



Evolutionary relationships of the ancient fern lineage the adder's tongues (Ophioglossaceae) with description of *Sahashia* gen. nov

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Abstract

As an ancient lineage of ferns, Ophioglossaceae are evolutionarily among the most fascinating because they have the highest chromosome count of any known organism as well as the presence of sporophores, subterranean gametophytes, eusporangioid sporangia without annuli, and endophytic fungi. Previous studies have produced conflicting results, identifying some lineages with unresolved relationships, and have paid much attention to the subfamily Botrychioideae. But the other species-rich subfamily, Ophioglossoideae, has remained largely understudied and only up to 12 accessions of Ophioglossoideae have been sampled. In this study, DNA sequences of seven plastid markers of 149 accessions (75 in Ophioglossoideae) representing approximately 82 species (approximately 74% of estimated species diversity sensu J. Syst. Evol., 2016, 54, 563) in the family, and two Marattiaceae and two Psilotaceae, are used to infer a phylogeny. Our major results include: (1) Ophioglossaceae are resolved as monophyletic with strong support, and so are all four subfamilies and genera sensu PPG I except *Botrypus* and *Ophioglossum*; (2) a new genus *Sahashia* is segregated from *Botrypus* so that the monophly of *Botrypus* can be retained; (3) the monophly of *Ophioglossum* in its current circumscription is uncertain in spite of our large character sampling; (4) there is substantial cryptic speciation in *Ophioderma* detected by our molecular and morphological study; (5) the recognition of *Holubiella* is advocated based on its morphology and its sister relationship with *Sceptridium*; and (6) a novel sister relationship between *Botrychium* and the JHS clade (*Japanobotrychium* + (*Holubiella* + *Sceptridium*)) is discovered.

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Introduction

Ophioglossaceae, known as the adder's tongues, are among the most fascinating fern lineages. They are

special in ferns and even in all organisms in several aspects. First, some species of *Ophioglossum* (e.g., *O. reticulatum*) have the highest chromosome count of any known organism, with up to 960 (Wang, 1986),

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1260 (Löve 1977), or even 1440 chromosomes (Ghatk, 1977), possibly representing an evolutionary dead-end evolved through repeated cycles of polyploidy (Khandelwal, 1990). Second, together with Psilotaceae they are the second earliest-diverging lineage of ferns (only after the horsetails) with its stem age having been dated to approximately 250 (Rothfels et al., 2015) or even 369 (Testo and Sundue, 2016) million years old. Third, some species of the family in their current definitions are among the most widely distributed vascular plants, and their morphology remains in almost the same form throughout the world, which is practically unparalleled in the other fern groups (Clausen, 1938; Wagner, 1990). Fourth, together with Psilotaceae and Marattiaceae, Ophioglossaceae are the only ferns with eusporangiate sporangia, subterranean gametophytes, and endophytic fungi. Fifth, the presence of sporophores in ferns is unique to Ophioglossaceae (Wagner, 1990; Sun et al., 2001; Hauk et al., 2003).

There are approximately 112 Ophioglossaceae species worldwide (PPG I, 2016), but there is no modern revision of the family and the last revision was published by Clausen (1938) 91 years ago. Species of Ophioglossaceae (except epiphytic ones) are often associated with disturbance and especially early to middle succession stages. Even those in mature forests are favoured by disturbances (Wagner, 1990).

Since the entire aerial plant is often represented only by a single leaf, few characters are available for taxonomy, which leads to extraordinary difficulty in taxonomy at nearly all levels. The taxonomy of this lineage has changed over time and different botanists have recognized different numbers of families. Some botanists recognized three families within the fern order Ophioglossales (e.g., Nishida, 1952; Ching et al., 1959; Ching, 1978) or the class Ophioglossopsida (Pichi Sermolli, 1977), whereas most researchers have accepted a broadly defined Ophioglossaceae *s.l.* (hereafter simply Ophioglossaceae) in the order (e.g., Presl, 1845; Clausen, 1938; Wagner, 1990; Smith et al., 2006; PPG I, 2016). The narrowly defined families, Botrychiaceae, Helminthostachyaceae, and Ophioglossaceae *s.s.*, recognized by Ching et al. (1959), Pichi Sermolli (1977), and Ching (1978), have been dated to have diverged approximately 154, 169, and 179 million years ago from their sisters, respectively (Testo and Sundue, 2016), ages older than those of approximately one-third of the families of ferns (Testo and Sundue, 2016) recognized by PPG I (2016).

Botanists have often disagreed on how many genera should be recognized in the order Ophioglossales. Presl (1845) recognized six genera: *Botrychium*, *Cheiroglossa*, *Helminthostachys*, *Ophioderma*, *Ophioglossum*, and *Rhizoglossum*. In his comprehensive monograph, Clausen (1938) recognized only the following three genera:

Botrychium *s.l.*, *Helminthostachys*, and *Ophioglossum* *s.l.*; he treated *Botrypus* and *Sceptridium* as subgenera of *Botrychium*, *Japanobotrychium* as a section of *B.* subg. *Osmundopteris* (Milde) R.T. Clausen (= *Botrypus*), and *Cheiroglossa*, *Ophioderma*, and *Rhizoglossum* as subgenera of *Ophioglossum* *s.l.* Nishida (1952) divided this group of ferns into eight genera: *Botrychium*, *Osmundopteris*, *Sceptridium* (Botrychiaceae), *Helminthostachys* (Helminthostachyaceae), and *Cheiroglossa*, *Ophioderma*, *Ophioglossum*, and *Rhizoglossum* (Ophioglossaceae). Ching et al. (1959) followed Presl's (1845) classification at generic level when treating species of China, then Ching (1978) adopted Nishida's (1952) classification but replaced the illegitimate *Osmundopteris* with *Botrypus*. Pichi Sermolli (1977) also accepted all genera except *Sceptridium* recognized by Nishida (1952). Kato (1987) basically agreed with Nishida's (1952) and Ching's (1978) subdivision of *Botrychium* *s.l.* into three genera and additionally advocated for the recognition of *Japanobotrychium*, but partially went back to Clausen (1938) by recognizing a broadly-defined *Ophioglossum* *s.l.* Wagner (1990) went back even further to Clausen (1938) by recognizing three genera only in the family. Later Wagner (1992) described a new section of *Botrychium*, *B.* sect. *Hiemobotrychium* W.H.Wagner, which was raised to a genus by Škoda (1997), *Holubiella* Škoda, but this genus has never been recognized by other botanists so far. The Korean endemic monospecific genus *Mankyua* was added to the family in 2001 (Sun et al., 2001). Smith et al. (2006) recognized the three classical genera and *Mankyua*. Christenhusz et al. (2011) added *Cheiroglossa* to Smith et al. (2006) classification of the family. PPG I (2016) accepted 10 genera in four subfamilies and one family: *Botrychium*, *Botrypus*, *Sceptridium* (Botrychioidae), *Helminthostachys* (Helminthostachyoidae), *Mankyua* (Mankyoideae), and *Cheiroglossa*, *Ophioderma*, *Ophioglossum*, and *Rhizoglossum* (Ophioglossoideae), which was very similar to Ching's (1978) classification but treated Ching's (1978) three families as subfamilies and added *Japanobotrychium* and *Mankyua*.

Molecular studies so far based on limited taxon and character sampling helped resolve some relationships in the family, but also produced some conflicting results and identified some lineages with unresolved relationships. The *rbcL* sequences of five samples representing five genera by Hasebe et al. (1995) resolved *Ophioglossum* as sister (MPBS: 65%) to the rest of the family, followed by *Helminthostachys* which was sister to *Botrychium* + (*Botrypus* + *Sceptridium*). Based on *rbcL* and *trnL-F* of 35 species Hauk et al. (2003) recovered the same results as those of Hasebe et al. (1995) in terms of the relationships among *Botrychium*, *Botrypus*, *Helminthostachys*, *Ophioglossum* *s.l.*, and *Sceptridium*, but made a number of new discoveries including (1) the sister relationship between *Cheiroglossa* and *Ophioderma*; (2) *Cheiroglossa*

and *Ophioderma* together being sister to *Ophioglossum* s.s.; and (3) the paraphyly of *Botrypus* sensu Kato (1987). In addition, Hauk et al. (2003) identified an unresolved trichotomy composed of *Ophioglossum crotalophoroides*, *Ophioglossum costatum* + *Ophioglossum gomezianum*, and the rest of *Ophioglossum* s.s. The paraphyly of *Botrypus* was confirmed by Sun et al. (2009) based on *rbcL* data who also found that *Mankyua* (included for the first time), *Helminthostachys*, and *Botrychium* s.l. formed a grade (without strong support) sister to *Ophioglossum* s.l., and that *Cheiroglossa* and *Ophioderma* were paraphyletic (low support) in relation to *Ophioglossum* s.s., in conflict with the resolution found by Hauk et al. (2003). Based on *rbcL* data of 19 species (22 accessions), Shinohara et al. (2013) found that (1) *Mankyua* was sister (MLBS: 66%; BIPP: 0.77) to the rest of the family, followed by *Helminthostachys* which was sister (MLBS: 64%; BIPP: 0.8) to a clade containing *Botrychium* s.l. and *Ophioglossum* s.l.; (2) *Japanobotrychium* (included for the first time) was sister to *Sceptridium*; and (3) *Ophioglossum costatum* and the rest of *Ophioglossum* s.s. were paraphyletic in relation to a clade containing *Cheiroglossa* and *Ophioderma*. However, based on *matK* data of the same samples, Shinohara et al. (2013) showed that *Mankyua*, *Helminthostachys*, and *Ophioglossum* s.l. formed a grade (strong support) sister to *Botrychium* s.l. and that *Ophioglossum costatum* and the rest of *Ophioglossum* s.s. were monophyletic (weak support). *Botrychium* s.s. was not included in the study of Shinohara et al. (2013).

The previous molecular studies paid a lot of attention to Botrychioideae (Hauk et al., 2012; Dauphin et al., 2014, 2016, 2017; Williams et al., 2016), but Ophioglossoideae has remained very much understudied and only up to 12 samples/species of Ophioglossoideae were included in previous studies (Hauk et al., 2003; Shinohara et al., 2013).

In this study, we aimed: (1) to reconstruct a new phylogeny of Ophioglossaceae based on a largely expanded taxon sampling, especially that of Ophioglossoideae (*Ophioglossum* s.l.); (2) to solve the previously reported conflicting relationships based on different molecular markers by largely expanding character sampling; (3) to resolve the relationships of major clades, with special attention paid to the relationships of *Helminthostachys* and *Mankyua*; and (4) to further test the monophyly and to resolve the relationships of individual genera in the family, especially those of *Botrypus*, *Holubiella*, and *Ophioglossum*.

Materials and methods

Taxon sampling

One hundred and forty-nine accessions representing approximately 84 species of nine out of the 10 genera in Ophioglossaceae covering

approximately 74% of the estimated 112 species in the family by PPG I (2016). In detail, our sampling included 21 accessions representing 19 species of *Botrychium*, 10 accessions representing approximately three species of *Botrypus*, two accessions representing the only two species of *Cheiroglossa*, seven accessions representing the only species of *Helminthostachys*, four accessions representing three species of *Japanobotrychium*, four accessions representing the only species of *Mankyua*, 19 accessions representing 10 species of *Ophioderma*, 54 accessions representing approximately 32 species of *Ophioglossum*, and 19 accessions representing approximately 11 species of *Sceptridium*. In addition, one accession of *Holubiella*, recognized by neither Hauk et al. (2003), nor Shinohara et al. (2013), nor PPG I (2016), was included. Multiple accessions of the widespread species in most genera were sampled. The only missing genus in the family recognized by PPG I (2016) and not sampled in our study was *Rhizoglossum* C. Presl. Two species of *Marattia* in Marattiaceae and one species in each genus (*Psilotum* and *Tmesipteris*) of Psilotaceae are used as outgroups following Hauk et al. (2003) and Shinohara et al. (2013).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from silica-dried material or sometimes from herbarium specimens using the TIANGEN plant genomic DNA extraction kit (TIANGEN Biotech., Beijing, China) or the DNeasy Plant Mini Kit (Qiagen, Shanghai, China) following the manufacturers' protocols.

Seven plastid markers (*atpB* gene, *matK* gene, *psbA-trnH* intergenic spacer, *rbcL* gene, *rps4* gene, *rps4-trnS* intergenic spacer, and *trnL-F* intergenic spacer) were selected for amplification and sequencing based on their resolving power as demonstrated in earlier studies (Hauk et al., 2003, 2012; Shinohara et al., 2013; Dauphin et al., 2014). The *atpB* gene was amplified with the primers ESATB672F (Wolf, 1997) and ESATPE384R (Pryer et al., 2004). The *matK* gene was amplified with the primers FERmatK fEDR^{+a}, FERmatK rAGK^{+b}, and Ophio matK rlAld (Kuo et al., 2011). The *psbA-trnH* region was amplified with the primers *psbA* (Sang et al., 1997) and *trnH* (Tate and Simpson, 2003). The *rbcL* gene was amplified with the primers F1 (Fay et al., 1997) and 1379R originally designed by Zurawski et al. (1984) and modified by Wolf et al. (1999). The *rps4* gene and *rps4-trnS* intergenic spacer were amplified with the primers *TRNS* (Souza-Chies et al., 1997) and an unnamed primer derived from Li and Lu (2006). The *trnL-F* intergenic spacer was amplified together using the primers FERN1 (Trewick et al., 2002) and F (Taberlet et al., 1991). The PCR conditions followed Zhang et al. (2001). Amplified fragments were purified with TIANquick Mini Purification Kits (Tiangen Biotech, Beijing, China) and purified polymerase chain reaction (PCR) products were sequenced by Invitrogen (Shanghai, China).

Sequence alignment and phylogenetic analysis

Sequences obtained for each marker were initially aligned using MAFFT ver. 7 (Katoh and Standley, 2013) and then manually adjusted in BioEdit (Hall, 1999). The data were partitioned by locus. Both the intergenic spacers *rps4-trnS* and *trnL-F* were too variable to be aligned with confidence among subfamilies and within Ophioglossoideae. We therefore conducted two sets of analyses: one with exons + *psbA-trnH* only included; and the other with exons + *psbA-trnH* + *rps4-trnS* and *trnL-F* spacers of Botrychioideae only following Hauk et al. (2003).

Equally weighted maximum parsimony (MP) analyses for each locus and the combined dataset were conducted in PAUP* ver. 4.0b10 (Swofford, 2002) using 1000 tree-bisection-reconnection (TBR) searches with MAXTREES set to increase without limit. Gaps were coded as missing data. Parsimony jackknife (JK) analyses

(Farris et al., 1996) were conducted using PAUP* with the removal probability set to approximately 37%, and “jac” resampling emulated. One thousand replicates were performed with 10 TBR searches per replicate and a maximum of 100 trees held per TBR search.

jModeltest2 (Darriba et al., 2012) was used to select the best-fitting likelihood model for maximum likelihood (ML; Felsenstein, 1973) and Bayesian analyses. The Akaike information criterion (Akaike, 1974) was used to select among models instead of the hierarchical likelihood ratio test, following Pol (2004) and Posada and Buckley (2004). The best-fitting models and parameter values are provided in Table 1.

For each marker and the combined analysis (Farris et al., 1996; Nixon and Carpenter, 1996) of all nucleotide characters, maximum likelihood tree searches and ML bootstrapping were conducted using RAxML-HPC2 on TG ver. 7.2.8 on CIPRES web server (Stamatakis et al., 2008; Miller et al., 2010), with 1000 rapid bootstrap (BS) analyses followed by a search for the best-scoring tree in a single run (Stamatakis et al., 2008).

Bayesian inference (BI) was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) on Cipres (Miller et al., 2010). Two independent runs, each with four chains (one cold, three heated) with temperature parameter set to 0.2, transition/transversion rate ratio set to beta, and priors set to their default values, were conducted, each beginning with a random tree and sampling one tree every 1000 generations of 10,000,000 generations. Convergence among generations and stationarity were assessed using Tracer version 1.4 (Rambaut and Drummond, 2007) and a burn-in (approximately 25%) was discarded. The remaining trees were used to calculate a 50% majority-rule consensus topology and posterior probabilities (PP).

Results

A total of 190 sequences were newly generated for this study (Appendix 1). A comparison of the trees resulting from MPJK analyses of the individual plastid markers and the combined plastid dataset, did not identify any well-supported conflicts (MPJK $\geq 70\%$; Mason-Gamer and Kellogg, 1996; Zhang and Simmons, 2006). For details regarding the data sets analysed and statistics for the resulting trees are given in Table 2. The alignments and ML tree are deposited at TreeBase with study #S24029 (<http://purl.org/phylo/treebase/phylows/study/TB2:S24029>).

The topology based on exons + *psbA-trnH* only (Fig. S1) and that based on exons + *psbA-trnH* + *rps4-trnS* and *trnL-F* spacers of Botrychiodae (Fig. 1) were similar to each other, but the latter analysis improved the support values from weak support to strong support in two important nodes at (*Holubiella* + *Sceptridium*) in Botrychiodae: sister relationship between *Holubiella* and *Sceptridium* and the monophyly of *Sceptridium*. Figure 1 shows the topology based on the second analysis.

Ophioglossaceae were resolved as monophyletic with strong support (MLBS: 100%, MPJK: 100%, BIPP: 1.0), and so were all four subfamilies sensu PPG I (2016) and genera recognized by Hauk et al. (2003) and Shinohara et al. (2013) except *Botrypus* sensu Hauk et al. (2003) which was resolved as paraphyletic

Table 1
Best-fitting models and parameter values for separate (*atpB*, *rbcL*, *rps4-trnS*, *trnL*, *trnL-F*, and *trnL & trnL-F*) and simultaneous plastid datasets in this study

Region	AIC selected model	Base frequencies			Substitution model (rate matrix)									
		A	C	G	T	A-C	A-G	A-T	C-G	C-T	G-T	T _i /T _v	I	G
Plastid <i>atpB</i> gene	TVM+G	0.3024	0.2039	0.1838	0.3098	0.2922	6.6252	0.2012	0.4076	6.6252	1.0000	—	0	0.1880
Plastid <i>matK</i> gene	TVM+I+G	0.3016	0.1818	0.1742	0.3424	1.8510	5.0629	0.2922	0.6693	5.0629	1.0000	—	0	4.3770
Plastid <i>rbcL</i> gene	GTR+I+G	0.2787	0.1854	0.2307	0.3052	2.5497	8.3820	1.0953	1.0821	15.6275	1.0000	—	0	1.2710
Plastid <i>psbA-trnH</i> spacer	TIM1+I+G	0.3087	0.1697	0.1735	0.3482	1.0000	2.6741	0.3215	0.3215	3.3109	1.0000	—	0	4.0580
Plastid <i>rps4</i> gene + <i>rps4-trnS</i> spacer	TPM1uf+I+G	0.3164	0.1940	0.2011	0.2884	1.0000	5.1929	0.1207	0.1207	5.1929	1.0000	—	0	3.2380
Plastid <i>trnL-F</i> spacer	TPM1uf+G	0.2806	0.2093	0.2186	0.2915	1.0000	5.0336	0.5164	0.5164	5.0336	1.0000	—	0	1.0880
Simultaneous	GTR+I+G	0.2986	0.1906	0.1970	0.3138	1.1990	5.0242	0.4279	0.5148	5.9301	1.0000	—	0	2.6910

“G” = gamma distribution shape parameter (Yang, 1994). “GTR” = general-time-reversible model (Tavaré, 1986). “T” = proportion of invariant sites. “T_i/T_v” = transition/transversion ratio.

Table 2

Data matrices and tree statistics for each of the analyses

Matrix	# accessions	# chars.	# PI chars. (%) ^a	MPT length	Missing data (%)	CI	RI
Plastid <i>atpB</i> gene	42	1186	343 (28.9)	694	73.8	0.6499	0.9073
Plastid <i>matK</i> gene	64	786	396 (50.4)	890	63.8	0.6560	0.9485
Plastid <i>rbcL</i> gene	134	1325	379 (28.6)	1084	17.7	0.4847	0.9433
Plastid <i>psbA-trnH</i> spacer	71	705	389 (55.2)	876	71.0	0.6815	0.9411
Plastid <i>rps4</i> gene + <i>rps4-trnS</i> spacer	53	961	276 (28.7)	593	75.3	0.7808	0.9542
Plastid <i>trnL-F</i> spacer	39	375	95 (25.3)	262	75.7	0.7901	0.9047
Simultaneous	149	5338	1877 (35.2)	4456	58.4	0.6499	0.9386

Missing data include missing sequences, uncertain bases (R, A or G; Y, C or T; S, G or C; W, A or T; K, G or T; M, A or C) and gaps (–). PI, parsimony-informative; MPT, most parsimonious trees; CI, consistency index; RI, retention index.

^aInclusive of outgroups.

in relation to a clade containing (*Botrychium* + JHS clade (*Japanobotrychium* + (*Holubiella* + *Sceptridium*))). The monophyly of *Ophioglossum* sensu Hauk et al. (2003) and Shinohara et al. (2013) received only weak support (<50%) in MLBS analysis, while MPJK and BIPP support values were high.

Within Ophioglossaceae, *Mankyua* was weakly or strongly (MLBS: 53%, MPJK: 67%, BIPP: 1.0) supported as sister to the rest, followed by a clade containing (*Helminthostachys* + Botrychioideae) + Ophioglossoideae. The sister relationship between *Helminthostachys* + Botrychioideae was weakly or strongly supported depending on the analyses (MLBS: 61%, MPJK: 96%, BIPP: 1.0). Within Botrychioideae, *Botrypus strictus* (Underw.) Holub was strongly (MLBS: 100%, MPJK: 96%, BIPP: 1.0) supported as sister to the rest, followed by *Botrypus virginianus*, which was strongly (MLBS: 100%, MPJK: 96%, BIPP: 1.0) supported as sister to a clade containing *Botrychium* + JHS clade (*Japanobotrychium* + (*Holubiella* + *Sceptridium*))). The JHS clade was weakly or strongly supported (MLBS: 60%, MPJK: 97%, BIPP: 1.0). Within Ophioglossoideae, *Ophioglossum* sensu Hauk et al. (2003) was resolved as sister to a strongly (MLBS: 100%, MPJK: 96%, BIPP: 1.0) supported clade containing *Cheiroglossa* and *Ophioderma*. Within *Ophioglossum*, the *O. costatum* clade and the *O. crotalophoroides* clade were paraphyletic in relation to the rest of *Ophioglossum* (Fig. 1).

Discussion

The resolution of *Mankyua*

It was quite striking that the Korean endemic monospecific genus *Mankyua* had not been described until 2001 (Sun et al., 2001). Morphologically this genus is very special by having a ternately divided compound trophophore, a linear and fleshy sporophore branched at base, and a creeping rhizome with

proliferous roots (Sun et al., 2001). Previous molecular studies reported inconsistent resolutions of the genus. Sun et al. (2009) found that *Mankyua* was sister to *Helminthostachys* + Botrychioideae with low support. Shinohara (2013) showed that *Mankyua* was either sister (moderate or strong support) to *Helminthostachys* + Ophioglossoideae (based on *matK* data) or sister (weak or moderate support) to the rest of the family (based on *rbcL* and combined *matK* + *rbcL* data). Our data weakly support *Mankyua* as sister to the rest of the family, consistent with the results based on *rbcL* and combined *matK* + *rbcL* data by Shinohara (2013).

The resolution of *Helminthostachys*

Since *Helminthostachys* was included in a molecular study for the first time (Hasebe et al., 1995), it has consistently been resolved as sister to Botrychioideae (Hauk et al., 2003; Sun et al., 2009; Shinohara et al., 2013 (based on *rbcL* and combined *matK* and *rbcL* data)), but the *matK* data of Shinohara et al. (2013) resolved it as sister to Ophioglossoideae with moderate or strong support. Our combined data of seven plastid markers resolved *Helminthostachys* as sister to Botrychioideae albeit with moderate support. This resolution is supported by their similar compound trophophores, branched sporophores, free venation, and exposed sporangia in both *Helminthostachys* and Botrychioideae. Wagner (1990) even placed them in Botrychiaceae. In contrast, there are very few characters supporting a sister relationship between *Helminthostachys* and Ophioglossoideae, e.g., the spikelike sporophores and the absence of vascular cambium (Kato, 1987).

The relationships within Botrychioideae

Our study is the first molecular analysis which included all genera in Botrychioideae recognized by PPG I (2016) in addition to the two controversial taxa

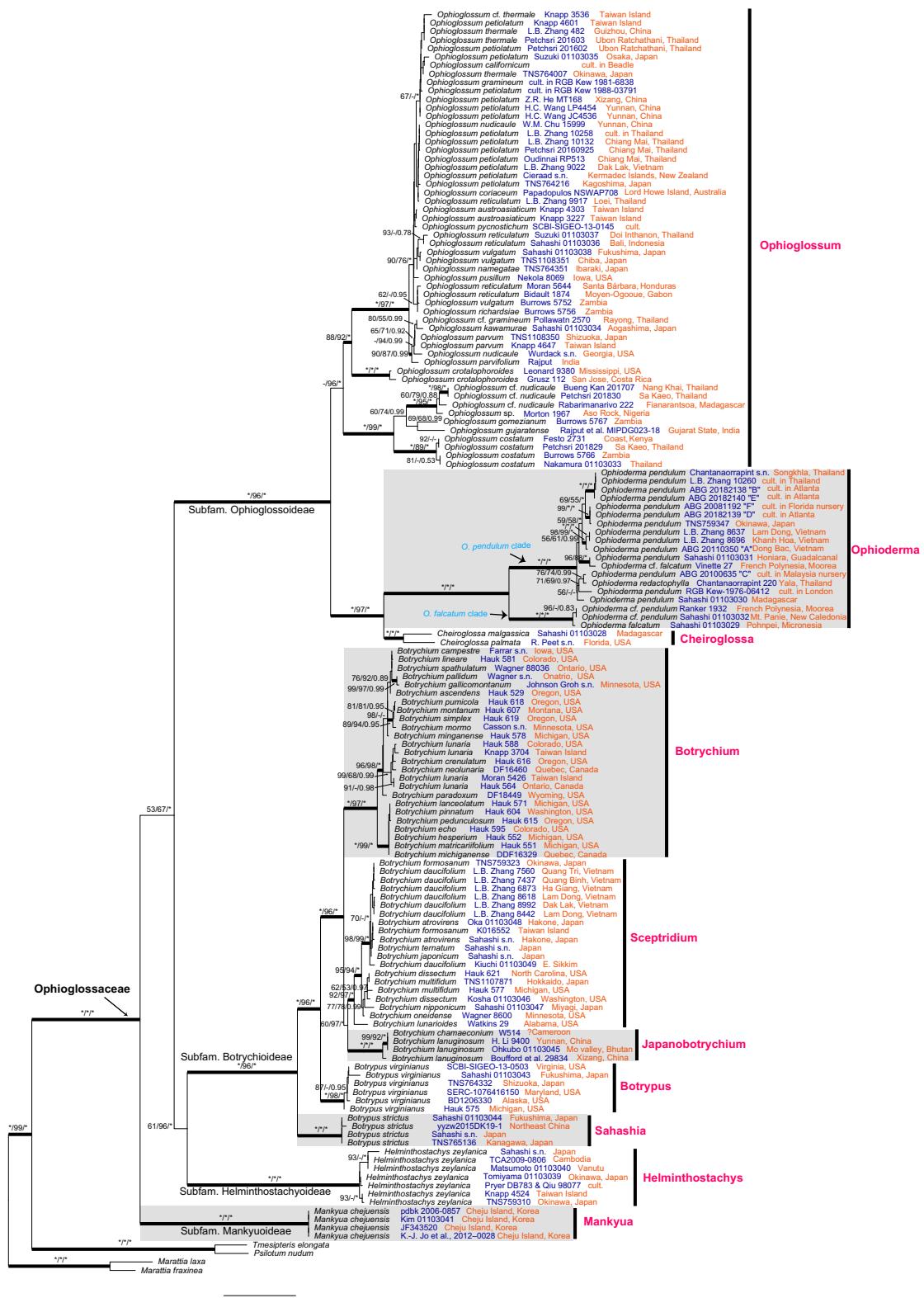


Fig. 1. Maximum likelihood phylogeny of Ophioglossaceae based on seven plastid markers (*atpB*, *rbcL*, *matK*, *psbA-trnH*, *rps4*, *rps4-trnS*, *trnLF*). Maximum likelihood bootstrap support (MLBS), maximum parsimony jackknife support (MPJK), and Bayesian inference posterior probability (BIPP) are on the left, middle, and right, respectively, along the branches. Thick horizontal lines indicate clades strongly supported all three analyses (MLBS $\geq 85\%$, MPJK $\geq 85\%$, and BIPP ≥ 0.95). Voucher information is indicated in blue. Geographical provenances are indicated in red. Black vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by current study. [Colour figure can be viewed at wileyonlinelibrary.com]

in phylogeny, *Botrypus strictus* and *Sceptridium lunarioides* (*Holubiella*; see below). Our study discovered some strongly supported novel relationships: the sister relationship between *Botrychium* and the JHS clade (*Japanobotrychium* + (*Holubiella* + *Sceptridium*)). This relationship is morphologically supported by the closed leaf sheaths, the lateral sporangial position, and the horizontally dehisced sporangia in these four genera, rather than the open leaf sheaths, the terminal sporangial position, and the longitudinally dehisced sporangia in *Botrypus strictus* and *B. virginianus* (Kato, 1987).

Our data resolved *Botrypus strictus* and *B. virginianus* as paraphyletic in relation to the rest of Botrychioideae, consistent with earlier results (Hauk et al., 2003; Shinohara et al., 2013).

The resolution of Japanobotrychium

Since *Japanobotrychium* was published by Masmune (1931) based on *J. arisanense* Masam. (= *J. lanuginosum*), the genus had been neglected and the type had commonly been included in *Botrypus* (e.g., Nishida, 1952; Holub, 1973; Kung, 1988) or simply in *Botrychium* s.l. (e.g., Clausen, 1938; Ching et al., 1959), until Kato (1987) demonstrated that *Japanobotrychium* was different from other Botrychioideae in a number of morphological features. For the first time Shinohara et al. (2013) included the type (one accession) in a molecular study and found that *Japanobotrychium* was sister to *Sceptridium* (four accessions sampled), but they did not sample any members of *Botrychium*. Our study with four accessions representing approximately three species of *Japanobotrychium* strongly supported the monophyly of *Japanobotrychium* and the sister relationship between *Japanobotrychium* and a clade containing *Holubiella* and *Sceptridium*. *Japanobotrychium* is well defined by having sporophores arising from the rachis instead of from the petiole in *Holubiella* and *Sceptridium* (Kato, 1987; Kung, 1988).

The resolution of Holubiella

Holubiella was published based on *Botrychium* sect. *Hiemobotrychium* (Škoda, 1997) and has never been recognized but has been included in *Sceptridium* since its publication (e.g., Hauk et al., 2003; PPG I 2016). For the first time Hauk et al. (2003) sampled the type of the genus (as *Sceptridium lunarioides*) in a molecular study and showed that *Sceptridium* sensu Hauk et al. (2003) was only moderately supported as monophyletic. Also, *Sceptridium* sensu Škoda (1997) was only weakly (BS: 58%) supported as monophyletic. Our study strongly supported *Sceptridium* sensu Škoda (1997) as monophyletic and *Holubiella* is

strongly supported as sister to *Sceptridium* sensu Škoda (1997).

The paraphyly of Botrypus

When *Botrypus* was first described (Michaux, 1803), two species, *B. virginianus* (L.) Michx. and *B. lunarioides* Michx., were included. Nishida (1952) transferred four species of *Botrychium* s.l. to *Osmundopteris*. Holub (1973) realized that *Osmundopteris* was a later synonym of *Botrypus* and transferred those species, which were transferred to *Osmundopteris* by Nishida (1952), to *Botrypus*. Kung (1988) transferred *Botrychium decurrens* Ching to *Botrypus*. Hauk et al. (2003) included two species of *Botrypus* sensu Holub (1973), the type of the genus, *B. virginianus* (one accession), and *B. strictum* (one accession), in a molecular study for the first time and they found *Osmundopteris* sensu Nishida (1952) or *Botrypus* sensu Holub (1973) was paraphyletic in relation to the 20 accessions of *Botrychium* + *Sceptridium* sampled. Shinohara (2013) added one more species of a member of *Botrypus* sensu Holub (1973), *B. lanuginosus* (one accession), in a molecular work and found that *B. lanuginosus*, together with *B. virginianus* and *B. strictus* (*Osmundopteris stricta*), was paraphyletic in relation to *Sceptridium* (4 spp. sampled). *Botrypus lanuginosus* was thus recognized as a member of *Japanobotrychium* by Shinohara et al. (2013).

Our study with 60 accessions of Botrychioideae sampled further corroborated the paraphyly of *Botrypus* sensu Holub (1973). This suggests that the sporophores arising near the base of trophophores which holds *B. strictus* and *B. virginianus* together are not a synapomorphy of *Botrypus*.

Sahashia gen. nov. segregated from *Botrypus*

Since *Botrychium strictum* is resolved as sister to the rest of Botrychioideae (Hauk et al., 2003; Shinohara et al., 2013; our Fig. 1), it cannot be included in any existing genera and a new genus, *Sahashia* gen. nov. (see also below), must be established so that other morphologically well-defined genera in the subfamily can be recognized. *Sahashia* is then defined by the sporophores that are a compact panicle (Kung, 1988). Geographically, the only species of *Sahashia* occurs in East Asia and the Far East of Russia, whereas those of the newly defined *Botrypus* are distributed in temperate regions within the Northern Hemisphere and Central and South Americas.

Within the newly defined *Botrypus*, there is molecular divergence in *B. virginianus* s.l., a species believed to be nearly cosmopolitan (Clausen, 1938), suggesting possible cryptic speciation in this species complex.

The relationships within Ophioglossoideae

The resolution of Cheiroglossa. With both species of *Cheiroglossa* sampled, our study showed that *Cheiroglossa* is monophyletic. Our large character sampling further corroborates the deep divergence between *Cheiroglossa* and *Ophioderma*. Morphologically, species of *Cheiroglossa* have scaly rhizomes and palmate trophophores that each have three or more sporophores. In contrast, species of *Ophioderma* have no scales on rhizomes and have ribbon-like trophophores that each have 1–2 sporophores.

Geographically, species of *Cheiroglossa* occur in Indian Ocean islands and Central to South America, whereas those of *Ophioderma* are distributed in Australasia. We doubt the distribution of *Cheiroglossa* in Vietnam as reported by Clausen (1938) and Wagner (1990).

The species diversity in *Cheiroglossa* remains unknown. Clausen (1938) only recognized one species with no infraspecific taxa at all. Christensen recognized a variety, *C. palmata* var. *malgassica* C.Chr., under the single species. Pichi Sermolli (1968) raised the variety to a species. Our study shows that there is substantial molecular divergence between these two taxa.

Resolution and cryptic speciation in Ophioderma. With our large sampling, *Ophioderma* is strongly supported as monophyletic and sister to *Cheiroglossa*, consistent with earlier findings (Hauk et al., 2003; Shinohara et al., 2013).

Ophioderma (or *Ophioglossum* subg. *Ophioderma* sensu Clausen, 1938) was believed to contain three species only by Clausen (1938) and PPG I (2016). Our largely expanded sampling in this genus showed that there are two deeply diverged clades within the genus: the *O. falcatum* clade and the *O. pendulum* clade (Fig. 1), which do not correspond to *O. subg. Ophioderma* and *O. subg. Simplexa* Nishida, respectively. The *O. pendulum* clade alone contains at least nine species (*O. intermedia* and *O. simplex* are not sampled in our study) judging from our molecular results and our preliminary morphological work. These species are distinct from one another in leaf margins, dissection, width, and texture of trophophores, and number of sporophores per trophophore, etc. Most of these species are currently recognized as "*O. pendulum*" and thus can be called cryptic species.

The most fascinating addition to the *Ophioderma pendulum* clade is the discovery of *O. redactophyllum* Chantanaorr. & Li Bing Zhang (Chantanaorraptin et al., 2019) which has terrestrial habit and scale-like trophophores, rather than the epiphytic habit and large and ribbon-like trophophores in *O. pendulum*. The two unsampled terrestrial species, *O. intermedium*

and *O. simplex*, might fall in the *O. pendulum* clade too.

The deep divergence between the *Ophioderma falcatum* clade and the *O. pendulum* clade suggests that the *O. falcatum* clade might deserve recognition at generic rank, but we failed to find morphological features to define this clade. More studies are needed.

The monophyly of Ophioglossum. In our ML tree based on *atpB* gene (Fig. S3), the clade containing *Ophioglossum costatum*, etc. (the *O. costatum* clade) is resolved as sister to the rest of Ophioglossoideae with moderate support (MLBS: 77%). In our ML trees based on either *rbcL* (Fig. S2) or *psbA-trnH* data (Fig. S7), the *O. costatum* clade is resolved as sister (MLBS < 50%) to *Cheiroglossa + Ophioderma*. In another word, *Ophioglossum* is resolved as paraphyletic in relation to the rest of Ophioglossoideae in *atpB*, *psbA-trnH*, and *rbcL* gene trees, whereas it is resolved as monophyletic in *matK* and *rps4* gene trees with low support (MLBS < 50%; Figs. S4 and S5). However, such conflict in the resolution of *Ophioglossum* is not well ($\geq 70\%$; see above) supported in MPJK analyses. Our ML analysis based on combined data of seven plastid markers (total aligned length: 5338 bp) failed to strongly support *Ophioglossum* as monophyletic (<50%), although BI and MP analyses based on combined data strongly support *Ophioglossum* as monophyletic (Fig. 1). Based on one or two plastid markers, neither Hauk et al. (2003) nor Shinohara et al. (2013) provided strong support for the monophyly of *Ophioglossum* (BS: 56% and 61%, respectively). Using GenBank data Testo and Sundue (2016) found that *Ophioglossum* was paraphyletic in relation to *Cheiroglossa + Ophioderma*. This strongly suggests that the monophyly of *Ophioglossum* sensu Hauk et al. (2003), Shinohara et al. (2013), and PPG I (2016) remains uncertain, although it can be defined by having sporophores erect and arising medianly from or below the base of trophophore blades ("*O. subg. Euophioglossum*" sensu Clausen, 1938).

Taxonomic treatment. Based on our molecular results and morphology, a new genus, *Sahashia* gen. nov., must be established.

Sahashia Li Bing Zhang & Liang Zhang, gen. nov.
Type: *S. stricta* (Underw.) Li Bing Zhang & Liang Zhang (\equiv *Botrychium strictum* Underw.).

Etymology:—In honour of Prof. Norio Sahashi based at TOHO for his contributions on the systematics of Ophioglossaceae (e.g., Sahashi, 1980; Shinohara et al., 2013; Zhang et al., 2013). The first DNA sequences of *Botrychium strictum* published by Hauk et al. (2003) were based on the material Prof. Sahashi collected.

Diagnosis: *Sahashia* is similar to *Botrypus* in having open leaf sheaths, sessile trophophores, and

sporophores arising from the base of trophophores, but it has a compact sporophore.

Plants terrestrial; trophophores deltoid, length nearly as large as width, 2–4-pinnate; sporophores sessile or short-stalked.

This genus contains one species distributed in East Asia and the Far East of Russia.

Sahashia stricta (L.Underw.) Li Bing Zhang & Liang Zhang, comb. nov. Basionym: *Botrychium strictum* Underw., Bull. Torrey Bot. Club 30: 52. 1903. 1903. *Osmundopteris stricta* (Underw.) Nishida, J. Jap. Bot. 27: 276. 1952. *Botrypus strictus* (L.Underw.) Holub, Preslia 45: 277. 1973.

It occurs in China (Chongqing, Gansu, Heilongjiang, Henan, Hubei, Jilin, Liaoning, Nei Mongol, Shaanxi, Sichuan), Korea, Japan, and Far East of Russia.

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References

- Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automat. Contr. 19, 716–723.
- Chantanaorapint, S., Petchsri, S., Zhang, L. and Zhang, L.-B., 2019. *Ophioderma redactophylla* sp. nov. (Ophioglossaceae), a new fern from the Malay Peninsula. Phytotaxa.
- Ching, R.-C., 1978. The Chinese fern families and genera: Systematic arrangement and historical origin (Cont.). Acta Phytotaxon. Sin. 16, 16–37.
- Ching, R.-C., Fu, S.-H., Wang, C.-H. and Shing, G.-H., 1959. Ophioglossaceae, Botrychiaceae, and Helminthostachyaceae: Volume 2. In: Ching, R.-C. (Ed.) Flora Reipublicae Popularis. Beijing: Sinicae. Science Press, pp. 6–26, 329–330.
- Christenhusz, M.J.M., Zhang, X.-C. and Schneider, H., 2011. A linear sequence of extant families and genera of lycophytes and ferns. Phytotaxa 19, 7–54.
- Clausen, R.T., 1938. A monograph of the Ophioglossaceae. Mem. Torrey Bot. Club 19, 1–177.
- Darriba, D., Taboada, G.L., Doallo, R. and Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772–772.
- Dauphin, B., Vieu, J. and Grant, J.R., 2014. Molecular phylogenetics supports widespread cryptic species in moonworts (*Botrychium* s.s. Ophioglossaceae). Amer. J. Bot. 101, 128–140.
- Dauphin, B., Grant, J.R. and Mráz, P., 2016. Ploidy level and genome size variation in the homosporous ferns *Botrychium* s.l. (Ophioglossaceae). Plant Syst. Evol. 302, 575–584.
- Dauphin, B., Farrar, D.R., Maccagni, A. and Grant, J.R., 2017. A worldwide molecular phylogeny provides new insight on cryptic diversity within the moonworts (*Botrychium* s. s., Ophioglossaceae). Syst. Bot. 42, 620–639.
- Dauphin, B., Grant, J.R., Farrar, D.R. and Rothfels, C.J., 2018. Rapid allopolyploid radiation of moonwort ferns (*Botrychium*; Ophioglossaceae) revealed by PacBio sequencing of homologous and homeologous nuclear regions. Mol. Phylogenet. Evol. 120, 342–353.
- Davis, C.C., Anderson, W.R. and Wurdack, K.J., 2005. Gene transfer from a parasitic flowering plant to a fern. Proc. R. Soc. B 272, 2237–2242.
- Ebihara, A., Nitta, J.H. and Ito, M., 2010. Molecular species identification with rich floristic sampling: DNA barcoding the pteridophyte flora of Japan. PLoS ONE 5, e15136.
- Farris, J.S., Albert, V.A., Källersjö, M., Lipscomb, D. and Kluge, A.G., 1996. Parsimony jackknifing outperforms neighbor-joining. Cladistics 12, 99–124.
- Fay, M.F., Swensen, S.M. and Chase, M.W., 1997. Taxonomic affinities of *Medusagynne oppositifolia* (Medusagynaceae). Kew Bull. 52, 111–120.
- Felsenstein, J., 1973. Maximum likelihood and minimum-steps methods for estimating evolutionary trees from data on discrete characters. Syst. Biol. 22, 240–249.
- de Gasper, A.L., Almeida, T.E., Dittrich, V.A.D.O., Smith, A.R. and Salino, A., 2017. Molecular phylogeny of the fern family Blechnaceae (Polypodiidae) with a revised genus-level treatment. Cladistics 33, 429–446.
- Ghatak, J., 1977. Biosystematic survey of pteridophytes from Shevaroy Hills, South India. Nucleus 20, 105–108.
- Gilman, A.V., Farrar, D.R. and Zika, P.F., 2015. *Botrychium michiganense* sp. nov. (Ophioglossaceae), a new North American moonwort. J. Bot. Res. Inst. Texas 9, 295–309.
- Grewe, F., Guo, W., Gubbels, E.A., Hansen, A.K. and Mower, J.P., 2013. Complete plastid genomes from *Ophioglossum californicum*, *Psilotum nudum*, and *Equisetum hyemale* reveal an ancestral land plant genome structure and resolve the position of Equisetales among monilophytes. BMC Evol. Biol. 13, 8.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
- Hasebe, M., Wolf, P.G., Pryer, K.M., Ueda, K., Ito, M., Sano, R., Gastony, G.J., Yokoyama, J., Manhart, J.R. and Murakami, N., 1995. Fern phylogeny based on *rbcL* nucleotide sequences. Amer. Fern J. 85, 134–181.
- Hauk, W.D., Parks, C.R. and Chase, M.W., 2003. Phylogenetic studies of Ophioglossaceae: evidence from *rbcL* and *trnL-F* plastid DNA sequences and morphology. Mol. Phylogenet. Evol. 28, 131–151.
- Hauk, W.D., Kennedy, L. and Hawke, H.M., 2012. A phylogenetic investigation of *Botrychium* s. s. (Ophioglossaceae): evidence from three plastid DNA sequence datasets. Syst. Bot. 37, 320–330.
- Holub, J., 1973. A note on the classification of *Botrychium* Sw. s. l. Preslia 45, 265–275.
- Kato, M., 1987. A phylogenetic classification of Ophioglossaceae. Gard. Bull. 40, 1–14.
- Katoh, K. and Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.
- Khandelwal, S., 1990. Chromosome evolution in the genus *Ophioglossum* L. Bot. J. Linnean Soc. 102, 205–217.
- Kim, H.T and Kim, K.J., 2018. Evolution of six novel ORFs in the plastome of *Mankyua chejuense* and phylogeny of euphorangioid ferns. Sci. Rep. 8, 16466.
- Knie, N., Fischer, S., Grewe, F., Polsakiewicz, M. and Knoop, V., 2015. Horsetails are the sister group to all other monilophytes

- and Marattiaceae are sister to leptosporangiate ferns. Mol. Phylogen. Evol. 90, 140–149.
- Kung, H.-S., 1988. Flora Sichuanica. Vol. 6. Chengdu: Science & Technology Press.
- Kuo, L.-Y., Li, F.-W., Chiou, W.-L. and Wang, C.-N., 2011. First insights into fern *matK* phylogeny. Mol. Phylogen. Evol. 59, 556–566.
- Li, C.-X. and Lu, S.-G., 2006. Phylogenetics of Chinese *Dryopteris* (Dryopteridaceae) based on the chloroplast *rps4-trnS* sequence data. J. Plant Res. 119, 589–598.
- Löve, A., 1977. IOPB chromosome number reports LVII. Taxon 26, 443–452.
- Masamune, G., 1931. Contributions to our knowledge of the flora of southern part of Japan. VI. J. Soc. Trop. Agric. 3, 246–247.
- Mason-Gamer, R.J. and Kellogg, E.A., 1996. Testing for phylogenetic conflict among molecular data sets in the tribe Triticeae (Gramineae). Syst. Biol. 45, 524–545.
- Michaux, A., 1803. Flora Boreali-Americana. Apud fratres Levrault.
- Miller, M.A., Pfeiffer, W. and Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), 14 November 2010, New Orleans, LA, pp. 1–8.
- Murdock, A.G., 2008. Phylogeny of marattioid ferns (Marattiaceae): inferring a root in the absence of a closely related outgroup. Amer. J. Bot. 95, 626–641.
- Nishida, M., 1952. A new system of Ophioglossales. J. Jap. Bot. 27, 271–278.
- Nitta, J.H., Meyer, J.Y., Taputuarai, R. and Davis, C.C., 2017. Life cycle matters: DNA barcoding reveals contrasting community structure between fern sporophytes and gametophytes. Ecol. Monogr. 87, 278–296.
- Nixon, K.C. and Carpenter, J.M., 1996. On simultaneous analysis. Cladistics 12, 221–241.
- Papadopoulos, A.S., Baker, W.J., Crayn, D., Butlin, R.K., Kynast, R.G., Hutton, I. and Savolainen, V., 2011. Speciation with gene flow on Lord Howe Island. Proc. Natl. Acad. Sci. USA 108, 13188–13193.
- Perrie, L. and Brownsey, P., 2007. Molecular evidence for long-distance dispersal in the New Zealand pteridophyte flora. J. Biogeogr. 34, 2028–2038.
- Pichi Sermolli, R.E.G., 1968. Fragmenta Pteridologiae—I. Webbia 23, 159–207.
- Pichi Sermolli, R.E.G., 1977. Tentamen pteridophytorum genera in taxonomicum ordinam redigendi. Webbia 31, 315–512.
- Pol, D., 2004. Empirical problems of the hierarchical likelihood ratio test for model selection. Syst. Biol. 53, 949–962.
- Posada, D. and Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.
- PPG I, 2016. A community-derived classification for extant lycophtyes and ferns. J. Syst. Evol. 54, 563–603.
- Presl, C.B., 1845. Supplementum tentaminis Pteridographiae. e typographia caes. reg. aulica filiorum Amadei Haase, Prague [Prague].<https://doi.org/10.5962/bhl.title.82188>
- Pryer, K.M., Schneider, H., Smith, A.R., Cranfill, R., Wolf, P.G., Hunt, J.S. and Sipes, S., 2001. Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. Nature 409, 618–622.
- Pryer, K.M., Schuettpelz, E., Wolf, P.G., Schneider, H., Smith, A.R. and Cranfill, R., 2004. Phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. Amer. J. Bot. 91, 1582–1598.
- Qiu, Y.-L., Li, L., Wang, B., Chen, Z., Knoop, V., Groth-Malonek, M., Dombrovská, O., Lee, J., Kent, L. and Rest, J., 2006. The deepest divergences in land plants inferred from phylogenomic evidence. Proc. Natl. Acad. Sci. USA 103, 15511–15516.
- Rai, H.S. and Graham, S.W., 2010. Utility of a large, multigene plastid data set in inferring higher-order relationships in ferns and relatives (monilophytes). Amer. J. Bot. 97, 1444–1456.
- Rambaut, A. and Drummond, A.J., 2007. Tracer 1.4. Available: <http://beast.bio.ed.ac.uk/Tracer>
- Ronquist, F. and Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
- Rothfels, C.J., Li, F.W., Sigel, E.M., Huiet, L., Larsson, A., Burge, D.O., Ruhsam, M., Deyholos, M., Soltis, D.E. and Stewart, C.N. Jr., 2015. The evolutionary history of ferns inferred from 25 low-copy nuclear genes. Amer. J. Bot. 102, 1089–1107.
- Sahashi, N., 1980. Morphological and taxonomical studies on Ophioglossales in Japan and the adjacent regions (4): Comparative morphology of spores of some species in Ophioglossales. J. Jap. Bot. 55, 73–80.
- Sang, T., Crawford, D.J. and Stuessy, T.F., 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Amer. J. Bot. 84, 1120–1136.
- Shinohara, W., Nakato, N., Yatabe-Kakugawa, Y., Oka, T., Kim, J.K., Murakami, N., Noda, H. and Sahashi, N., 2013. The use of *matK* in Ophioglossaceae phylogeny and the determination of *Mankyua* chromosome number shed light on chromosome number evolution in Ophioglossaceae. Syst. Bot. 38, 564–570.
- Škoda, B., 1997. Taxonomic comments on the " Flora of North America north of Mexico," vol. 2, with some nomenclatural combinations for Pteridophyta. Preslia Praha 68, 341–359.
- Small, R.L., Lickey, E.B., Shaw, J. and Hauk, W.D., 2005. Amplification of noncoding chloroplast DNA for phylogenetic studies in lycophytes and monilophytes with a comparative example of relative phylogenetic utility from Ophioglossaceae. Mol. Phylogen. Evol. 36, 509–522.
- Smith, A.R., Pryer, K.M., Schuettpelz, E., Korall, P., Schneider, H. and Wolf, P.G., 2006. A classification for extant ferns. Taxon, 55, 705–731.
- Souza-Chies, T.T., Bittar, G., Nadot, S., Carter, L., Besin, E. and Lejeune, B., 1997. Phylogenetic analysis of Iridaceae with parsimony and distance methods using the plastid gene *rps4*. Plant Syst. Evol. 204, 109–123.
- Stamatakis, A., Hoover, P. and Rougemont, J., 2008. A rapid bootstrap algorithm for the RAxML Web servers. Syst. Biol. 57, 758–771.
- Sun, B.Y., Kim, M.H., Kim, C.H. and Park, C.W., 2001. *Mankyua* (Ophioglossaceae): a new fern genus from Cheju Island, Korea. Taxon 50, 1019–1024.
- Sun, B.Y., Baek, T.G., Kim, Y.D. and Kim, C.S., 2009. Phylogeny of the family Ophioglossaceae with special emphasis on genus *Mankyua*. Korean J. Pl. Taxon. 39, 135–142.
- Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony, Version 4.0 b10. Sinauer, Sunderland, Massachusetts.
- Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J., 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17, 1105–1109.
- Tate, J.A. and Simpson, B.B., 2003. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. Syst. Bot. 28, 723–737.
- Tavare, S. (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura, R.M. (Ed.), Some Mathematical Questions in Biology—DNA Sequence Analysis. Providence, RI: Am. Math. Soc. pp. 57–86.
- Testo, W. and Sundue, M., 2016. A 4000-species dataset provides new insight into the evolution of ferns. Mol. Phylogen. Evol. 105, 200–211.
- Trewick, S., Morgan-Richards, M., Russell, S., Henderson, S., Rumsey, F., Pinter, I., Barrett, J., Gibby, M. and Vogel, J., 2002. Polyploidy, phylogeography and Pleistocene refugia of the rockfern *Asplenium ceterach*: evidence from chloroplast DNA. Mol. Ecol. 11, 2003–2012.
- Wagner, W.H., 1990. Ophioglossaceae. In: Kramer, K.U., Green, P.S. (Eds.), The Families and Genera of Vascular Plants-Volume 1: Pteridophytes and Gymnosperms. Springer, Berlin, pp. 193–197.
- Wagner, W.H., 1992. *Hiemobrychium*, a new section of *Botrychium* subgenus *Sceptridium* from the southeastern United States. Novon 2, 267–268.

- Wang, Z.R., 1986. The polymorphy of fronds and chromosome number of *Ophioglossum petiolatum* Hook. *Acta Bot. Sin.* 28, 472–476.
- Williams, E.W., Farrar, D.R. and Henson, D., 2016. Cryptic speciation in allotetraploids: Lessons from the *Botrychium matricariifolium* complex. *Amer. J. Bot.* 103, 740–753.
- Wolf, P.G., 1997. Evaluation of *atpB* nucleotide sequences for phylogenetic studies of ferns and other pteridophytes. *Amer. J. Bot.* 84, 1429–1440.
- Wolf, P.G., Sipes, S.D., White, M.R., Martines, M.L., Pryer, K.M., Smith, A.R. and Ueda, K., 1999. Phylogenetic relationships of the enigmatic fern families Hymenophyllaceae and Lophosoriaceae: Evidence from *rbcL* nucleotide sequences. *Plant Syst. Evol.* 219, 263–270.
- Yang, Z. (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J. Mol. Evol.*, 39, 306–314.
- Zhang, L.-B. and Simmons, M.P., 2006. Phylogeny and delimitation of the *Celastrales* inferred from nuclear and plastid genes. *Syst. Bot.* 31, 122–137.
- Zhang, L.-B., Comes, H.P. and Kadereit, J.W., 2001. Phylogeny and quaternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on *ITS* and AFLP variation. *Amer. J. Bot.* 88, 2331–2345.
- Zhang, X.-C., Liu, Q.-R. and Sahashi, N., 2013. Ophioglossaceae. In: Wu, Z.-Y., Raven, P.H., Hong, D.-Y. (Eds.), *Flora of China*, Vol. 2–3 (Pteridophytes). Science Press, Beijing; Missouri Botanical Garden Press, St. Louis, pp. 73–80.
- Zhong, B., Fong, R., Collins, L.J., McLennan, P.A. and Penny, D., 2014. Two new fern chloroplasts and decelerated evolution linked to the long generation time in tree ferns. *Genome Biol. Evol.* 6, 1166–1173.
- Zurawski, G., Clegg, M.T. and Brown, A.H.D., 1984. The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. *Genetics* 106, 735–749.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Maximum likelihood phylogeny of Ophioglossaceae based on five plastid markers (*atpB*, *rbcL*, *matK*, *psbA-trnH*, *rps4*) with maximum likelihood bootstrap support (MLBS) along the branches. Voucher information is indicated in blue. Geographical provenances are indicated in red. Black vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by the current study.

Fig. S2. Maximum likelihood phylogeny of Ophioglossaceae based on plastid *rbcL* data with maximum likelihood bootstrap support (MLBS) along the branches. Voucher information is indicated in blue. Geographical provenances are indicated in red. Black vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by current study.

Fig. S3. Maximum likelihood phylogeny of Ophioglossaceae based on plastid *atpB* data with maximum likelihood bootstrap support (MLBS) along the branches. Voucher information is indicated in blue. Geographical provenances are indicated in red. Black

vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by the current study.

Fig. S4. Maximum likelihood phylogeny of Ophioglossaceae based on plastid *matK* data with maximum likelihood bootstrap support (MLBS) along the branches. Voucher information is indicated in blue. Geographical provenances are indicated in red. Black vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by the current study.

Fig. S5. Maximum likelihood phylogeny of Ophioglossaceae based on plastid *rps4* data with maximum likelihood bootstrap support (MLBS) along the branches. Voucher information is indicated in blue. Geographical provenances are indicated in red. Black vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by the current study.

Fig. S6. Maximum likelihood phylogeny of Botrychioideae based on plastid *trnL-F* data with maximum likelihood bootstrap support (MLBS) along the branches. Voucher information is indicated in blue. Geographical provenances are indicated in red. Black vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by the current study.

Fig. S7. Maximum likelihood phylogeny of Ophioglossaceae based on plastid *psbA-trnH* data with maximum likelihood bootstrap support (MLBS) along the branches. Voucher information is indicated in blue. Geographical provenances are indicated in red. Black vertical bars on the rightmost indicate the genera of Ophioglossaceae recognized by the current study.

Appendix 1

List of taxa sampled with information related to taxonomy, GenBank accession numbers, references, and voucher information. Herbarium acronyms follow *Index Herbariorum* (Thiers, 2015).

Botrychium ascendens W.H. Wagner, *Hauk* 529 (NCU), Oregon, USA: *rbcL* L40982 (Hauk et al., 2003), *trnL-F* AY138422 (Hauk et al., 2003). **Botrychium campestre** W.H. Wagner & Farrar, *Farrar* s.n. (ISC), Iowa, USA: *rbcL* L40961 (Hauk et al., 2003), *trnL-F* AY138426 (Hauk et al., 2003), *rps4-trnS* AY870427 (Small et al., 2005). **Botrychium crenulatum** W.H. Wagner, *Hauk* 616 (NCU), Oregon, USA: *rbcL* L40959 (Hauk et al., 2003), *trnL-F* AY138431 (Hauk et al., 2003). **Botrychium echo** W.H. Wagner, *Hauk* 595 (NCU), Colorado, USA: *rbcL* L40962 (Shinohara et al., 2013), *trnL-F* DQ849131 (Hauk et al., 2012). **Botrychium gallicomontanum** Farrar & Johnson-Groh, *Johnson Groh* s.n., Minnesota, USA: *rbcL* DQ849133 (Hauk et al., 2012), *trnL-F* DQ849134 (Hauk et al., 2012). **Botrychium hesperium** (Maxon & R.T. Clausen) W.H. Wagner & Lellinger, *Hauk* 552 (NCU), Michigan, USA: *rbcL* L40960 (Hauk et al., 2012), *trnL-F* DQ849158. **Botrychium lanceolatum** (S.G. Gmel.) Ångstr., *Hauk* 571 (NCU), Michigan, USA: *rbcL* L40963 (Hauk et al., 2003), *trnL-F* AY138432 (Hauk et al., 2003), *rps4-trnS* AY870428 (Small et al., 2005). **Botrychium lineare** W.H. Wagner, *Hauk* 581 (NCU), Colorado, USA: *rbcL* L40964 (Hauk et al., 2003), *trnL-F* AY138425 (Hauk et al., 2003). **Botrychium lunaria** (L.) Sw., *Hauk* 564 (NCU), Ontario, Canada: *rbcL* L40965 (Hauk et al., 2003), *trnL-F* AY138430 (Hauk et al., 2003), *rps4-trnS* AY870429 (Small et al., 2005). *Hauk* 588 (NCU), Colorado, USA: *rbcL*

- DQ849146 (Hauk et al., 2012), *trnL-F* DQ849147 (Hauk et al., 2012). *R. Knapp* 3704 (P), Taiwan Island: *trnL-F* MN524663 (this study). *Moran* 5426 (MO), Taiwan Island, China: *rbcL* L40966 (Hauk et al., 2012), *atpB* U93826 (Wolf, 1997), *matK* KP757848 (Knie et al., 2015), *rps4-trnS* AF313595 (Pryer et al., 2001). *Botrychium matricariifolium* (Retz.) A. Braun ex W.D.J. Koch, *Hauk* 551 (NCU), Michigan, USA: *rbcL* L40967 (Hauk et al., 2012), *trnL-F* DQ849155 (Hauk et al., 2012). *Botrychium michiganense* W.H. Wagner ex A.V. Gilman, Farrar & Zika, *DDFI6329* (ISC), Quebec, Canada: *matK* KY191217 (Dauphin et al., 2018), *trnL-F* KY191850 (Dauphin et al., 2018), *psbA-trnH* KY192136 (Dauphin et al., 2018). *Botrychium minganense* Vict., *Hauk* 578 (NCU), Michigan, USA: *rbcL* L40970 (Hauk et al., 2012), *trnL-F* DQ849160 (Hauk et al., 2012). *Botrychium montanum* W.H. Wagner, *Hauk* 607 (NCU), Montana, USA: *rbcL* L40916 (Hauk et al., 2003), *trnL-F* AY138429 (Hauk et al., 2003). *Botrychium mormo* W.H. Wagner, *Casson* s.n., Minnesota, USA: *rbcL* DQ849169 (Hauk et al., 2012), *trnL-F* DQ849170 (Hauk et al., 2012). *Botrychium neolunaria* Stensvold & Farrar, *DFI6460* (ISC), Quebec, Canada: *matK* KY191254 (Dauphin et al., 2018), *trnL-F* KY191888 (Dauphin et al., 2018), *psbA-trnH* KY192174 (Dauphin et al., 2018). *Botrychium pallidum* W.H. Wagner, *Wagner* s.n. (MICH), Ontario, Canada: *rbcL* DQ849172 (Hauk et al., 2012), *trnL-F* DQ849173 (Hauk et al., 2012). *Botrychium paradoxum* W.H. Wagner, *DFI8449* (ISC), Wyoming, USA: *matK* KY191298 (Dauphin et al., 2018), *psbA-trnH* KY192201 (Dauphin et al., 2018). *Botrychium pedunculosum* W.H. Wagner, *Hauk* 615 (NCU), Oregon, USA: *rbcL* L40973 (Hauk et al., 2003), *trnL-F* AY138434 (Hauk et al., 2003). *Botrychium pinnatum* H. St. John, *Hauk* 604 (NCU), Washington, USA: *rbcL* L40974 (Hauk et al., 2003), *trnL-F* AY138433 (Hauk et al., 2003). *Botrychium pumicola* Coville ex Underw., *Hauk* 618 (NCU), Oregon, USA: *rbcL* L40976 (Hauk et al., 2003), *trnL-F* AY138428 (Hauk et al., 2003). *Botrychium simplex* E. Hitchc., *Hauk* 619 (NUC), Oregon, USA: *rbcL* L40977 (Hauk et al., 2012), *trnL-F* DQ849180 (Hauk et al., 2012), *rps4-trnS* AY870430 (Small et al., 2005). *Botrychium spathulatum* W.H. Wagner, *Wagner* 88036 (MICH), Ontario, Canada: *rbcL* L40980 (Hauk et al., 2003), *trnL-F* AY138423 (Hauk et al., 2003). *Botrypus virginianus* (L.) Michx., *Hauk* 575 (NCU), Michigan, USA: *rbcL* AY138407 (Hauk et al., 2003), *trnL-F* AY138443 (Hauk et al., 2003), *rps4-trnS* AY870434 (Small et al., 2005). *Sahashi* 01103043 (TNS), Fukushima, Japan: *rbcL* AB626650 (Shinohara et al., 2013). *J. Shue SERC-1076416150*, Maryland, USA: *rbcL* KP402672, *psbA-trnH* KP402494. *TNS764332* (TNS), Shizuoka, Japan: *rbcL* AB574673 (Ebihara et al., 2010), *psbA-trnH* AB575338 (Ebihara et al., 2010). *BD1206330* (NEU), Alaska, USA: *matK* KY191410 (Dauphin et al., 2018), *trnL-F* KF700455 (Dauphin et al., 2016), *psbA-trnH* KY192273 (Dauphin et al., 2018). *N.A. Bourg & M. Belovitch SCBI-SIGEO_13_0503*, Virginia, USA: *rbcL* KP643988 (Erickson et al., direct submission), *psbA-trnH* KP643498 (Erickson, direct submission). *Cheiroglossa malgassica* (C.Chr.) Pic. Serm., *Sahashi* 01103028 (TNS), Madagascar: *rbcL* AB626642 (Shinohara et al., 2013), *matK* AB716733 (Shinohara et al., 2013). *Cheiroglossa pal mata* (L.) C. Presl, *R. Peet* s.n. (NCU), Florida, USA: *rbcL* AY138421 (Hauk et al., 2003). *Chingioglossum costatum* (R.Br.) Li Bing Zhang & Petchsri, *Burrows* 5766 (K), Zambia: *rbcL* AY138418 (Hauk et al., 2003). *L. Festo* et al. 2731 (EA, MO, NHT), Coast, Kenya: *psbA-trnH* MN524683 (this study). *Nakamura* 01103033, Thailand: *rbcL* AB626643 (Shinohara et al., 2013), *matK* AB716734 (Shinohara et al., 2013). *S. Petchsri* 2018-29 (BCU), Sa Kaeo, Thailand: *rbcL* MN524768 (this study), *atpB* MN524736 (this study), *matK* MN524826 (this study), *rps4-trnS* MN524788 (this study), *psbA-trnH* MN524684 (this study). *Chingioglossum gomezianum* (Welw. ex A. Braun) Li Bing Zhang & Liang Zhang, *Burrows* 5767 (K), Zambia: *rbcL* AY138419 (Hauk et al., 2003). *Chingioglossum gujaratense* (S.M. Patil, R.N. Kachhiyapatel, R.S. Patel & K.S. Rajput) Li Bing Zhang & Liang Zhang, *K.S. Rajput* et al. *MIPDG023-18*, Gujarat State, India: *rbcL* MH229473, *psbA-trnH* MH229474.
- Chingioglossum* sp., *Buong Kan* 201707, Nang Khai, Thailand: *rbcL* MN524771 (this study), *atpB* MN524739 (this study), *rps4-trnS* MN524791 (this study), *psbA-trnH* MN524687 (this study). *Morton* 1967 (MO), Nigeria: *atpB* MN524752 (this study), *rps4-trnS* MN524806 (this study), *psbA-trnH* MN524706 (this study). *Petchsri* 201830 (BCU), Sa kaeo, Thailand: *rbcL* MN524773 (this study), *atpB* MN524740 (this study), *rps4-trnS* MN524793 (this study), *psbA-trnH* MN524689 (this study). *M. Rabarimanarivo* et al. 222 (MO), Fianarantsoa, Madagascar: *psbA-trnH* MN524690 (this study). *Haukia crotalophoroides* (Walter) Li Bing Zhang & Liang Zhang, *A. Grusz* 112 (DUKE), San Jose, Costa Rica: *rbcL* MN524769 (this study), *atpB* MN524737 (this study), *matK* MN524827 (this study), *rps4-trnS* MN524789 (this study), *psbA-trnH* MN524685 (this study). *Leonard* 9380 (NCU), Mississippi, USA: *rbcL* AY138417 (Hauk et al., 2003). *Holubiella lunarioides* (Michx.) Škoda, *Watkins* 29 (ISC), Alabama, USA: *rbcL* AY138406 (Hauk et al., 2003), *trnL-F* AY138442 (Hauk et al., 2003), *rps4-trnS* AY870431 (Small et al., 2005). *Japanobotrychum chamaeconium* (*Bitter & Hieron. ex Bitter*) *Nishida ex Tagawa*, *W514*, ?Cameroon: *rbcL* DQ849188 (Hauk et al., 2012), *trnL-F* DQ849189 (Hauk et al., 2012). *Japanobotrychum lanuginosum* (Wall. ex Hook. & Grev.) *Nishida* ex *Tagawa*, *Boufford* et al. 29834 (GH), Bomi, Xizang, China: *rbcL* DQ110378 (Davis et al., 2005). *Liheng* 9400 (MO), Yunnan, China: *rbcL* JN628840 (Hauk et al., 2012), *trnL-F* JN628839 (Hauk et al., 2012). *Ohkubo* 01103045 (TNS), Mo valley, Bhutan: *rbcL* AB626653 (Shinohara et al., 2013). *Helminthostachys zeylanica* (L.) Hook., *R. Knapp* 4524 (P), Taiwan Island: *rbcL* MN524758 (this study), *atpB* MN524725 (this study), *matK* MN524813 (this study), *psbA-trnH* MN524670 (this study). *Matsumoto* 01103040 (TNS), Vanuatu: *rbcL* AB626636 (Shinohara et al., 2013), *matK* AB716727 (Shinohara et al., 2013). *Pryer* DB783 (UC) & Qiu 98077 (Z), cult.: *rbcL* EU352293 (Rai & Graham, 2010), *atpB* DQ646095 (Qiu et al., 2006), *matK* KP757847 (Knie et al., 2015). *Sahashi* s.n. (TOHO), Japan: *rbcL* AY138409 (Hauk et al., 2003), *trnL-F* AY138445 (Hauk et al., 2003), *rps4-trnS* AY870436 (Small et al., 2005). *TCA2009_0806*, Cambodia: *rbcL* JQ349077 (Kim & Kim, direct submission), *rps4-trnS* KF006816 (Kim & Kim, direct submission). *TNS759310* (TNS), Okinawa, Japan: *rbcL* AB574674 (Ebihara et al., 2010), *psbA-trnH* AB575339 (Ebihara et al., 2010). *Tomiyama* 01103039 (TNS), Okinawa, Japan: *rbcL* AB626637 (Shinohara et al., 2013), *matK* AB716728 (Shinohara et al., 2013). *Mankya chejuensis* B.Y. Sun, M.H. Kim & C.H. Kim, Jeju Island, Korea: *rbcL* JF343520 (Kim & Kim, direct submission), *atpB* JF343520 (Kim & Kim, direct submission), *rps4-trnS* JF343520 (Kim & Kim, direct submission). *psbA-trnH* KJ415171 (Kim & Kim, direct submission). *pdkb* 2006-0857, Jeju Island, Korea: *rbcL* JQ349078 (Kim & Kim, direct submission), *psbA-trnH* KJ415174 (Kim & Kim, direct submission). *Kim* 01103041, Jeju Island, Korea: *rbcL* AB626635 (Shinohara et al., 2013), *matK* AB716726 (Shinohara et al., 2013), *psbA-trnH* KJ415173 (Kim & Kim, direct submission). *K.-J. Jo* et al., 2012-0028 (KUS), Jeju Island, Korea: *rbcL* KP205433 (Kim & Kim, 2018), *atpB* KP205433 (Kim & Kim, 2018), *rps4-trnS* KP205433 (Kim & Kim, 2018), *psbA-trnH* KP205433 (Kim & Kim, 2018). *Marattia fraxinea* Sm., *K. Roux* s.n. (UC), South Africa: *rbcL* EU439088 (Murdock, 2008), *atpB* EU439067 (Murdock, 2008). *Marattia laxa* Kunze, *Christenhusz* 1313 (TUR), Mexico: *rbcL* EU439084 (Murdock, 2008), *atpB* EU439062 (Murdock, 2008). *Ophioderma falcatum* Deg., *Sahashi* 01103029 (TNS), Pohnpei, Federated States Of Micronesia: *rbcL* AB626641 (Shinohara et al., 2013), *matK* AB716732 (Shinohara et al., 2013). *Vinette* 27 (UC), Moorea, French Polynesia: *rbcL* KY099838 (Nitta et al., 2017), *psbA-trnH* KY099969 (Nitta et al., 2017). *Ophioderma pendulum* C. Presl, *ABG* 20182138 "B", cult. in ABG: *rbcL* MN524759 (this study), *atpB* MN524726 (this study), *matK* MN524814 (this study), *rps4-trnS* MN524709 (this study), *psbA-trnH* MN524671 (this study). *ABG* 20182139 "D", cult. in ABG: *rbcL* MN524760 (this study), *atpB* MN524727 (this study), *matK* MN524815 (this study), *rps4-*

trnS MN524710 (this study), *psbA-trnH* MN524672 (this study). *ABG 20182140 "E"*, cult. in ABG: *rbcL* MN524761 (this study), *atpB* MN524728 (this study), *matK* MN524816 (this study), *rps4-trnS* MN524711 (this study), *psbA-trnH* MN524673 (this study). *ABG 20081192 "F"*, cult. in ABG from Florida nursery: *atpB* MN524729 (this study), *matK* MN524817 (this study), *rps4-trnS* MN524712 (this study), *psbA-trnH* MN524674 (this study). *ABG 20100635 "C"*, cult. in ABG from Malaysia nursery 2010: *rbcL* MN524762 (this study), *atpB* MN524730 (this study), *matK* MN524818 (this study), *rps4-trnS* MN524713 (this study), *psbA-trnH* MN524675 (this study). *ABG 20110350 "A"*, cult. in ABG from Dong Bac, Vietnam: *atpB* MN524731 (this study), *matK* MN524819 (this study), *rps4-trnS* MN524714 (this study), *psbA-trnH* MN524676 (this study). *Chantanaorrapint & Sangrattanaprasert s.n.*, Songkhla, Thailand: *rbcL* MN524763 (this study), *atpB* MN524732 (this study), *matK* MN524820 (this study), *rps4-trnS* MN524715 (this study), *psbA-trnH* MN524677 (this study). *L.B.Zhang et al. 10260* (KUN, CDBI, BCU, PYU, MO, XTBG), cult. in Thailand: *rbcL* MN524764 (this study), *atpB* MN524733 (this study), *matK* MN524821 (this study), *rps4-trnS* MN524716 (this study), *psbA-trnH* MN524678 (this study). *L.B.Zhang et al. 8637* (CDBI, MO, PHH), Lam Dong, Vietnam: *rbcL* MN524765 (this study), *atpB* MN524734 (this study), *matK* MN524822 (this study), *rps4-trnS* MN524717 (this study), *psbA-trnH* MN524679 (this study). *L.B.Zhang et al. 8696* (CDBI, MO, PHH), Khanh Hoa, Vietnam: *rbcL* MN524766 (this study), *atpB* MN524735 (this study), *matK* MN524823 (this study), *rps4-trnS* MN524718 (this study), *psbA-trnH* MN524680 (this study). *Ranker 1932* (COLO), Moorea, French Polynesia: *rbcL* KY099839 (Nitta et al., 2017), *psbA-trnH* KY099970 (Nitta et al., 2017). *RGB Kew 1976-06412* (K), Kew Gardens, London, UK: *rbcL* AY138420 (Hauk et al., 2003), *matK* JF303896. *Sahashi 01103030* (TNS), Madagascar: *rbcL* AB626639 (Shinohara et al., 2013), *matK* AB716730 (Shinohara et al., 2013). *Sahashi 01103031* (TNS), Honiara, Guadalcanal: *rbcL* AB626638 (Shinohara et al., 2013), *matK* AB716729 (Shinohara et al., 2013). *Sahashi 01103032* (TNS), Mt. Panie, New Caledonia: *rbcL* AB626640 (Shinohara et al., 2013), *matK* AB716731 (Shinohara et al., 2013). *TNS759347* (TNS), Okinawa, Japan: *rbcL* AB574677 (Ebihara et al., 2010), *psbA-trnH* AB575341 (Ebihara et al., 2010). *Ophioderma redactophylla* Chantanaorr. & Li Bing Zhang, *Chantanaorrapint & Sangrattanaprasert 220* (BKF, MO, PSU), Yala, Thailand: *rbcL* MN524767 (this study), *matK* MN524824 (this study), *rps4-trnS* MN524719 (this study), *psbA-trnH* MN524681 (this study). *Ophioglossum austroasiaticum* Nishida, R. Knapp 3227 (P), Taiwan Island: *matK* MN524825 (this study), *psbA-trnH* MN524682 (this study). *R. Knapp 4303* (P), Taiwan Island: *rbcL* MN524785 (this study), *atpB* MN524751 (this study), *matK* MN524841 (this study), *rps4-trnS* MN524804 (this study), *psbA-trnH* MN524702 (this study). *Ophioglossum californicum* Prantl, cult. in Beadle Center Greenhouse in Univ. of Nebraska-Lincoln: *rbcL* KC117178 (Grewe et al., 2013), *atpB* KC117178 (Grewe et al., 2013), *matK* KC117178 (Grewe et al., 2013), *rps4-trnS* KC117178 (Grewe et al., 2013). *Ophioglossum coriaceum* A.Cunn., *Papadopoulos NSWAP708* (NSW), Lord Howe Island, Australia: *rbcL* JF950813 (Papadopoulos et al., 2011). *Ophioglossum cf. gramineum* Willd., R. Pollawat 2570 (BCU), Jumrung Swamp Forest, Rayong Botanical Garden, Klaeng District, Rayong Province, Thailand: *rbcL* MN524770 (this study), *atpB* MN524738 (this study), *matK* MN524828 (this study), *rps4-trnS* MN524790 (this study), *psbA-trnH* MN524686 (this study). *RGB Kew 1981-6838* (K), London, UK: *rbcL* AY138412 (Hauk et al., 2003). *Ophioglossum kawamurae*, *Sahashi 01103034* (TNS), Aogashima, Japan: *rbcL* AB626648 (Shinohara et al., 2013), *matK* AB716739 (Shinohara et al., 2013). *Ophioglossum namegatae* Nish. & Kurita, *TNS764351* (TNS), Ibaraki, Japan: *rbcL* AB574675 (Ebihara et al., 2010), *psbA-trnH* AB575340 (Ebihara et al., 2010). *Ophioglossum nudicaule* L. f., W. M. Chu et al. 15999 (PYU), Yunnan, China: *rbcL* MN524772 (this study), *matK* MN524829 (this study), *rps4-trnS* MN524792 (this study), *psbA-trnH* MN524688 (this study). *Wurdack s.n.* (NCU), Georgia, USA: *rbcL* AY138416 (Hauk et al., 2003). *Ophioglossum parvifolium* Grev. & Hook. in Hook., K.S. Rajput et al. s.n., India: *rbcL* MH205947 (Rajput et al., direct submission), *psbA-trnH* MG875322 (Rajput et al., direct submission). *Ophioglossum parvum* M.Nishida & S.Kurita (original determination by R. Knapp as *O. sp.*), *TNS1108350* (TNS), Shizuoka, Japan: *rbcL* AB574676 (Ebihara et al., 2010). *R. Knapp 4647* (P, MO), Taiwan Island: *rbcL* MN524787 (this study), *matK* MN524843 (this study), *rps4-trnS* MN524805 (this study), *psbA-trnH* MN524704 (this study). *Ophioglossum petiolatum* Hook., R. Knapp 4601 (P), Taiwan Island: *rbcL* MN524786 (this study), *matK* MN524842 (this study), *psbA-trnH* MN524703 (this study). *Z.R.He et al. MT-168* (PYU), Xizang, China: *rbcL* MN524774 (this study), *atpB* MN524741 (this study), *matK* MN524830 (this study), *rps4-trnS* MN524794 (this study), *psbA-trnH* MN524691 (this study). *L.B.Zhang et al. 10132* (KUN, CDBI, BCU, PYU, MO, XTBG), cult. in Thailand: *rbcL* MN524775 (this study), *atpB* MN524742 (this study), *matK* MN524831 (this study), *rps4-trnS* MN524795 (this study), *psbA-trnH* MN524692 (this study). *L.B.Zhang et al. 10258* (KUN, CDBI, BCU, PYU, MO, XTBG), cult. in Thailand: *rbcL* MN524776 (this study), *atpB* MN524743 (this study), *matK* MN524832 (this study), *rps4-trnS* MN524796 (this study), *psbA-trnH* MN524693 (this study). *L.B.Zhang et al. 9022* (CDBI, MO, PHH), Dak Lak, Vietnam: *rbcL* MN524777 (this study), *matK* MN524833 (this study), *rps4-trnS* MN524797 (this study), *psbA-trnH* MN524694 (this study). *Oudinnai 2018/7/3 RP513*, Chiang Mai, Thailand: *rbcL* MN524778 (this study), *atpB* MN524744 (this study), *matK* MN524834 (this study), *rps4-trnS* MN524798 (this study), *psbA-trnH* MN524695 (this study). *S. Petchsri 2016-2* (BCU), Ubon Ratchathani, Thailand: *rbcL* MN524779 (this study), *atpB* MN524745 (this study), *matK* MN524835 (this study), *rps4-trnS* MN524799 (this study), *psbA-trnH* MN524696 (this study). *S. Petchsri 2016/9/25* (BCU), Chiang Mai, Thailand: *rbcL* MN524780 (this study), *atpB* MN524746 (this study), *matK* MN524836 (this study), *psbA-trnH* MN524697 (this study). *RGB Kew 1988-03791(K)*, London, UK: *rbcL* AY138411. *Suzuki 01103035* (TNS), Osaka, Japan: *rbcL* AB626647 (Shinohara et al., 2013), *matK* AB716738 (Shinohara et al., 2013). *TNS764216* (TNS), Kagoshima, Japan: *rbcL* AB574678 (Ebihara et al., 2010), *psbA-trnH* AB575342 (Ebihara et al., 2010). *H.C.Wang et al. JC4536* (PYU), Yunnan, China: *rbcL* MN524781 (this study), *atpB* MN524747 (this study), *matK* MN524837 (this study), *rps4-trnS* MN524800 (this study), *psbA-trnH* MN524698 (this study). *H.C.Wang et al. LP4454* (PYU), Yunnan, China: *rbcL* MN524782 (this study), *atpB* MN524748 (this study), *matK* MN524838 (this study), *rps4-trnS* MN524801 (this study), *psbA-trnH* MN524699 (this study). *E. Cieraad s.n.* (WELT P20751), Kermadec Islands, Raoul Island, New Zealand: *rbcL* EF469946 (Perrie & Brownsey, 2007). *Ophioglossum pusillum* Raf., *Nekola 8069* (COE), Iowa, USA: *rbcL* AY138413 (Hauk et al., 2003). *Ophioglossum pycnostichum* (Fernald) Á. Löve & D. Löve, *SCBI-SIGEO_13_0145*: *rbcL* KP644048 (Erickson et al., direct submission), *psbA-trnH* KP643548 (Erickson et al., direct submission). *Ophioglossum reticulatum* L., *E. Bidault et al. 1874* (MO), Moyen-Ogooué, Gabon: *rbcL* MN524783 (this study), *atpB* MN524749 (this study), *matK* MN524839 (this study), *rps4-trnS* MN524802 (this study), *psbA-trnH* MN524700 (this study). *L.B.Zhang et al. 9917* (KUN, CDBI, BCU, PYU, MO, XTBG), Loei, Thailand: *rbcL* MN524784 (this study), *atpB* MN524750 (this study), *matK* MN524840 (this study), *rps4-trnS* MN524803 (this study), *psbA-trnH* MN524701 (this study). *R. Moran 5644* (MO), Santa Bárbara, Honduras: *rbcL* AY138410 (Hauk et al., 2003), *rps4-trnS* AF313594 (Pryer et al., 2001). *Sahashi 01103036* (TNS), Bali, Indonesia: *rbcL* AB626646 (Shinohara et al., 2013), *matK* AB716737 (Shinohara et al., 2013). *Suzuki 01103037* (TNS), Doi Inthanon, Thailand: *rbcL* AB626645 (Shinohara et al., 2013), *matK* AB716736 (Shinohara et al., 2013). *Opioglossum richardsiae* J.E. Burrows, *Burrows 5756* (K), Zambia: *rbcL* AY138415 (Hauk et al., 2003). *Ophioglossum cf. thermale* Kom., *R. Knapp 3536* (P), Taiwan Island:

psbA-trnH MN524707 (this study). *Ophioglossum thermale* Kom., L.B.Zhang et al. 482 (CDBI, MO), Guizhou, China: *psbA-trnH* MN524705 (this study). *S. Petchsri* 201603 (BCU, CDBI), Ubon Ratchathani, Thailand: *psbA-trnH* MN524708 (this study). *TNS764007* (TNS), Okinawa, Japan: *rbcL* AB574679 (Ebihara et al., 2010), *psbA-trnH* AB575343 (Ebihara et al., 2010). *Ophioglossum vulgatum* L., Burrows 5752 (K), Zambia: *rbcL* AY138414 (Hauk et al., 2003). *Sahashi 01103038* (TNS), Fukushima, Japan: *rbcL* AB626644 (Shinohara et al., 2013), *matK* AB716735 (Shinohara et al., 2013). *TNS1108351* (TNS), Chiba, Japan: *rbcL* AB574681 (Ebihara et al., 2010), *psbA-trnH* AB575344 (Ebihara et al., 2010). *Psilotum nudum* (L.) P. Beauv., *rbcL* AP004638 (Wakasugi et al., direct submission), *atpB* AP004638 (Wakasugi et al., direct submission). *Sahashia stricta* (L.Underw.) Li Bing Zhang & Liang Zhang, *Sahashi 01103044* (TNS), Fukushima, Japan: *rbcL* AB626649 (Shinohara et al., 2013). *Sahashi s.n.* (OS), Japan: *rbcL* AY138408 (Hauk et al., 2003), *trnL-F* AY138444 (Hauk et al., 2003), *rps4-trnS* AY870435 (Small et al., 2005). *TNS765136* (TNS), Kanagawa, Japan: *rbcL* AB574669 (Ebihara et al., 2010), *psbA-trnH* AB575335 (Ebihara et al., 2010). China: Northeast, YYZW2015DK19-1: *rbcL* KY014896, *psbA-trnH* KX068968 (unpublished). *Sceptridium atrovirens* *Sahashi*, *Oka 01103048* (TNS), Hakone, Japan: *rbcL* AB626652 (Shinohara et al., 2013), *matK* AB716743 (Shinohara et al., 2013). *Sahashi s.n.* (TOHO), Japan: *rbcL* AY138402 (Hauk et al., 2003), *trnL-F* AY138438 (Hauk et al., 2003). *Sceptridium daucifolium* (*Wall. ex Hook. & Grev.*) Lyon, *Kiuchi 01103049*, E. Sikkim, India: *rbcL* AB626651 (Shinohara et al., 2013), *matK* AB716742 (Shinohara et al., 2013). L.B.Zhang et al. 6873 (CDBI, MO, VNMN), Ha Giang, Vietnam: *matK* MN524807 (this study), *trnL-F* MN524660 (this study), *rps4-trnS* MN524654 (this study), *psbA-trnH* MN524664 (this study). L.B.Zhang et al. 7437 (CDBI, MO, VNMN), Quang Binh, Vietnam: *rbcL* MN524753 (this study), *atpB* MN524720 (this study), *matK* MN524808 (this study), *trnL-F* MN524661 (this study), *rps4-trnS* MN524655 (this study), *psbA-trnH* MN524665 (this study). L.B.Zhang et al. 7560 (CDBI, MO, VNMN), Quang Tri, Vietnam: *rbcL* MN524754 (this study), *atpB* MN524721 (this study), *matK* MN524809 (this study), *trnL-F* MN524662 (this study), *rps4-trnS* MN524656 (this study), *psbA-trnH* MN524666 (this study). L.B.Zhang et al. 8442 (CDBI, MO, PHH), Lam Dong, Vietnam: *rbcL* MN524755 (this study), *atpB* MN524722 (this study), *matK* MN524810 (this study), *rps4-trnS* MN524657 (this study), *psbA-trnH* MN524667 (this study). L.B.Zhang et al. 8618 (CDBI, MO, PHH), Lam Dong, Vietnam: *rbcL* MN524756 (this study), *atpB* MN524723 (this study), *matK* MN524811 (this study), *rps4-trnS* MN524658 (this study), *psbA-trnH* MN524668 (this study). L.B.Zhang et al. 8992 (CDBI, MO, PHH), Đák Lák, Vietnam: *rbcL* MN524757 (this study), *atpB* MN524724 (this study), *matK* MN524812 (this study), *rps4-trnS* MN524659 (this study), *psbA-trnH* MN524669 (this study). *Sceptridium dissectum* (Spreng.) Lyon, *Hauk 621* (NCU), North Carolina, USA: *rbcL* AY138401 (Hauk et al., 2003), *trnL-F* AY138436 (Hauk et al., 2003), *rps4-trnS* AY870432 (Small et al., 2005). *Kosha 01103046*, Washington, USA: *rbcL* AB626655 (Shinohara et al., 2013), *matK* AB716746 (Shinohara et al., 2013). *Sceptridium formosanum* (Tagawa) Holub, *K013186c* (KBCC), Taiwan Island, China: *rbcL* JF303963 (Kuo et al., 1988), *matK* JF303897 (Kuo et al., 1988). *TNS759323* (TNS), Okinawa, Japan: *rbcL* AB574662 (Ebihara et al., 2010), *psbA-trnH* AB575328 (Ebihara et al., 2010). *Sceptridium japonicum* (Prantl) Lyon, *Sahashi s.n.* (TOHO), Japan: *rbcL* AY138403 (Hauk et al., 2003), *trnL-F* AY138439 (Hauk et al., 2003), *rps4-trnS* AY870433 (Small et al., 2005). *Sceptridium multifidum* (S.G.Gmel.) M.Nishida, *Hauk 577* (NCU), Michigan, USA: *rbcL* AY138400 (Hauk et al., 2003), *trnL-F* AY138435 (Hauk et al., 2003). *TNS1107871* (TNS), Hokkaido, Japan: *rbcL* AB574666 (Ebihara et al., 2010), *psbA-trnH* AB575332 (Ebihara et al., 2010). *Sceptridium nipponicum* (Makino) Holub, *Sahashi 01103047* (TNS), Miyagi, Japan: *rbcL* AB626654 (Shinohara et al., 2013), *matK* AB716745 (Shinohara et al., 2013). *Sceptridium oneidense* (Gilbert) Holub, *Wagner 8600* (MICH), Michigan, USA: *rbcL* AY138898 (Hauk et al., 2003), *trnL-F* AY138437 (Hauk et al., 2003). *Sceptridium ternatum* (Thunb.) Lyon, *Sahashi s.n.* (TOHO), Japan: *rbcL* AY138404 (Hauk et al., 2003), *trnL-F* AY138440 (Hauk et al., 2003). *Tmesipteris elongata* P.A. Dang., *MPN47838*, Palmerston North, New Zealand: *rbcL* KJ569699 (Zhong et al., 2014), *atpB* KJ569699 (Zhong et al., 2014).