizations among *Asplenium ruprechtii* and related taxa in Korea

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(Received 30 May 2019; Revised 14 June 2019; Accepted 21 June 2019)

ABSTRACT: The purported four hybrid origins of *Asplenium* in Korea were tested based on morphological, cytological and DNA sequence data. *Asplenium castaneo-viride*, *A. × uiryeongse*, *A. × montanus*, and *A. × kitazawae* share several morphological characteristics with the Asian walking fern *A. ruprechtii* and related taxa as parents and show a sympatric distribution with the putative parents, raising the possibility of hybrid origins: *A. castaneo-viride* (*A. ruprechtii* and *A. incisum*), *A. × uiryeongse* (*A. ruprechtii* and *A. pekinense*), *A. × montanus* (*A. ruprechtii*, *A. trichomanes*, and *A. incisum*), and *A. × kitazawae* (*A. ruprechtii* and *A. sarelii*). We investigated flow cytometry and chloroplast DNA sequence data (*rbcL*, *rps4-trnS*, and *rps4-trnS* intergenic spacer) to clarify the hybridization and origin of each hybrid. In the flow cytometry analyses, *A. ruprechtii* shows diploid (2x) only, whereas *A. castaneo-viride* (3x, 4x), *A. × uiryeongse* (3x), *A. × montanus* (3x, 4x), and *A. × kitazawae* (2x, 4x) exhibit polyploidy, suggesting hybrid events along speciation. The *rbcL* and *rps4-trnS* and *rps4-trnS* intergenic spacer data suggest that *A. ruprechtii* is one of the maternal ancestors of all four hybrids. In addition, the *rps4-trnS* and *rps4-trnS* intergenic spacer data indicate that *A. incisum* is also the maternal ancestor of *A. × kitazawae* and *A. × montanus*, proposing multiple hybridization events for these two hybrids. In *A. × montanus*, morphological features such as the leaf forms and sympatric distributions of the species also support the multi-maternal hypothesis, but the morphological features of *A. × kitazawae* must be examined with consideration of hybrid events. To clarify the complex hybrid evolutionary lineages of the four *Asplenium* hybrids, further research with taxon sampling and molecular markers should be conducted.

Keywords: *Asplenium ruprechtii*, *Asplenium* hybrids, hybrid origin, cpDNA, flow cytometry, Aspleniaceae

The genus *Asplenium* L. (Aspleniaceae) is widely distributed throughout the temperate and tropical regions of all the continents except Antarctica, and comprises of approximately 700 species worldwide (Schneider et al., 2004a; Chang et al., 2013). Distinguishing characteristics of the genus are erect or shortly creeping rhizomes, dense scales throughout or on stipe bases, clustered or remote fronds, simple to 4-pinnate lamina and costa usually with all basal basiscopic veins, linear indusia, echinate spores, and $n = 36$ (Murakami and Schaal, 1994; Iwatsuki, 1995; Lin and Viane, 2012), especially compared with its related genus, *Hymenasplenium* (Murakami and Schaal, 1994; Hasebe et al., 1995; Murakami et al., 1999; Schneider et al., 2004b; Perrie and Brownsey, 2005). Fifteen

to twenty taxa of *Asplenium* are known in Korea (Park, 1975; T. B. Lee, 1980; Y. N. Lee, 2006; Kim and Sun, 2007; Lee and Lee, 2015, 2018). Among them, autopolyploid and allopolyploid in *Asplenium* are common and have been investigated on the possibility of hybrid speciation in some taxa (Rumsey et al., 2004; Ekrt and Štech, 2008; Chang et al., 2013).

Interspecific gene flow may proceed only to the formation of F_1 due to factors such as hybrid sterility. If hybrids reproduce, they may backcross with parents at the site of hybridization and create a hybrid swarm. Long-term hybrid swarms can be maintained in different ways (Anderson, 1949; Arnold, 1997). Spontaneous hybridization between the parental taxa might occur at a sufficiently high frequency to

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counterbalance selection against the hybrids and hybrid derivatives (Ellstrand et al., 2007). Alternatively, hybridization might be very rare, but the individuals of hybrid ancestry might be maintained largely by selective advantages (Ellstrand et al., 2007; Lee et al., 2012).

Asian walking fern, *Asplenium ruprechtii* Sa. Kurata, is characterized by simple leaf, almost smooth leaf margin, reticulate leaf vein; and produces a sterile hybrid or fertile allotetraploid hybrid with *A. incisum* Thunb. (Lovis et al., 1972; Ching and Iwatsuki, 1982). In Korean populations, it is hypothesized to form a hybrid, *A. castaneo-viride* Baker, between *A. ruprechtii* and *A. incisum*. Diverse ploidy levels of *A. castaneo-viride* has been reported: the populations of *A. castaneo-viride* in Mt. Mai and Mt. Duryun were diploid ($2n = 72$), whereas the population in Mt. Buram was tetraploid ($2n=144$) (Kwon et al., 2009). In addition to the above hybrid taxon, we have also found two new natural hybrids, *A. × uiryeongse* Lee & Lee between *A. ruprechtii* and *A. pekinense* Hance, and *A. × montanus* Lee & Lee which was postulated to be double hybrids between *A. ruprechtii* and *A. trichomanes* L., and one more with *A. incisum* (Lee et al., 2015). More recently, we have revealed unrecorded hybrid, *A. × kitazawae* Sa. Kurata & Hutoh, between *A. ruprechtii* and *A. sarelii* Hooker among 19 taxa in Korea (Lee et al., 2015).

Typical *A. × uiryeongse* has intermediate characteristics between the parents (*A. ruprechtii* and *A. pekinense*) such as a narrowly winged upper rachis, 1–2 pinnatifid lamina, shorter pinna stalk, shape of pinna and pinnule, and serration of indusium margins. *A. castaneo-viride* (*A. ruprechtii* and *A. incisum*) also exhibits parental morphology such as shape of basal pinna of lamina and preference of habitats (soils or rocks with soils). Intermediate characteristics of typical *A. × kitazawae* between the parents (*A. ruprechtii* and *A. sarelii*) and sympatric distribution with the parental species support hybrid origin. Shapes of scale in stipe bases, winged in upper rachis, 1–2 pinnatifid lamina, lamina quality, and shapes and margins of pinna strengthen the hybrid origin hypothesis (Lee and Lee, 2015, 2018; Lee et al., 2015).

Gross morphology of *A. × montanus* includes a winged upper rachis, one pinnate lamina, lamina quality, shape of pinna, and serration of indusium margins which are intermediate characteristics of the postulated parents (*A. ruprechtii*, *A. trichomanes*, and *A. incisum*). Documentation of hybridization has traditionally been based on morphological characters that are intermediate between the parental types or combine parental characters (Grant, 1981; Rieseberg et al., 1993). Hybrids can be identified easily by their aborted spores and intermediate morphology (Lovis and Reichstein, 1985;

Jessen, 1995; Ekrt and Štech, 2008).

DNA content is often used as a proxy for ploidy levels in comparative studies of chromosome number variation (e.g., Ceccarelli et al., 1995; Suda et al., 2006; Suda and Trávníček, 2006). Based on the assumption that DNA content varies with chromosome numbers when chromosome number increases due to whole- or partial-genome duplications (polyploidy or aneuploidy), this coupling of genome size with chromosome number should help to explain the ploidy levels of taxa and potential parental taxa of hybrids (Chung et al., 2012). Flow cytometry estimates reliable, comparative DNA content among samples using fresh leaves (Doležel and Bartoš, 2005).

DNA sequence data have been utilized to clarify parental taxa of hybrid origin groups. Chloroplast DNA has been instrumental in revealing interspecific hybridization and in documenting the hybrid origin region of several vascular plant species (Rieseberg, 1995; Arnold, 1997; Cronn et al., 2003; Lee et al., 2012). To infer maternal genetic histories *rbcL*, *rps4-trnS*, and *rps4-trnS* Intergenic spacers have provided critical information in many fern groups (Murakami et al., 1999; Skog et al., 2004; Wei et al., 2013).

In the present study, the postulated parental species of *Asplenium* hybrids are evaluated and hybrid processes are specified using cpDNA and cytological data. Putative parental species of *Asplenium* hybrid taxa have been hypothesized based on sympatric species and morphological characteristics: *A. × uiryeongse* (*A. ruprechtii* and *A. pekinense*), *A. castaneo-viride* (*A. ruprechtii* and *A. incisum*), *A. × kitazawae* (*A. ruprechtii* and *A. sarelii*), and *A. × montanus* (*A. ruprechtii*, *A. trichomanes*, and *A. incisum*). Flow cytometry evaluates a ploidy level for each taxon to clarify hybridization events, and cpDNA data reveal maternal lineages of each hybrid taxon. In addition, to reexamine the taxonomic delimitations and relationships of these taxa, we analyzed morphological, flow cytometry, and chloroplast DNA sequences data.

Materials and Methods

The sources of plant materials for morphology and DNA analysis including GenBank accession numbers are listed in Appendix 1. All voucher specimens were deposited at Ewha Womans University Herbarium (EWH). It was analyzed fourteen morphological characters observed and measured from mature individuals between each hybrid and the postulated parents of *Asplenium* (Lee et al., 2015).

Materials

Leaves used for DNA analyses were collected from the

natural populations that encompass all morphological and habitat diversity. A total of five species of *Asplenium* and four putative hybrid, *A. × castaneo-viride*, *A. × uiryeongse*, *A. × montanus*, and *A. × kitazawae* were sampled. Three species of *Asplenium*, *A. oblongifolium* (from New Zealand AY283231/EU240026), *A. lamprophyllum* (from New Zealand AY283230/EU240021), and *A. yoshinagae* (from China, AY725030/AY725045) were used as outgroup, we got each three accessions of *rbcL* and *rps4-trnS* regions from DNA bank (Perrie and Brownsey, 2005; Li and Lu, 2006; Shepherd et al., 2008).

Cytological data (flow cytometry)

Young, fresh leaves of each samples, which they were same populations for DNA samples as Appendix 2, were stored in plastic bags at 4°C and their DNA ploidy level was determined. Diploid sample of *Asplenium ruprechtii*, verified by chromosome counting, was used as an internal standard. About 100 mg of fresh young leaf tissue was chopped using a fresh razor blade, in a Petri dish containing 500 µL ice-cold nucleic extraction buffer (solution B kit, Partec, Münster, Germany). The suspension was filtered through CellTris (Partec). After incubation period (10 min at room temperature), the staining solution containing 2 mL of DAPI (solution B kit, Partec) was added. After staining, data analyzing was recorded using the cytometer, CyFlow Ploidy Analyser (Partec GmbH).

Ploidy levels size were estimated as the mean of individual counts for *A. × castaneo-viride*, *A. × uiryeongse*, *A. × kitazawae* and *A. × montanus*, and related parents' taxa comparing with means values of standard (*Asplenium ruprechtii*, 2x) (Yan et al., 2016). The coefficients of variation for each analyzed sample were calculated. Moreover, we selected less than 3 of coefficient of variation (CV), and less than 0.2 of relative standard error (RSE). Measurements were performed at least two to three times per individual on different days, and in each run 5,000 nuclei were recorded within both the standard and sample peaks. Mean and CV values of each peak were calculated using WinMDI 2.8 (Purdue University Cytometry Laboratories, West Lafayette, IN, USA). The Appendix 2 presents means and standard deviations of Mean peak, CV, and RSE values of the samples.

Molecular data (DNA extraction, sequencing, and analyses)

Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification, purifications, and DNA sequencing strategies followed (White et al., 1990). The amplification reaction

mixture was prepared using TaKaRa Ex Taq DNA polymerase (Takara Bio, Tokyo, Japan). For the cpDNA phylogeny, a total of 30 accessions, including three or two individuals of *A. × castaneo-viride*, *A. × uiryeongse*, *A. × kitazawae*, and *A. × montanus* for two cpDNA regions, *rbcL*, *rps4-trnS*, and *rps4-trnS* intergenic spacer, was analyzed.

Primers used for amplification and sequencing were *trnS* (TAC CGA GGG TTC GAA TC) (Souza-Chies et al., 1997; Smith and Cranfill, 2002) the *rps4F1* (RPS4F1 GCC GCT AGA CAA TTA GTC AAT C) (Hennequin et al., 2003) for part of the *rps4-trnS* gene and *rps4-trnS* intergenic spacer (Schneider et al., 2004a), *rbcL*-TKT-F1 and *rbcL*-TKT-R3N-2 for the *rbcL* gene (Ebihara et al., 2005). Amplification was conducted using a PTC-100 thermal cycler (MJ Research, USA) with the following temperature profile for all regions: a 32 to 37 cycle reaction with denaturalization at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 to 3 min. In addition, an initial denaturalization at 94°C for 2.5 min and a final extension at 72°C for 10 min were performed. PCR products were purified with AccuPrep PCR Purification Kit (Bioneer Inc, Daejeon, Korea). Automated sequencing analysis was performed using a sequencer (Base station, MJ Research, USA). Chloroplast DNA regions were each aligned with gap adjustments, using the Clustal X program, followed by manual adjustment (Gibson et al., 1994; Thompson et al., 1997). Each cpDNA region was analyzed using maximum parsimony (MP) by PAUP* (Swofford, 2002). Bootstrap values were calculated from 5,000 replicate analyses using tree bisection and reconnection branching swapping and simple stepwise addition of taxa (Felsenstein, 1985). PAUP* evaluated congruence the cpDNA sequence data using the incongruence length difference (ILD) test (Farris et al., 1994, 1995).

Results and Discussion

Morphological analysis

Asplenium × uiryeongse and its putative parental species, *A. ruprechtii* and *A. pekinense*, in natural habitats are shown in Fig. 1. Typical *A. × uiryeongse* has the intermediate characters of the parents such as 1–2 pinnatifid lamina, 1–1.5 mm stalk length of pinna, short toothed pinna, and slightly lacerate margins. Furthermore, *A. × uiryeongse* has some characteristics of *A. ruprechtii*, such as almost entire scale margins and linear lanceolate lamina. It also has characters similar to *A. pekinense*, such as lanceolate, long tailed apex of scales in stipe bases, firmly herbaceous lamina, and no gemma (Table 1) (Lee et al., 2015). Therefore, morphological data support *Asplenium*

Table 1. Comparative morphological characters of four hybrids and putative parents of *Asplenium*.

Characters	<i>A. ruprechtii</i>	<i>A. pekinense</i>	<i>A. x uiryongense</i>	<i>A. incisum</i>	<i>A. x castaneo-viride</i>	<i>A. sarelii</i> complex	<i>A. x kitazawae</i>	<i>A. trichomanes</i>	<i>A. x montanum</i>
Presence of gemma	Present	Absent	Absent	Absent	Absent	Absent	Absent	Present	Absent
Scale shape in stipe base	Deltoid-lanceolate, acuminate apex	Lanceolate, long tailed apex	Lanceolate, long tailed apex	Narrowly lanceolate, acute apex	Narrowly lanceolate, acute apex	Deltoid lanceolate, long tailed apex	Lanceolate, acute apex	Narrowly lanceolate, acute apex	Lanceolate, acute apex
Pinnation of lamina	NA	2-3 pinnatifid	1-2 pinnatifid	1-2 pinnate	1 pinnate	2-3 pinnatifid	1-2 pinnatifid	1 pinnate	1-pinnated
Shape of lamina	Linear or lanceolate	Deltoid or oblong	Linear lanceolate	Linear lanceolate	Oblanceolate to linear lanceolate	Deltoid or oblong	Oblong lanceolate	Linear lanceolate	Linear lanceolate
Quality of lamina	Sub-coriaceous	Firmly herbaceous	Firmly herbaceous	Thinly herbaceous	Firmly herbaceous	Herbaceous	Firmly herbaceous	Herbaceous	Firmly herbaceous
Stalk length of pinna (mm)	0	2-3	1-1.5	1-1.5	0-0.7	2-4	1-1.2	1-1.5	1-1.5
Shape of pinna	NA	Oblong or oblong-ovate	Oblong	Oblong or oblong-ovate	Oblong to oblong-ovate	Oblong or oblong-ovate	Oblong or oblong-ovate	Oblong or oblong-ovate	Oblong or oblong-ovate
Apex of pinna	NA	Shallowly acuminate	Obtuse	Obtuse	Obtuse	Shallowly acuminate	Shallowly acuminate or obtuse	Obtuse	Obtuse
Stalk length of pinnule (mm)	0	>1	>1	0-1	0	>1	0	0	0
Margin of pinnae or pinnule segment	None	Needle shaped, long toothed	Short toothed	Crenate	Crenate	Short toothed	Crenate	Crenate	Crenate
Length of indusium (mm)	1.5-4	1.5-3	1.5-3	1.5-3	1.5-3.5	1.0-1.5	1.0-1.5	1-1.5	1.5-3
Margin of indusium	Entire	Lacerate	Slightly lacerate	Almost entire	Entire	Entire	Entire	Lacerate	Lacerate
Shape of sorus	Linear	Linear	Linear	Oblong	Linear to oblong	Linear	Linear	Oblong-linear	Oblong-linear
Habitat	Rock	Rock	Rock	Soils or soiled rock	Rock	Rock	Rock	Rock	Soils or soiled rock

Fig. 1. Sympatric natural habitats of four *Asplenium* hybrids and putative parental species. **A.** *A. × uiryeongse* (1) with *A. ruprechtii* (2) and *A. pekinense* (3). **B.** *A. × castaneo-viride* (4) with *A. ruprechtii* (2) and *A. incisum* (5). **C.** *A. × kitazawae* (6) with *A. ruprechtii* (2) and *A. sarelii* complex (7). **D.** *A. × montanus* (8) with *A. ruprechtii* (2), *A. trichomanes* (9), and *A. incisum* (5).

× uiryeongse as hybrid between *A. ruprechtii* and *A. pekinense*.

A. castaneo-viride (*A. ruprechtii* and *A. incisum*) has intermediate characters of the parental species such as herbaceous lamina, short toothed margin of pinnae, and almost entire margins of indusia (Fig. 1, Table 1). In the previous study, the morphological characters such as involving leaves, spores, epidermal cells, stomata and chromosome number support the hybrids between *A. ruprechtii* and *A. incisum* (Kwon et al., 2009). The accumulated morphological data clarify the hybrid origin of *A. castaneo-viride* from *A. ruprechtii* and *A. incisum*.

A. × kitazawae and its putative parental species, *A. ruprechtii* and *A. sarelii*, are presented in Fig. 1 and Table 1. Typical *A. × kitazawae* has intermediate characters of the parents such as acute apex of scale in stipe, 1–2 pinnatifid lamina, firmly herbaceous lamina, 1 mm pinna length, and crenate pinnae. The hybrid has both parental characters. Some characteristics such as no pinnule and lanceolate lamina share with of *A.*

ruprechtii, and oblong or oblong-ovate pinna, shallowly acuminate pinna apex, 1–1.5 mm length of indusia, and missing gemma are similar to *A. sarelii* complex (Table 1) (Lee et al., 2015). Therefore, morphological data support *A. × kitazawae* as the hybrid between *A. ruprechtii* and *A. sarelii*.

A. × montanus and its putative three parental species, (*A. ruprechtii*, *A. trichomanes*, and *A. incisum*) in natural habitats are exhibited in Fig. 1. Typical *A. × montanus* has intermediate characters of the parents such as firmly herbaceous lamina, 1–3 mm length of indusia. *A. × montanus* has some characteristics of *A. incisum*, such as no gemma, habitat on soils or rock. It also has characters similar to *A. trichomanes*, such as 1-pinnated lamina, no pinnule, lacerate margin of indusia, and oblong-linear sori (Table 1) (Lee et al., 2015). Therefore, morphological data support *A. × montanus* as hybrid between *A. ruprechtii* and *A. trichomanes*, and once more hybridization between *A. incisum*.

Hybrids are known to have morphological characters intermediate between their parents and/or combining characters

from each of the parents, and sometimes the hybrids display characters not found in either parent (Grant, 1981; Rieseberg et al., 1993). Our morphological results could conserve the hybridization from putative parental taxa.

Ploidy level analysis

Flow cytometry analysis detected diploid, triploid, tetraploid, and hexaploid plants (Figs. 2, 3, Table 2, Appendices 1, 2). Ploidy levels were estimated as the mean

Fig. 2. Flow cytometry histograms of two hybrids, *Asplenium* × *uiryeongse* (*A. ruprechtii* and *A. pekinense*), and *A. × castaneo-viride* (*A. ruprechtii* and *A. incisum*).

Fig. 3. Flow cytometry histograms of two hybrids, *Asplenium* × *kitazawae* (*A. ruprechtii* and *A. sarelii*) and *A. × montanus* (*A. ruprechtii*, *A. trichomanes*, and *A. incisum*).

Table 2. Nuclear DNA content and diploid level in examined populations of between the four hybrid taxa and related taxa within the genus *Asplenium*.

Taxon	Population (number of individuals)	Peak (mean $\pm$ SD)	Ploidy level estimated
<i>A. ruprechtii</i>	Bulamsan Mt. (1), Bukhansan Mt. (4)	9,390.40 $\pm$ 370.92	2x
<i>A. pekinense</i>	Bukhansan Mt. (3), Goryeong (2)	26,726.00 $\pm$ 2,421.59	4x
<i>A. $\times$ uiryeongse</i>	Bukhansan Mt. (1)	16,551.00 $\pm$ na	3x
<i>A. incisum</i>	Bukhansan Mt. (2), Bulamsan Mt. (1)	15,002.00 $\pm$ 2,775.49	2x
<i>A. $\times$ castaneo-viride</i>	Bulsamsan Mt. (2), Bulamsan Mt. (2)	20,080.25 $\pm$ 3,414.04	4x
<i>A. sarelii</i> complex	Daegu (2), Cheongdo (2)	30,740.50 $\pm$ 1,840.66	6x
<i>A. $\times$ kitazawae</i>	Daegu (2)	11,378.00 $\pm$ 193.75	2x
<i>A. $\times$ kitazawae</i>	Daegu (1)	23,194.00 $\pm$ na	4x
<i>A. trichomanes</i>	Yeoncheon (2)	26,412.50 $\pm$ 673.87	4x
<i>A. incisum</i>	Yeoncheon (1)	15,952.50 $\pm$ 3,160.06	2x
<i>A. $\times$ montanus</i>	Yeoncheon (6)	23,509.33 $\pm$ 379.89	4x

of individual counts for each taxon.

Five samples of *Asplenium ruprechtii* from two localities (Bulamsan Mt. and Bukhansan Mt.) were determined as diploids (Fig. 2, Table 2, Appendix 2). The species occurs throughout the Korea peninsula and is hypothesized as one of parental species of all the hybrids (Lee et al., 2015; Lee and Lee, 2018). The flow cytometry analyses clarified the ploidy level of the species as only a diploid.

The hybrid, *A. $\times$ uiryeongse* observed in the Bukhansan Mt. site, was a triploid (Fig. 2, Table 2, Appendix 2). Five samples of *A. pekinense*, the sympatric species, from two natural populations (Bukhansan Mt. and Goryeong) were tetraploids. This result fail to reject the hybrid hypothesis of *A. $\times$ uiryeongse*, derived from its putative and sympatric parental species, *A. ruprechtii* and *A. pekinense* (Lee et al., 2015).

Four samples of *A. $\times$ castaneo-viride* from two localities (Bulamsan Mt. and Bukhansan Mt.) were tetraploids in the flow cytometry analyses (Fig. 2, Table 2, Appendix 2). The results are incongruent from the previous reports. The hybrid was known as two ploidy levels: $2n = 72$ (2x) from Maisan Mt. and Dureunsan Mt. populations (Kwon et al., 2009) and $2n = 144$ (4x) from Bulamsan Mt. populations (Lee et al., 2015). Five samples of *A. incisum* from the same two localities (Bulamsan Mt. and Bukhansan Mt.) show diploids in the flow cytometry analyses (Fig. 2, Table 2, Appendix 1).

A. $\times$ kitazawae and its putative parental species, *A. ruprechtii* (2x) and *A. sarelii* complex (6x), were diploids or allotetraploids. Four samples of *A. sarelii* complex from two localities (Daegu and Cheongdo) were hexaploidy (Fig. 3, Table 2, Appendix 1). *A. sarelii* has been known as a diploid

in China and tetraploids in Korea and Japan, but tetraploid *A. anogrammoides* only distributes in Korea (Lin and Viane, 2012). However, *A. anogrammoides* known as tetraploid in Korea were observed as a hexaploid in this study. It needs to conduct further research on Korean *A. sarelii* complex, including *A. sarelii*, *A. anogrammoides*, and *A. pekinense* covering all the distribution areas.

Six samples of *A. $\times$ montanus* from one locality (Yeoncheon) and its putative three parental species (*A. trichomanes*, *A. ruprechtii*, and *A. incisum*) were tetraploids (Fig. 3, Table 2, Appendix 2). Based on individuals with intermediate characters between *A. ruprechtii* and *A. trichomanes* and hybrid individuals with ecological characters of with *A. incisum*, double hybridization events have been hypothesized (Lee et al., 2015). These FCM results fail to deny the hypotheses that the hybrid, *A. $\times$ montanus* came out from its putative parental species, *A. ruprechtii* and *A. trichomanes*; and once more hybridized with *A. incisum* (Lee et al., 2015).

These flow cytometry results could sustain the hybridization among putative, sympatric parental taxa based on comparative genome size (ploidy levels).

Molecular data analysis

The aligned length of *rbcL* region is 1,205 bp, and the aligned length of *rps4* gene and *rps4-trnS* intergenic spacer (IGS) region is 753bp. Of the 1,205 aligned *rbcL* region, 1,051 sites (87.2%) are identical, 43 sites (3.56%) are parsimony uninformative, and 111 sites (9.2%) provide phylogenetic information. MP analysis of the entire *rbcL* sequences finds 248 equally most parsimonious trees with a tree length (TL)

Fig. 4. Phylogenetic tree of four hybrids of *Asplenium* and related taxa deduced from *rbcL* data. Bootstrap values (>50%) by maximum parsimony method are shown above branches. *Asplenium oblongifolium* and *A. lamprophyllum* are used as outgroups.

of 191, a consistency index (CI) of 0.8534 (0.8100 excluding uninformative characters) and a retention index (RI) of 0.9622. One of 191 equally most parsimonious trees is shown in Fig. 4 and identical to the ones treating gaps with missing data.

In the 753 bp aligned *rps4* gene and *rps4-trnS* IGS region, 550 sites (73%) are identical, 43 sites (5.7%) are parsimony uninformative, and 160 sites (21.2%) provide phylogenetic information. MP analysis of the entire *rps4* gene and *rps4-trnS* IGS sequences finds 267 equally most parsimonious trees with a TL of 6, with a high CI (0.8764 or 0.8520 excluding uninformative characters) and RI (0.9513). One of 6 equally most parsimonious trees is Fig. 5.

The bounded DNA data of chloroplast genes *rbcL* and *rps4* gene and *rps4-trnS* IGS for 25 accessions are ranged from 1,839 bp in *A. trichomanes* to 1,920 bp in *A. incisum*. The ILD p-value for the combined data set = 0.01, failing to prove that the data sets are homologous. The incongruence of the two cpDNA data sets suggests diverse, complex evolutionary

history of maternal lineages. For the incongruence of the data sets, the combined cpDNA data is excluded in further phylogenetic analyses.

In the *rbcL* analyses, *A. x ruprechtii* and four hybrids (*A. x uiryeongse*, *A. castaneo-viride*, *A. x kitazawae*, and *A. x montanus*) form a clade with the strong support of a 100% bootstrap value (Fig. 4). However, *A. ruprechtii* exhibits three haplotypes refracting geographic patterns: Japan, Germany, and Korea types. Among the three haplotypes, Germany and Japan types form polytomy with *A. x uiryeongse*, *A. x kitazawae*, and *A. x montanus*; and Korean type is nested with *A. x castaneo-viride*. Especially, in MP trees, *A. x castaneo-viride* nested strongly with Korean *A. ruprechtii* with a 100% bootstrap value (Fig. 4). In addition, the same haplotype is found in *A. ruprechtii* (individuals 11, 19), which is identical to three individuals of *A. x castaneo-viride* (individuals 12, 15, and 16). From these results, we suggest that *A. ruprechtii* is a maternal parent of four hybrids.

Fig. 5. Phylogenetic tree of four hybrids of *Asplenium* and related taxa deduced from *rps4-trnS* and *rps4-trnS* intergenic spacer data. Bootstrap values (> 50%) by maximum parsimony method are shown above branches. *Asplenium oblongifolium* and *A. lamprophyllum* are used as outgroups.

A. incisum shows two haplotypes whereas only one haplotype is found in *A. trichomanes*. The sequencing results obtained from DNA bank are similar to the newly generated data from a Korean population, but one accession (AB574866) of *A. pekinense* from Japan is nested with *A. anogrammoides* (AB853879) from Japan. Two accessions of *A. sarelii* (AB574873 and AB014693) from Japan are nested with Korean *A. anogrammoides* (30 and 33). It suggests that *A. sarelii* and *A. anogrammoides* from Japan might be confused. Two species has been recognized as *A. sarelii* only. However, *A. sarelii* exhibits 2x (China), and *A. anogrammoides* is 4x (Japan) (Wang et al., 2003). In addition, it is revealed for the first time that *A. anogrammoides* in Korea is 6x. In the phylogenetic tree, *A. sarelii* and *A. anogrammoides* fail to prove the monophyly (Fig. 5). The taxa have been problematic in terms of morphological delimitation of the species. These results show to need the reexamination of these groups.

As in the *rbcL* data, *A. ruprechtii* forms a clade with *A. x uiryeongse* and *A. castaneo-viride* in the *rps4* gene and *rps4-trnS* IGS region. The haplotype of two hybrids (*A. castaneo-*

viride and *A. x uiryeongse*) make a monophyletic group with a 100% bootstrap value (Fig. 5). From these results, we confirm that *A. ruprechtii* is the maternal parent of *A. x uiryeongse* and *A. castaneo-viride*. However, not like in the *rbcL* topology, *A. x montanus* is distinctly nested with *A. incisum* as well as *A. x kitazawae*. It suggests that *A. incisum* should be another maternal parent of *A. x montanus* and *A. x kitazawae*. *A. x kitazawae* has been considered as the hybrid of *A. ruprechtii* and *A. sarelii* complex (*A. sarelii* and *A. anogrammoides*), but our results suggest another maternal lineage of *A. incisum*. In addition, because of unclear taxon delimitation of *A. sarelii* complex, it is crucial to examine *A. x kitazawae* hybrid origins including *A. sarelii* complex. To clarify taxonomic statuses of *A. x kitazawae* and *A. sarelii*, further study with broader samplings covering distribution areas are preferred.

The cpDNA data suggest that maternal parents of four hybrids (*A. castaneo-viride*, *A. x uiryeongse*, *A. x kitazawae* and *A. x montanus*) are *A. ruprechtii* in common, and *A. incisum* should be another maternal parent of *A. x kitazawae* and *A. x montanus*. *A. x kitazawae* and *A. x montanus* might

have been hybridized more than once. These results are also supported by morphological features (Lee et al., 2015), chromosome numbers, and spore characters (Kwon et al., 2009), except for *A. × kitazawae*. Further research with molecular markers and taxon sampling should be conducted to clarify both paternal and maternal lineages of the hybrids.

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Acknowledgments

We thank Dr. Adjoa Ridhardson Ahedor at Rose State College for critical reading and comments on the earlier version of the manuscript. Flow Cytometry analyses were conducted at National Institute of Horticultural and Herbal Science with the technical assistance of H.-So. Kim (Breeding Technology Service Team).

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Appendix 1. Collection data of four natural hybrid *Asplenium* taxa and related taxa studied in molecular analyses (*DNA accession No. used from GenBank). Voucher specimens were deposited at Ewha Womans University Herbarium (EWH). Numbers in bold after each accession number are the same as in Figs. 2–4. GenBank accession numbers listed in the following order: *rbcL*, *rps4-trnS*, and *rps4-trnS* intergenic spacer.

- Asplenium ruprechtii* Sa. Kurata: [11], KOREA. Seoul: Bulamsan Mt., 16 Apr 2014, C. S. Lee 14041601 (EWH), MK774627, *MK774644; [19], Seoul: Bukhansan Mt., 19 May 2014, C. S. Lee 14051904 (EWH), MK774628, *MK774645.
- A. × castaneo-viride* Baker: [12, 15, 16], KOREA. Seoul: Bulamsan Mt., 16 Apr 2014, C. S. Lee 14041611-13 (EWH), MK774629, MK774630, MK774631, *MK774646, *MK774647, *MK774648.
- A. × uiryeongse* C. S. Lee & K. Lee; [21], KOREA. Seoul: Bukhansan Mt., 19 May 2014, C. S. Lee 14051906 (EWH), MK774632, *MK774649; [29], 12 Jun 2014, C. S. Lee & K. Lee 14061201 (EWH), MK774632, *MK774649.
- A. × montanus* C. S. Lee & K. Lee; [35, 36], KOREA. Gyeonggi-do: Yeoncheon-gun, 20 Sep 2014, C. S. Lee & K. Lee 14092005-6 (EWH), MK774615, MK774616, *MK774636, *MK774637.
- A. × kitazawae* Sa. Kurata & Hutoh in Kurata; [42, 43], KOREA. Gyeongsangbuk-do: Daegu, 25 Sep 2014, C. S. Lee & K. Lee 14092505-6 (EWH), MK774634, MK774635, *MK774651, *MK774652.
- A. incisum* Thunb.; [13, 14], KOREA. Seoul: Bulamsan Mt., 16 Apr 2014, C. S. Lee 14041605-06 (EWH), MK774620, MK774621, *MK995098, *MK995099; [41], Gyeongsangbuk-do: Daegu, 25 Sep 2014, C. S. Lee & K. Lee 14092508-09 (EWH), *MK995100; [73], Gyeonggi-do: Yeoncheon-gun, 20 Sep 2014, C. S. Lee & K. Lee 14092011 (EWH), MK774622, *MK774639.
- A. pekinense* Hance; [17, 18], KOREA. Seoul: Bukhansan Mt., 19 May 2014, C. S. Lee 140519010-11 (EWH), MK774623, MK774624, *MK774640, *MK774641.
- A. trichomanes* L.; [39, 40, 72], KOREA. Gyeonggi-do: Yeoncheon-gun, 20 Sep 2014, C. S. Lee & K. Lee 14092015-7 (EWH), MK774618, MK774619, MK774617, *MK774638.
- A. anogrammoides* (*A. sarelii* complex); [30, 33], KOREA. Gyeongsangbuk-do: Daegu, 25 Sep 2014, C. S. Lee & K. Lee 14092511-12 (EWH), MK774625, MK774626, *MK774642, *MK774643.

Appendix 2. Flow cytometry data of the four natural hybrids *Asplenium* taxa and related taxa studied in molecular analyses.

Taxon	Individual (Voucher)	Peak	CV (%)	RSE
<i>A. ruprechtii</i>	Bulamsan Mt. (20140401)	9,043	2.63	0.0797
	Bukhansan Mt. (20140501)	9,278	2.76	0.0413
	Bukhansan Mt. (20140502)	9,288	2.45	0.037
	Bukhansan Mt. (20140503)	9,319	2.5	0.043
	Bukhansan Mt. (20140504)	10,024	2.3	0.0506
<i>A. pekinense</i>	Bukhansan Mt. (20140505)	28,496	1.9	0.0458
	Goryeong (20140901)	28,370	1.75	0.0408
	Goryeong (20140902)	28,138	1.74	0.0306
	Bukhansan Mt. (20140506)	25,724	1.83	0.1114
	Bukhansan Mt. (20140507)	22,902	2.69	0.1474

Appendix 2. Continued.

Taxon	Individual (Voucher)	Peak	CV (%)	RSE
<i>A. × uiryongse</i>	Bukhansan Mt. (20140508)	16,551	2.76	0.0955
<i>A. incisum</i>	Bukhansan Mt. (20140509)	18,187	1.83	0.0288
	Bukhansan Mt. (20140510)	13,718	2.37	0.0509
	Bulamsan Mt. (20140402)	13,101	2.7	0.1214
<i>A. × castaneo-viride</i>	Bulamsan Mt. (20140403)	17,039	2.87	0.1208
	Bulamsan Mt. (20140404)	17,211	2.91	0.1014
	Bulamsan Mt. (20140511)	23,131	1.81	0.0411
	Bulamsan Mt. (20140512)	22,940	1.62	0.0417
<i>A. sarelii</i> complex	Daegu (20140903)	32,320	2.02	0.0653
	Daegu (20140904)	31,117	2.21	0.0542
	Cheongdo (20140905)	28,087	2.52	0.0541
	Cheongdo (20140906)	31,438	1.62	0.0376
<i>A. × kitazawae</i>	Daegu (20140907)	11,515	2.2	0.0359
	Daegu (20140908)	11,241	2.36	0.0398
	Daegu (20140909)	23,104	2.4	0.0754
<i>A. trichomanes</i>	Yeoncheon (20140911)	26,889	2.8	0.0872
	Yeoncheon (20140912)	25,936	2.65	0.084
<i>A. incisum</i>	Bukhansan Mt. (20140511)	18,187	1.83	0.0288
	Bukhansan Mt. (20140512)	13,718	2.37	0.0509
<i>A. × montanus</i>	Yeoncheon (20140913)	24,116	1.73	0.0663
	Yeoncheon (20140914)	23,153	1.89	0.0302
	Yeoncheon (20140914-1)	23,153	1.89	0.0302
	Yeoncheon (20140915)	23,308	2.18	0.0448
	Yeoncheon (20140916)	23,740	2.02	0.0436
	Yeoncheon (20140913-1)	23,586	1.61	0.0419

CV, coefficient of variation; RSE, relative standard error.