Monachosorum arakii Tagawa (Dennstaedtiaceae) is a Relict “International” Hybrid: A Reassessment of the Monachosorum Species

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Monachosorum arakii Tagawa (Dennstaedtiaceae) is a Relict “International” Hybrid: A Reassessment of the Monachosorum Species

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Abstract—Monachosorum arakii Tagawa is a plant species endemic to the western part of the main island of Japan. It is characterized by large bulbils on the rachises and is a close relative of M. henryi Christ, which can be found in the Sino-Himalayan region and is not present in Japan. Although M. arakii was reported to be a hexaploid, we determined that it is a pentaploid based on chromosome counts. All of the herbarium specimens examined, including the holotype, had irregularly shaped spores, suggesting that this is a sterile hybrid species. Analysis of the nuclear gapCp sequences also supported its hybrid origin from M. henryi (tetraploid) and M. nipponicum Makino (hexaploid). It should be noted that the parental species of M. arakii, which are endemic to Japan, only co-occur in China. It is possible that the hybrids are relicts from the time when M. henryi were also present in Japan, and are now reproducing only vegetatively by rhizome division and bulbil production. The updated taxonomic treatments for Monachosorum species provided in the current study recognize four species and two hybrid taxa.

Keywords—Asia, bulbil, chromosome, Japan, ploidy level, spore.

Monachosorum Kunze (Dennstaedtiaceae) is a small Asiatic genus comprising approximately six species that predominantly grow in temperate regions (Kramer 1990; Yan et al. 2013; Fig. 1). Monachosorum maximowiczii (Baker) Hayata is sometimes regarded as the independent genus Ptilopteris Hance according to its 1-pinnate, lanceolate fronds, but its sister relationship to members of Monachosorum (M. arakii Tagawa and M. nipponicum Makino [known as M. flagellare (Maxim. ex Makino) Hayata in previous publications; see Taxonomic Treatment]) has been confirmed by chloroplast phylogeny (Ebihara 2011). Monachosorum arakii, which is found in Japan’s Kyoto Prefecture, has features, such as large bulbils on the rachis (Tagawa 1935), similar to those of M. henryi Christ, which is distributed in the Sino-Himalayan region. Monachosorum arakii differs from M. henryi by its smaller fronds, and “narrower and more sharply apiculate-acute teeth or [of] ultimate segments.” Tagawa (1935) described M. arakii as a new species with a morphology intermediate to that of M. henryi and M. nipponicum. However, it has never been treated as a hybrid taxon, probably due to the lack of widespread M. henryi distribution in Japan.

Monachosorum arakii is a species endemic to Japan that is found in approximately 18 locations along 15 river systems in Honshu, Shikoku, and Kyushu, usually in high-humidity environments (Kurata and Nakaike 1979; A. Ebihara, unpublished data; Fig. 2). As a result of environmental changes caused largely by human activity, the number of locations where it can be found has decreased, and it was recently placed in the category EN in the Red Data Book of Japan (Japanese Ministry of Environment 2015).

Hirabayashi (1968) observed irregular meiosis with n = ca. 156 II + ca. 24 I, n = ca. 162 II + ca. 12 I chromosomes in M. arakii, and identified it as a hexaploid species. Despite his observation of irregular meiosis with univalent chromosomes, suggesting sterility, he did not consider the possibility of it being a hybrid. This only could have occurred as a result of hybridization between hexaploid M. nipponicum (Mitui 1967, 1968; Hirabayashi 1968) and diploid M. maximowiczii (Kurita 1967; Mitui 1968; Hirabayashi 1968), which seems unrealistic both morphologically and cytologically. To clarify the origin and phylogeny of M. arakii, we used molecular and cytological approaches to carefully compare all members of the genus Monachosorum distributed in Japan and Asia.

Materials and Methods

Materials—Two or more plants were sampled from each of the widely accepted species in Monachosorum [M. arakii, M. nipponicum, M. henryi, M. maximowiczii, and M. subdigitatum (Blume) Kuhn], and from a putative hybrid between M. nipponicum and M. maximowiczii. A total of 40 samples were subjected to molecular analysis and chromosome counts: 23 from Japan, 9 from Taiwan, 3 from China, 3 the Philippines, and 2 from Indonesia (Table 1; Appendix I).

Chromosome Counts and Spore Observations—The root tips of cultivated stocks of M. henryi, some of which were grown from bulbils, were fixed, and chromosomes in mitotic metaphase were observed according to the method of Ebihara et al. (2014). Spores of the herbarium specimens (M. arakii and M. subdigitatum) and voucher specimens were observed after being embedded in Boleit (Ohken Co., Tokyo, Japan). The length of the spores was averaged from 30 mature spores.

Molecular Analyses—DNA was extracted from silica-dried leaf tissues, using a DNaseq plant mini kit (Qiagen, Hilden, Germany). DNA collection from Japanese ferns (Ebihara et al. 2010) in the Center for Molecular Biodiversity Research, National Museum of Nature and Science were also included in this study. Two markers, the chloroplast rbcL gene with 1,187 base pairs (bp) and the nuclear gapCp region of approximately 600 bp, were used. For the rbcL region, methods for amplification and sequencing were as described by Ebihara et al. (2010). For the gapCp region, fragments including introns 8–10 and exons 9 and 10 were amplified using the ESGAPCP8F1 and ESGAPCP11R1 primers, respectively (Schuettelpelz et al. 2008).

Multiple sequences were identified by electrophoresis using the single strand conformation polymorphism (SSCP) method in a mutation detection enhancement (MDE) gel (Cambio, East Rutherford, NJ, USA) containing 2% glycerol, which was run at 20°C for 12 h at 350 V, followed by silver staining. After comparing banding patterns, each band was excised and stored at –80°C for 2 h, and then stored again at
−80°C for 2 h in 20 μL water. These solutions were used as templates for PCR with the ESGAPCP8F1 and ESGAPCP11R1 primers, followed by sequencing according to the same method used for amplification of *rbcL*.

The sequences were typified and made non-redundant by removing duplicate sequences (Appendix 2). They were aligned using ClustalX 2.1 with sequences of *Dennstaedtia scabra* (Wall. ex Hook.) T. Moore from GenBank (accession numbers EU551392–EU551401) as an outgroup. Maximum likelihood (ML) model testing was performed using jModelTest (Posada 2008), with the appropriate substitution models selected based on the Akaike information criterion (Akaite 1974). Garli 2.0 (Zwickl 2006) was used to reconstruct the ML phylogeny. The matrix was partitioned into four groups (i.e. first, second, and third codons, and introns), and each group had its own substitution model. The proportions of invariant sites and state frequencies were estimated, and the “genthreshfortopoterm” option was set to 20,000. To calculate ML bootstrap support (BS) values, 1,000 replicates were run under the same criteria. The posterior probability (PP) of Bayesian inference was determined using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist

**Fig. 1.** Representative members of the genus *Monachosorum*. A. *M. arakii* (Mie Pref., Japan). B. bulbils on the rachis of *M. arakii* (Kumamoto Pref., Japan). C. *M. henryi* (Kaohsiung Co., Taiwan). D. *M. nipponicum* (Kumamoto Pref., Japan). E. *M. maximowiczii* (Saitama Pref., Japan).

**Fig. 2.** General distribution of the five *Monachosorum* species. The enlarged map of Japan indicates known locations of *M. arakii* (dots) and the sampled sites (arrowheads) with major river systems.
Table 1. Monachosorum material used in this study. The gapCp alleles and genotypes in square brackets show those obtained by comparison of SSCP banding positions. Tentatively assumed as octoploid.

<table>
<thead>
<tr>
<th>Species Locality Sample No.</th>
<th>Sample No.</th>
<th>nC/L</th>
<th>gapCp alleles</th>
<th>gapCp genotype</th>
<th>Ploidy</th>
<th>Spore size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan, Mie Pref., Owase-shi AE3454</td>
<td>seq. 1</td>
<td>[A/B₁/B₂/C₁]</td>
<td>[AB,BC₃C₄]</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ibid. AE3455</td>
<td>seq. 1</td>
<td>[A/B₁/B₂/C₁]</td>
<td>AB,BC₃C₄</td>
<td>5x (2n = 280)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Japan, Hyogo Pref., Sasayama-shi ibid. Nakato2867</td>
<td>seq. 1</td>
<td>[A/B₁/B₂/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ibid. Nakato3004</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>5x (2n = ca. 280)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Japan, Kumamoto Pref., Itsuki-mura ibid. KS2007-226</td>
<td>N/A</td>
<td>[A/B₁/B₂/C₃/C₄]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ibid. KS2007-232</td>
<td>AB57490</td>
<td>[A/B₁/B₂/C₃/C₄]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ibid. AE3041</td>
<td>seq. 1</td>
<td>[A/B₁/B₂/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>M. maximoviczii Japan, Satsuma Pref., Chichibu-shi ibid. AE322</td>
<td>A/b</td>
<td>A</td>
<td>[AA]</td>
<td>2x (2n = 112)</td>
<td>Fig. 3A, F</td>
<td>25.0 ± 2.3 μm</td>
</tr>
<tr>
<td>Japan, Wakayama Pref., Nachikatsuura-cho ibid. AE322</td>
<td>N/A</td>
<td>A</td>
<td>[AA]</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>M. nipponicum (= ‘M. flagellare’). Japan, Tochigi Pref, Kanuma-shi ibid. AE2609</td>
<td>N/A</td>
<td>[A/B₁/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Japan, Satsuma Pref., Chichibu-shi ibid. AE322</td>
<td>A/b</td>
<td>A</td>
<td>[AA]</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Japan, Wakayama Pref., Shingu-shi ibid. AE2609</td>
<td>N/A</td>
<td>[A/B₁/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Japan, Kumamoto Pref., Itsuki-mura ibid. AE322</td>
<td>N/A</td>
<td>[A/B₁/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Japan, Kagoshima Pref., Ohkuchi-shi ibid. AE322</td>
<td>N/A</td>
<td>[A/B₁/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>M. maximoviczii x M. nipponicum (= ‘M. flagellare’). Japan, Mie Pref., Owase-shi KI2007-1281</td>
<td>seq. 1</td>
<td>[A/B₁/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Japan, Wakayama Pref., Nachikatsuura-cho ibid. AE322</td>
<td>seq. 1</td>
<td>[A/B₁/C₃]</td>
<td>AB,BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>M. henryi Taiwan, Kaohsiung Co., Sanpin TW2006-65</td>
<td>seq. 1</td>
<td>B₁/B₃/C₂</td>
<td>BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taiwan, Pingtung Co., Jinshui-ying TW2006-137</td>
<td>seq. 1</td>
<td>B₁/B₃/C₂/C₁</td>
<td>BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taiwan, Kaohsiung Co., Taoyuan Hsian TW2008-1812</td>
<td>seq. 1</td>
<td>B₂/C₀/C₁</td>
<td>BC₃C₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taiwan, Ilan Co., Taipin-shan GK10400</td>
<td>seq. 1</td>
<td>B₁/B₃/C₀</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taiwan, Ilan Co., Fushan ibid. Kuo3781</td>
<td>seq. 1</td>
<td>B₁/C₃</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taiwan, Chiayi Co., Alishan Lu29337</td>
<td>seq. 1</td>
<td>B₁/C₃</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taiwan, Chiayi Co., Tefuye Lu29514</td>
<td>seq. 1</td>
<td>B₁/C₃</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ibid. AE3398</td>
<td>seq. 1</td>
<td>B₁/C₃</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taiwan, Chiayi Co., Tashan AE3414</td>
<td>seq. 1</td>
<td>B₁/B₃/C₀</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>China, Guangdong, Xingyi City Chang, 7003</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>China, Yunnan, Malipo Co. Kuo3113</td>
<td>seq. 1</td>
<td>B₁/C₃</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>China, Yunnan, Pingbian Co. Kuo3232</td>
<td>B₁/C₆</td>
<td>BC₃C₄</td>
<td>4x (2n = ca. 224)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. subdigitation Indonesia, Java, Gede-Pangrango National Park Wadi1884</td>
<td>seq. 1</td>
<td>B₁/C₆/C₇/D₁</td>
<td>BC₃C₄</td>
<td>-</td>
<td>32.4 ± 2.4 μm</td>
<td></td>
</tr>
<tr>
<td>Indonesia, Java, Halimun National Park Wadi2054</td>
<td>seq. 1</td>
<td>B₁/C₆/C₇/D₁</td>
<td>BC₃C₄</td>
<td>-</td>
<td>32.4 ± 2.4 μm</td>
<td></td>
</tr>
<tr>
<td>The Philippines, Mindanao, Mt. Abo Kuo2736</td>
<td>seq. 2</td>
<td>B₁/C₇/D₁</td>
<td>BC₃C₄</td>
<td>-</td>
<td>32.4 ± 2.4 μm</td>
<td></td>
</tr>
<tr>
<td>The Philippines, Mindanao, Mt. Kitanglad Kuo3484</td>
<td>seq. 2</td>
<td>C₁/D₂/D₃/D₄</td>
<td>BC₃C₄</td>
<td>-</td>
<td>32.4 ± 2.4 μm</td>
<td></td>
</tr>
</tbody>
</table>
and Huelsenbeck 2003). Two simultaneous runs involved four chains (1,000,000 generations each), in which each chain was sampled every 1,000 generations. The first 25% of a sample was discarded as burn-in, and the remainder was used to calculate the 50% majority-rule consensus tree. All of the gaps were treated as missing data.

**RESULTS**

**Ploidy Levels**—The chromosome numbers $2n = 112$ ($2x$, $x = 56$) and $2n = 336$ / ca. 336 (6x) were counted for *M. maximowiczii* (one sample from Wakayama Prefecture, Japan) and *M. nipponicum* (two samples from Wakayama Prefecture), respectively (Fig. 3A, B, F, G). These results matched previous cytological records. Counts of $2n = 224$ / ca. 224 ($4x$, $x = 56$) were obtained in three samples of putative hybrids between *M. maximowiczii* and *M. nipponicum* (three samples from Wakayama Prefecture, Fig. 3C, H). Six *M. henryi* samples (one each from five different locations in Taiwan and Guandong Province, China) showed $2n = 224$ / ca. 224 ($4x$) (Fig. 3D, I), which is consistent with previous counts (Hirabayashi 1968, based on a Taiwanese sample, as 'M. subdigitatum'). In *M. arakii*, two samples, one each from two different locations (Mie Prefecture and Hyogo Prefecture), showed $2n = 280$ / ca. 280 (5x) (Fig. 3E, J) which is inconsistent with previous haploid counts.

**Spore Observations**—Normal, regular-shaped spores were observed in the voucher specimens or living stocks of *M. maximowiczii*, *M. nipponicum*, and *M. henryi*, whereas irregular spores were observed in *M. arakii* and the putative hybrid of *M. maximowiczii* and *M. nipponicum* (Fig. 4). In *M. arakii*, only irregular-shaped spores were observed in all of the 71 specimens, with mature fronds deposited in the herbarium of the National Museum of Nature and Science, Japan (TNS), as well as in 21 specimens, including the holotype and isotype (Y. Araki 1372) in the herbarium of Kyoto University (KYO). Mean spore length of the voucher specimens were approximately 25.0 μm for *M. maximowiczii* ($N = 1$), 35.3–35.9 μm for *M. nipponicum* ($N = 3$), 32.4–33.2 μm for *M. henryi* ($N = 2$), and 38.9–40.0 μm for *M. subdigitatum* ($N = 3$; Appendix 3). We also observed a spore size of 36.4–38.6 μm for specimens of *M. subdigitatum* in KYO ($N = 3$), which was the largest spore size among the *Monachosorum* species.

**DNA Sequences**—In the 1,187 bp of the chloroplast gene *rbcL*, the same sequence (*rbcL* seq. 1’ in Appendix 2) was observed in *M. nipponicum*, *M. henryi*, *M. arakii*, and the putative hybrid of *M. maximowiczii* and *M. nipponicum*. This sequence matched sequences for *M. nipponicum* (GenBank accession No. AB574791 as *M. flagellare*) and *M. arakii* (GenBank accession No. AB574790) generated during a DNA barcoding study of Japanese ferns (Ebihara et al. 2010). Infraspecific variation was found in *M. subdigitatum*; i.e. two samples from Indonesia had the same sequence as ‘rbcL’ seq. 1’ above, whereas three samples from the Philippines had ‘rbcL’ seq. 2’ (see Appendix 2) which differs from ‘rbcL’ seq. 1’ by a 3 bp substitution. Thus, only three different *rbcL* sequences were found in *Monachosorum* species: *M. maximowiczii*, Filipino populations of *M. subdigitatum*, and all of the remaining samples.

For the nuclear gapCp marker, two to five sequences were identified in every individual, except for a single sequence in *M. maximowiczii* (Table 1). Not all of the sequences were definitively isolated from *M. subdigitatum*, probably due to its complicated genotype often consisting of more than five alleles. Infraspecific variation was not detected by SSCP analysis of *M. maximowiczii*, *M. nipponicum*, and their putative hybrid. On the other hand, banding patterns varied among *M. henryi*, *M. arakii*, and *M. subdigitatum*. In the phylogenetic tree (Fig. 5; Appendix 4), robustly supported clades were identified as B, C, C’, C”, and D. The sequences included in clade B were found in *M. arakii*, *M. nipponicum*, and the putative hybrid. Clade C sequences were found in *M. arakii*, *M. nipponicum*, the putative hybrid, a part of *M. henryi*, and a part of *M. subdigitatum*. Clade C’ sequences were found in a part of *M. arakii* and a part of *M. henryi*. Clade C” sequences were found in a part of *M. subdigitatum*. Clade D sequences were found in *M. subdigitatum*. Sequence A was found in *M. arakii*, *M. nipponicum*, *M. maximowiczii*, and the putative hybrid. The C’ sequences, instead of the C sequences, appeared in *M. henryi*. The clade consists of the C sequences were clearly sister to that of the C’ sequences. Thus, even though the monophyly of a superclade that consists of the sequences C, C’, and C” is not robustly supported, they are considered alleles at the same locus.

**Taxonomic Treatment**

**Keys to Species**

1. Bulbil present on adaxial side of rachis ................................................................. 2
2. Apex of ultimate segments acute to apiculate; spores irregular .................................................. 2
3. Bulbil absent on adaxial side of rachis ........................................................................ 3
4. Frond tripinnate, subtriangular; pinnules of lower pinna usually anadromously divided ............................................................. M. subdigitatum
5. Frond pinnate to tripinnate, lanceolate to subtriangular-lanceolate; pinnules of lower pinna catadromously divided ............................................................. 4
6. Frond pinnate, less than 5 cm wide; spores normal ............................................................. 5
7. Spores normal; frond usually more than 10 cm wide; hexaploid ............................................................. M. nipponicum
8. Spores irregular; tetraploid ............................................................................. M. *flagellare*


Monachosorum maximowiczii var. melanocaulon Hayata, Icon. Pl. Formosan. 6: 160. 1916.—TYPE: TAIWAN. Nokozan, Apr 1916, B. Hayata s.n. (holotype: TI!).

**Distribution**—Japan (Honshu, Shikoku, and Kyushu), China (Anhui, Guizhou, Hubei, Hunan, Jiangxi, Sichuan, Yunnan, and Zhejiang Prov.), and Taiwan.

**Ploidy and Reproductive Mode**—This species is a sexual diploid.

**Note**—This is the solely known diploid species in the genus.


**Distribution**—China (Chongqing, Guangdong, Guangxi, Guizhou, Hunan, Jiangxi, Sichuan, Xizang, and Yunnan Prov.), Taiwan, Philippines (Luzon), Nepal, India, Bhutan, Myanmar, Thailand, and Vietnam.

**Ploidy and Reproductive Mode**—This species is a sexual tetraploid.

**Note**—Characterized by large bulbils on their rachises.

**Monachosorum davallioides** Kunze, Bot. Zeit. 6: 119. 1848.—
_TYPE: INDONESIA. Java, Tankuwan-prau [Tankuban-prahu], Zöllinger s.n. (syntype: L?, n. v.); Junghuhn s.n. (syntype: L?, n. v.).


_Distribution_—Peninsular Malaysia, Philippines (Mindanao), Sumatra, Java, Borneo, Sulawesi, Moluccas, and New Guinea.

_Ploidy and Reproductive Mode_—This species is a octoploid or higher, presumably sexual reproduction.

>Note—This is the Malesian counterpart of _M. henryi_. The frond shape is similar to that of _M. henryi_, and is distinguishable by the absence of bulbils on the rachis. The origin,
ploidy level, and infraspecific variation were not fully clarified in this study.


*Monachosorum flagellare* auct non. (Maxim. ex Makino) Hayata.

**Distribution**—Japan (Honshu, Shikoku, and Kyushu) and China (Guangxi, Guizhou, Hubei, Hunan, Jiangxi, Sichuan, Yunnan, and Zhejiang Prov.).

**Ploidy and Reproductive Mode**—This species is a sexual hexaploid.

**Note**—We frequently found putative hybrids with *M. maximowiczii* in the specimens identified as *M. flagellare*. A total of 76 sheets of hybrids (vs. 435 sheets of *M. flagellare*) were deposited in TNS. Even in the syntypes of *M. flagellare*, more than half were putative hybrids, producing only irregular spores. Makino (1909) described the five syntypes from different locations in the Kochi Prefecture in Shikoku, Japan: (1) Tsubayama-mura (T. Makino, Aug 1885); (2) Mt. Yokogura-yama (T. Makino, 28 Aug 1887); (3) Mt. Kuishi-yama (T. Makino, 7 Oct 1892); (4) Nanokawa-mura (K. Watanabe, Nov 1889); and (5) Yasui-mura (S. Yano, 16 Aug 1890). The first, second, and third specimens were found in MAK, the second one was also found in TNS, and the fifth one was found in TI. We could not find specimens matching the fourth specimen in MAK, TI, or TNS. We examined the spores of five syntype sheets in MAK (one sheet each of the first and the third specimens, three sheets of the second specimen), and only the third syntype produced normal spores. The remaining syntypes produced irregular spores. The result was that the five syntypes consisted of two hybrids, a non-hybrid, and undetermined and missing specimens. The comparison of frond sizes and lamina dissection among the syntypes showed that a typical individual of *M. flagellare* in the protologue corresponded to the hybrid, whereas the non-hybrid, which has wider (approximately 28 cm broad) and more dissected (tripinnatifid) fronds, was recognized as only being at the extreme of the variation range. In summary, it is appropriate to apply the name *M. flagellare* to the hybrid taxon, and *M. nipponicum* is the correct name for the non-hybrid. The latter name was originally given to those that do not produce gemmae, but this characteristic was variable within this species and could not be used for identification.

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**Fig. 6.** An explanatory figure of relationships among *Monachosorum* species. The vertical axis represents the ploidy level. The bold arrow shows allopolyploidization.

Distribution—Japan.

Ploidy and Reproductive Mode—This species is a sterile tetraploid.

Note—A hybrid taxon between M. maximowiczii and M. nipponicum produced irregularly shaped spores. This is common in mixed populations of the two parental species in Japan, and the same is expected in China. The lectotype of M. kweichowense looks very similar to this hybrid, in terms of frond shape, but we will suspend judgment until the spores are observed.


Distribution—Japan (Honshu, Shikoku, and Kyushu), and possibly in China.

Ploidy and Reproductive Mode—This is a sterile pentaploid.

Note—A sterile hybrid taxon between M. nipponicum and M. henryi.

Discussion

Biological Status of the Taxa Examined—As a consequence of the results from the current and previous studies, M. maximowiczii, M. nipponicum, and M. henryi were considered diploid, hexaploid, and tetraploid sexual species, respectively. Assuming that the genomic formula of M. maximowiczii was “AA” and M. henryi was “BBCC,” M. nipponicum (genomic formula: AABBCC) can be described as an allopolyploid, with M. henryi as the maternal progenitor (Fig. 6). Due to the allopolyploid origin of M. nipponicum, the SSCP banding pattern of the putative hybrid between M. maximowiczii and M. nipponicum was not distinguishable from that of M. nipponicum. This hybrid combination was highly probable, due to its ploidy level (tetraploid) and irregular spores. Monachosorum subdigitatum is a close relative to (or sometimes considered conspecific with [e.g. Roskov et al. 2015]) M. henryi, and is only distinguishable by the lack of bulbils (Yan et al. 2013). Our measurement of spore size suggested that spores of M. subdigitatum were larger than that of any of its congeners, and its ploidy level was possibly octoploid. Several unique gapCp alleles were also detected in M. subdigitatum (the C” and D clades). Although our sampling did not cover the wide distribution range of M. subdigitatum, currently available information suggests that it is an independent species and distinct from M. henryi.

For M. arakii, we counted pentaploid chromosome numbers in two individuals collected from two locations distant from each other; one location was in the same area as the hexaploid that was reported by Hirabayashi (1968). However, after careful reexamination of those reported chromosome numbers, we found that a count of chromosome number led to the incorrect record of M. arakii as hexaploid instead of pentaploid, due to the difficulty of distinguishing between univalents and bivalents. All of the information on M. arakii, including irregular meiosis (Hirabayashi 1968), irregularly shaped spores, and pentaploidy (odd ploidy) suggests that it is a sterile hybrid taxon. When gapCp genotypes and the cytotypes were considered, the origin of M. arakii was best explained by hybridization between M. henryi (genomic formula: BBCC) and M. nipponicum (genomic formula AABBCC). This combination was already presumed by Tagawa (1935) based on the morphology in its original description.

Ferns are well known for their high rate of polyploids, which are mostly allopolyploids derived from hybridization and subsequent chromosome doubling (Lovis 1977, Walker 1984, Wood et al. 2009). Reticulate evolution, particularly in species complexes comprising morphologically ill-defined species, may become evident by genome analyses and/or biparentally inherited DNA markers (e.g. Wagner 1954, Reichstein 1981, Ebihara et al. 2005, Chang et al. 2013, Jaruwattanaphan et al. 2013). Given the unexpected result that reticulate evolution occurred in such a small group as Monachosorum, we recommend that researchers should always explore the presence of a common genome in morphologically distinct species pairs in fern polyploids.

Origin of M. arakii—Three M. arakii samples, collected from three locations distant from each other (i.e. different prefectures), had different gapCp genotypes. Because most of the alleles were also found in Taiwanese M. henryi, genetic variation in M. arakii was probably derived from alleles possessed by its parental species. Therefore, the hybrid taxon M. arakii originated independently at least three times. There remain uncertainties surrounding M. arakii, regarding the lack of current distribution of M. henryi in Japan, and the overlapping distribution of the two parental species only in the Yunnan-Guizhou Plateau of southern China.

Interspecific, sterile hybrids are commonly found in ferns (Knobloch et al. 1984, Barrington et al. 1989, Knobloch 1996), and more than 300 combinations have been found in Japan (Nakaike 2004). In general, sterile hybrid taxa grow sympatrically with their parental fertile species. Even if they are unaccompanied by one or both parental species, the parents are usually found in nearby habitats, or in an extreme case, elsewhere within the country.

The existence of sterile hybrids growing far from their parents can sometimes be explained by their gametophyte behavior. For example, Ebihara et al. (2009) discovered evidence in the filmy fern genus Vandenboschia of Japan filamentous gametophytes growing independently from their counterpart sporophytes, which contributed to the creation of hybrids. It is unlikely that a similar phenomenon occurs in the Monachosorum genus because gametophytes of this genus are cordate (Momose 1967), usually known as short-lived, and never become independent gametophytes.

Bulbils produced by M. arakii and M. henryi are outstanding in size among ferns. Although their large bulbils have an advantage in vegetative reproduction, their dispersibility has not been tested. In addition, it cannot be assumed that the bulbils could cross the sea from the continent to the Japanese archipelago and arrive in their current habitat (inland riverside...
Standards and Petitions Subcommittee 2010), which excludes observation by Momose (1967) of [Fig. 2], mostly in upstream areas), considering their size. M. arakii reproduction is quite limited, as suggested by the presence of dominant irregular spores in the specimens. Currently, we have no evidence of apogamous reproduction, either in Monachosorum or across the entire Dennstaedtiaceae.

The following scenario, assuming M. arakii is a relict hybrid, might best fit the results from our study: 1) M. henryi was previously distributed in Japan; 2) the hybrid M. arakii was produced recurrently from the parents; 3) M. henryi became extinct in Japan; and 4) M. arakii reproduced vegetatively by its bulbils and rhizomes. The evidence is too scarce to discuss the cause and time of M. henryi extinction. We estimated the time of extinction as occurring from thousands to millions of years ago, because vegetatively reproduced species may survive for more than a million years, as in the case of Appalachian independent gametophytes (Farrar 1990).

A hybrid that does not produce functional spores could behave as a typical species, with well-established alternative strategies of propagation such as bulbil production and rhizome division. To precisely describe the taxon entity, we transferred the name M. arakii to a hybrid status. If we strictly follow the guideline of the Red List by IUCN (IUCN Standards and Petitions Subcommittee 2010), which excludes non-apomictic hybrids from its scope, M. arakii may not be categorized as a globally threatened species. However, special care is needed to conserve such relict hybrids, which would never be formed in the future and could never colonize distant habitats.

M. arakii has only been found in Japan, but it has the potential to grow in southern China (Guangxi, Guizhou, Hunan, Jiangxi, Sichuan, and Yunnan Provinces), where the distributions of M. nipponicum and M. henryi overlap (Yan et al. 2013). The specimen of M. elegans Ching from Guangxi, China and a plant photographed as M. flagellare in the Chinese Flora (Zhang 2012) exhibit frond morphology quite similar to that of M. arakii. Further studies on Chinese materials might extend the distribution range of this international hybrid.

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Literature Cited


APPENDIX 1. Information of voucher specimens. Species—sample ID; locality; voucher specimen.

*M. arakii*—AE3454; Japan, Mie Pref., Owase-shi; TNS VS-1219649.


*M. maximoviczii*—Ichikawa 605231; Japan, Saitama Pref., Chichibu-shi; TNS VS-763926. AE3282; Japan, Wakayama Pref., Nachikatsuura-cho; TNS VS-1184930. AE3283; Japan, Wakayama Pref., Nachikatsuura-cho; TNS VS-1184931.


*M. henryi*—TW-2006-65; Taiwan, Kaohsiung Co., Sanpin; TNS VS-761631. TW-2006-137; Taiwan, Pingtung Co., Jinshui-ying; TNS VS-762079. TW-2008-1812; Taiwan, Kaohsiung Co., Taoeyuan Hsian; TNS VS-775697. GK10400; Taiwan, Ilan Co., Taipin-shan; TNS VS-9542673. Kuo2781; Taiwan, Ilan Co., Fushan; TNS VS-1209690. Lu25837; Taiwan, Chiayi Co., Alishan; TNS VS-1209689. Lu25914; Taiwan, Chiayi Co., Tefuye; TNS VS-1209688. AE3398; Taiwan, Chiayi Co., Tefuye; TNS VS-1219681. AE3414; Taiwan, Chiayi Co., Tshash; TNS VS-1219669. Chang 7003; China, Guangdong, Xingyi City; TAIF. Kuo3113; China, Yunnan, Malipo Co.; TAIF. Kuo3232; China, Yunnan, Pingbian Co.; TAIF.

*M. subdigitatum*—Wade1884; Indonesia, Java, Gede-Pangrango National Park; TAIF. 388729–388731. Wade2054; Indonesia, Java, Halimun National Park; TAIF. 388853–388855. Kuo2756; The Philippines, Mindanao, Mt. Apo; TAIF. Kuo3494; The Philippines, Mindanao, Mt. Kitanglad; TAIF. Kuo3635; The Philippines, Mindanao, Mt. Kitanglad; TAIF.

APPENDIX 2. GenBank accessions of obtained sequences.

*Cp rbcL*—seq. 1, LC137746; seq. 2, LC137747.

nr gapCp—A, LC137748; B1, LC137749; B2, LC137750; B3, LC137751; B4, LC137752; B5, LC137753; B6, LC137754; B7, LC137755; C1, LC137756; C2, LC137757; C3, LC137758; C4, LC137759; C5, LC137760; C6, LC137761; C7, LC137762; C8, LC137763; C9, LC137764; C1′, LC137765; C2′, LC137766; C1′, LC137767; C2′, LC137768; D1, LC137769; D2, LC137770; D3, LC137771; D4, LC137772.

APPENDIX 3. Spore sizes of herbarium specimens of *M. subdigitatum*. Averages of length of 30 spores and standard deviations are shown.

Species, collection No., locality, spore size; *M. subdigitatum*, Wilde & W-Duyfies 16365 (KOYO), Indonesia (N. Sumatra), 37.5 ± 2.4 μm; *M. subdigitatum*, Iwatsuki et al. p-829 (KOYO), the Philippines (Luzon), 38.6 ± 2.8 μm; *M. subdigitatum*, Kokawa & Hotta 4093 (KOYO), Malaysia (Sabah), 36.4 ± 1.6 μm.

APPENDIX 4. Substitution models selected for phylogenetic analyses.

Method, 1st codon position, 2nd codon position, 3rd codon position, Introns;

Bayesian, HKY, HKY, HKY+I, HKY+G; ML, TrN, K80, TPM2+I, TPM2+G.