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Resurrection of *Spicantopsis hancockii* (Blechnaceae) as an endemic species to Taiwan - Reidentification of *Spicantopsis* in the Tokara Islands, Japan

Atsushi EBIHARA[1,*], Shuichiro TAGANE[2], Shun K. HIROTA[3,4], Yoshihisa SUYAMA[4], Narumi NAKATO[5], Li-Yaung KUO[6]

1. *Department of Botany, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba-shi, Ibaraki 305-0005, Japan.* ***2.*** *The Kagoshima University Museum, Kagoshima University, 1-21-30 Korimoto, Kagoshima-shi, Kagoshima 890-0065, Japan.* ***3.*** *Botanical Gardens, Osaka Metropolitan University, 2000 Kisaichi, Katano-shi, Osaka 576-0004, Japan.* ***4***. *Field Science Center, Graduate School of Agricultural Science, Tohoku University, 232-3 Yomogida, Naruko-onsen, Osaki, Miyagi 989-6711, Japan* ***5.*** *1-363 Narahashi, Higashiyamato-shi, Tokyo 207-0031, Japan.* ***6***. *Institute of Molecular and Cellular Biology, National Tsing Hua University, Hsinchu City, Taiwan.* [*]*Corresponding author's email: ebihara@kahaku.go.jp; Tel: +81-29-853-8988*

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ABSTRACT: *Spicantopsis hancockii* (Blechnaceae) was once thought to be endemic to Taiwan, and later known to occur in the Tokara Islands in southern Japan. Because of its high morphological similarity to *S. niponica* endemic to Japan, the identification of the Tokara Archipelago populations has been controversial. MIG-seq analysis using 2324 SNPs revealed that *S. niponica* and the samples of *S. hancockii* from Taiwan formed two distinct clusters, while the population of *S. hancockii* in the Tokara Islands was included in the cluster of *S. niponica.* Micromorphological traits of sporangia observed in the Tokara populations coincide with those of *S. niponica* reported by the previous study. Therefore, it is appropriate to treat the Tokara populations of "*S. hancockii*" as the southernmost range of *S. niponica*, not as *S. hancockii*. As a result, *S. hancockii* is resurrected as an endemic species to Taiwan.

KEY WORDS: Blechnaceae, *Blechnum*, Ryukyu, *Spicantopsis*, *Spicantopsis niponica*, *Struthiopteris*, Tokara Islands.

INTRODUCTION

Spicantopsis Nakai, a small East Asian genus of the Blechnaceae comprising three species recently segregated from *Struthiopteris* Scop. has a confined distribution, occurring only in the Japanese Archipelago and Taiwan (Ebihara, 2016; Molino *et al.*, 2019). Prior to the detailed elucidation of phylogenetic relationships within the family, *Struthiopteris* and *Spicantopsis* were often included in the broadly circumscribed genus *Blechnum*. Two of the three constituent species of *Spicantopsis* (*S. niponica* (Kunze) Nakai [Fig. 1A] and *S. amabilis* (Makino) Nakai) are endemic to Japan, both widely distributed in the country except in subtropical regions (Ebihara, 2016). The third species, *S. hancockii* (Hance) Nakai, occurs in Taiwan (Fig. 1B) and is also believed to be distributed in the Tokara Islands of Japan (Ebihara, 2016; Molino *et al.*, 2019). *Spicantopsis hancockii* was described based on a specimen from northern Taiwan (Hance, 1883) and is known to occur in mountainous areas above ca. 1000 m elevation (Chiou *et al.*, 1994). The morphological difference between *S. hancockii* and *S. niponica* is very small. In fact, Nakai (1933) proposed a combination for *Blechnum* (= *Struthipoteris*) *hancockii* as a variety of *Blechnum* (= *Struthipoteris*) *niponicum* and DeVol (1975) stated that "the differences are so few and so small that *B. niponicum* should probably be reduced to a synonym". In recent years, however, a prevailing idea has been to treat each as an independent species (Iwatsuki *et al.*, 1995; TPG, 2019). Nakaike (1982) noted about their differences in multiple characters as follows: "[*S. hancockii* is] very similar to *S. niponica*, but the fertile fronds are slightly shorter than sterile ones. The margins of the pinnae are recurved into the abaxial side. The scales of the lateral segments are said to have long marginal hairs. The spores are said to be larger." - the last two characteristics are presumably based on observation by Ogata (1941). Ebihara (2016) observed lamina scales of the two species, but any obvious difference was not found (i.e., long marginal hairs were found not only in *S. hancockii* but also in *S. niponica*). Molino *et al.* (2020) observed sporangium traits in *Spicantopsis* and *Struthiopteris*, and found that sporangia having posterior basal cells are a unique trait in *S. niponica*. Their results of spore measurement showing that spores of *S. niponica* were slightly larger than those of *S. hancockii* did not support Ogata (1941)'s observation. Molino *et al.* (2019) conducted a phylogenetic analysis of *Struthiopteris* sensu lato using three plastid DNA regions (*rbcL*, *trnL-trnF* and *psbA-trnH*). The results supported a clade consisting of *S. niponica* (three samples) and *S. hancockii* (three samples) with high probability, while the subclades within the clade are without support.

The occurrence of the *Spicatopsis hancockii* in the Tokara Islands, located in the northern Ryukyu of Japan, was first reported by Hatsushima (1962). The Tokara Islands consist of seven inhabited islands and several uninhabited ones, most of which are with active volcanoes. The islands are located approximately 60 km

Fig. 1. **A**. ***Spicantopsis niponica*** in Hokkaido, Japan (photographed by A. Ebihara, September 14, 2020); **B**. ***Spicantopsis hancockii*** in Alishan, Taiwan (photographed by A. Ebihara, July 2, 2014) **C**. "***Spicantopsis hancockii***" in Suwanosejima Isl., the Tokara Islands, Kagoshima Pref., Japan (photograph by S. Tagane, May 4, 2019).

southwest of Yakushima Island, the southern distribution limit of *S. niponica*. He identified a specimen collected by himself in 1952 near the ninth station (580 m a.s.l.) of Mt. Maedake, Kuchinoshima Island, as *S. hancockii*, and introduced another specimen that was collected near the summit (ca. 900 m a.s.l.) of Nakanoshima Island in the Tokara Islands. In addition, a third specimen was collected in Suwanosejima Island (e.g., KAG 131526 in 1975). As a result, *S. hancockii* was recorded from a total of three islands in the Tokara Islands (Hatusima, 1986). Some researchers believe that Kuroshima and Kuchino-erabu Islands in the Osumi Islands, located between Kyushu and Yakushima Island, are also within the native range of *S. hancockii* (Sako, 1978; Shiuchi and Hotta, 2015; Suzuki *et al.*, 2022), but the majority's idea is that the population in the Osumi Islands is *S. niponica* (Fig. 2; Kurata and Nakaike, 1987; Ebihara, 2016).

On a global scale, there is no consensus on the identification of the Tokara Islands population of *S. hancockii.* Even after Hatsushima (1962) reported the species from the Tokara Islands, many publications in Taiwan did not accept his report and treated *S. hancockii* as endemic to Taiwan (e.g., DeVol, 1975; Chiou *et al.*, 1994; Kuo, 1997, 2001). Although recent publications on the flora of Japan (Nakaike, 1982; Iwatsuki *et al.*, 1995) accepted *S. hancockii* as a native species in Japan, the authors posed the necessity of careful consideration about identity of its Tokara population. This taxonomically controversial situation caused inconvenience in conservation - *Blechnum* [= *Spicantopsis*] *hancockii* is classified in the DD (Data Deficient) category in the national red list of Japan (Ministry of the Environment, Japan, 2020). Its recent growth condition in Kuchinoshima and Nakanoshima Islands has not been investigated (the latest collections were in 1988 and 1983, respectively), but a population was confirmed in Suwanosejima Island in 2019 (Fig. 1C). In order to judge the conservation priority of the Tokara Archipelago population of *S. hancockii*, it is necessary to obtain clear evidence for its identity.

It is well-known that a high proportion of polyploids of ferns is allopolyploid originating from interspecific hybridization (reviewed in Ebihara and Nitta, 2019 for Japanese ferns). In the case of species complexes containing allopolyploids, particularly of hexaploid or higher ploidy levels, single- or low-copy nuclear DNA markers are commonly used to elucidate the complicated reticulate process of species formation (e.g., Ebihara *et al.*, 2005; Schuettpelz *et al.*, 2008). In such allopolyploids, there is technical difficulty in identifying the same copy of genes from DNA sequence data including a large number of loci when they are obtained by assembling numerous short reads by high-throughput sequencing (see Rothfels *et al.*, 2017). On the other hand, *Spicantopsis* is known to be sexually reproducing diploid with $n = 31$ / $2n = 62$ chromosomes in all three species (Nakato, 1987; Ebihara *et al.*, 2014; Nakato *et al.*, 2020) - an exceptional report of tetraploid from Taiwan for *S. hancockii* (Tsai, 1973) needs careful verification. Given that previous phylogenetic analyses using a total of 2538-bp length plastid DNA (Molino *et al.*, 2019) detected only a small

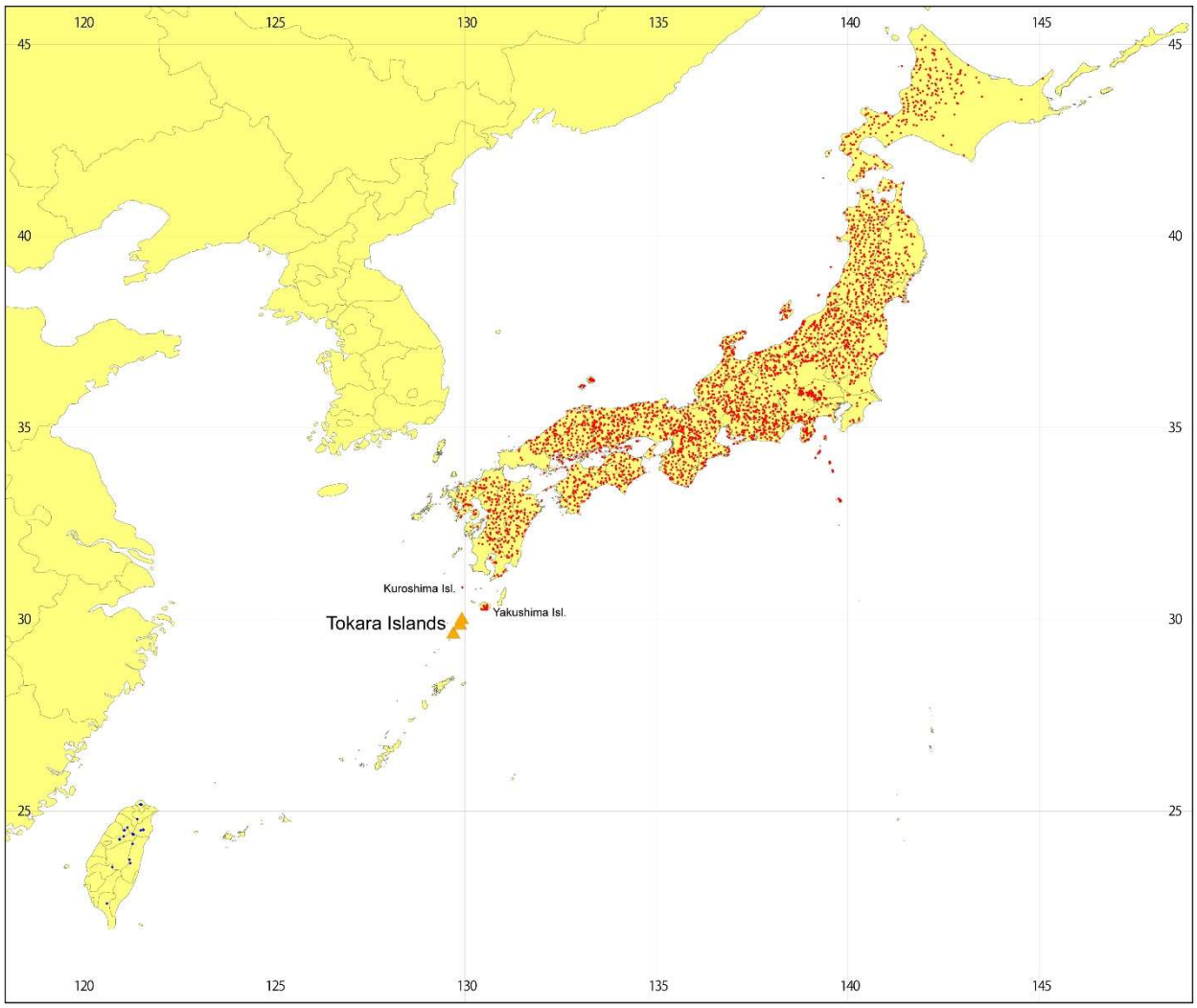

Fig. 2. A distribution map of ***Spicantopsis hancockii*** and ***S. niponica***. Orange triangle: "***S. hancockii***" in the Tokara Islands. Blue dot: ***S. hancockii*** of Taiwan based on the GBIF database (https://doi.org/10.15468/dl.4bzxua). Red dot: ***S. niponica*** of Japan based on 3588 herbarium specimens deposited at National Museum of Nature and Science (TNS).

amount of variation in these two species and that this genus is essentially composed of diploids, we decided to use the MIG-seq method, which provides information on more loci in a simple manner (Suyama and Matsuki, 2015).

MATERIALS AND METHODS

Materials for DNA analysis are one *Spicantopsis hancockii* collected by Tagane *et al.* in Suwanosejima Island, the Tokara Islands in 2019, four *S. hancockii* from Taiwan, seven *S. niponica* from Japan, and one *S. amabilis* from Japan for comparison (Table 1). One of the seven *S. niponica* samples (GC055) is the "var. *minima*" form from Yakushima Island, the same sample as that one used in the molecular analysis by Molino *et al.* (2019), but the taxon is often regarded as an extreme form of *S. niponica* and is not recognized as an independent taxon (e.g., Ebihara, 2016).

Total DNA was extracted using the Qiagen DNeasy Plant Mini Kit following the provided protocol. Library preparation for MIG-seq was performed a two-step PCR method according to Suyama *et al.* (2022). The first PCR was performed using the total DNA diluted to 10 ng/ μ L with MIG-seq primer set 1 (Suyama and Matsuki, 2015). Then, Illumina sequencing adapter and indices were added to the first PCR products by second PCR. The second PCR products were mixed with equal volume and size-selected (> 350 bp). Sequencing was performed using Illumina MiSeq system and MiSeq Regent kit v3 150 cycle (Illumina, San Diego, California, USA). All raw MIG-seq data were deposited at the DDBJ Sequence Read Archive (DRA) with accession number DRA015380.

Low-quality reads and extremely short reads containing adapter sequences were removed using Trimmomatic 0.39 (Bolger *et al.*, 2014). The Stacks 2.60

Table 1. Samples of ***Spicantopsis*** used in the present study.

Species	Sample ID	Locality	Voucher specimen (Herbarium & Reg. No.)
Spicantopsis hancockii			
	GC988	Taiwan. Ilan Co., Taipingshan	TNS VS-776516
	EY114	Taiwan. Taoyuan City, Mt. Peichatien (cultivated in Dr. Cecilia Koo Botanic Conservation Center)	TAIF 447323; TNS VS-1181066
	EY115	Taiwan. Taoyuan City, Mt. Peichatien (cultivated in Dr. Cecilia Koo Botanic Conservation Center)	TAIF 447324; TNS VS-1181101
	EY116	Taiwan. Jiayi Co., Tashan	TNS VS-1219667
	EY117	Japan. Kagoshima Pref., Suwanose-jima Isl.	TNS VS-1313834
Spicantopsis niponica			
	EY112	Japan. Hokkaido Pref., Yubari-shi	TNS VS-765676
	EY111	Japan, Akita Pref., Daisen-shi	TNS VS-765259
	EY110	Japan. Yamagata Pref., Yamagata-shi	TNS VS-765232
	EY113	Japan. Tokyo Pref., Niijima Isl.	TNS VS-1024486
	EY109	Japan. Kagoshima Pref., Ohkuchi-shi	TNS VS-762631
	GC055	Japan. Kagoshima Pref., Yakushima Isl.	TNS VS-763250
	GC884	Japan. Kagoshima Pref., Yakushima Isl.	TNS VS-773471
Spicantopsis amabilis			
	GC076	Japan. Kagoshima Pref., Yakushima Isl.	TNS VS-763339

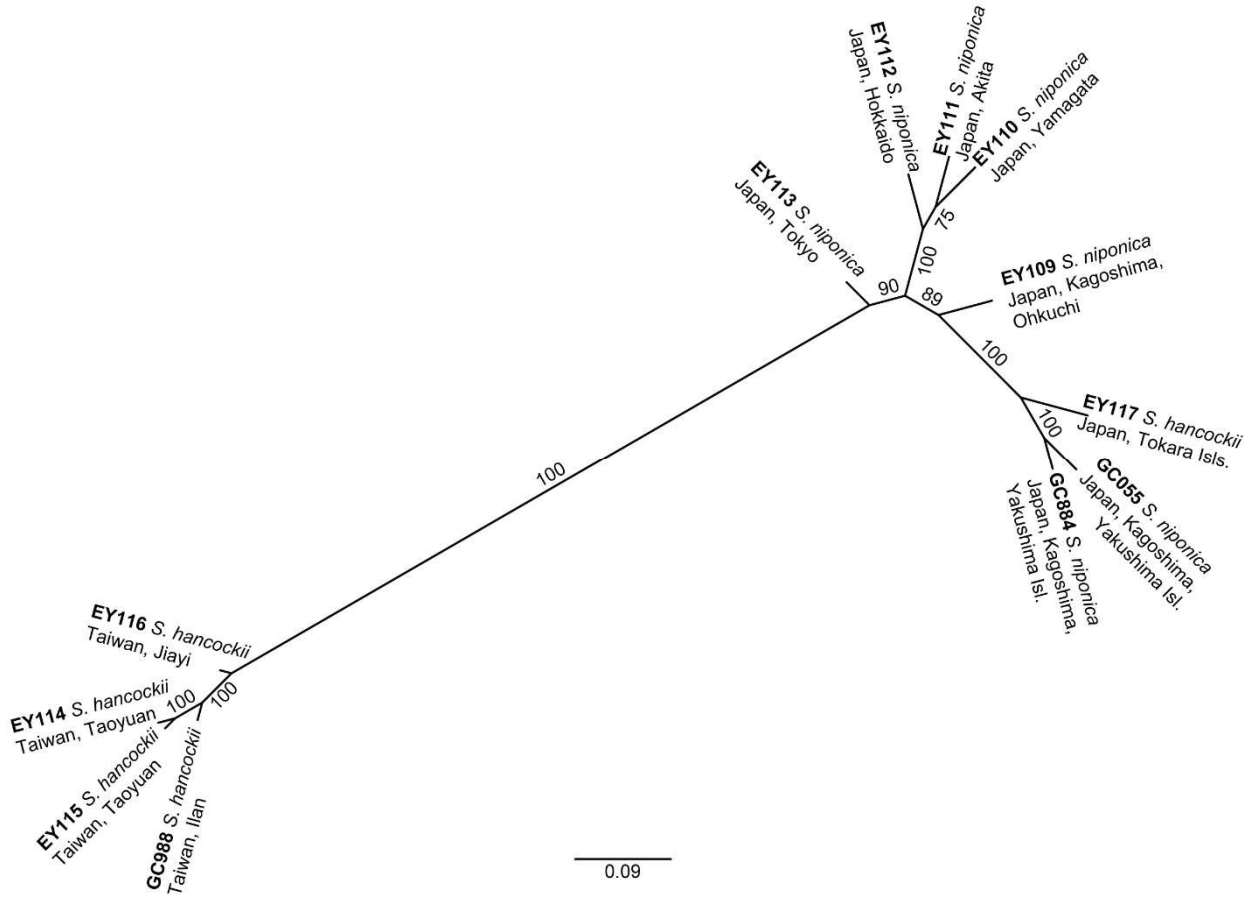

Fig. 3. An unrooted Maximum-Likelihood (ML) tree of ***Spicantopsis hancockii*** and ***S. niponica*** based on 2324 SNPs.

pipeline software (Catchen *et al.*, 2013; Rochette *et al.*, 2019) was used for de novo SNP discovery and genotyping with the following parameters: minimum depth of coverage required to create a stack (m) = 3, maximum distance between stacks (M) = 2, maximum mismatches between loci when building the catalog (n) = 2. Three different filtering criteria were considered for quality control of the SNP data. First, any SNP site where one of two alleles had less than three counts was filtered out. Second, SNPs with high heterozygosity (Ho ≥ 0.6) were

Table 2. A list of examined specimens of ***Spicantopsis hancockii*** collected from Tokara Islands, Japan.

Herbarium	Reg. No.	Collector & No.	Coll. Date [YYYY-MM-DD]	Locality	Comparative length of fertile and sterile fronds
TNS	VS-365778	*S. Sako 7267*	1968-12-18	Kuchinoshima Isl.	sterile > fertile
KAG	131524	*S. Sako 7267*	1968-12-18	Kuchinoshima Isl., alt. 550 m	NA (sterile only)
KAG	131525	*S. Maeda s.n.*	1988-10-04	Mt. Yokodake, Kuchinoshima Isl.	sterile > fertile
TNS	VS-832511a	*F. Miyamoto 443*	1976-08-22	Mt. Ontake, Nakanoshima Isl.	fertile > sterile
TNS	VS-832511b	*F. Miyamoto 443*	1976-08-22	Mt. Ontake, Nakanoshima Isl.	NA (sterile only)
KAP	0083683f	*M. Daikuzono s.n.*	1983-08-02	Nakanoshima Isl.	NA (sterile only)
KAP	0008353101f	*M. Daikuzono s.n.*	1983-08-02	Nakanoshima Isl.	NA (sterile only)
KAG	084262	*K. Maruno s.n.*	2001-09-24	Suwanosejima Isl.	fertile > sterile
KAG	131526	*Kirino s.n.*	1975------	Suwanosejima Isl.	NA (sterile only)
KAP	00100124f2	*H. Kirino s.n.*	2001-09-23	near Nabedao, Suwanosejima Isl.	fertile > sterile

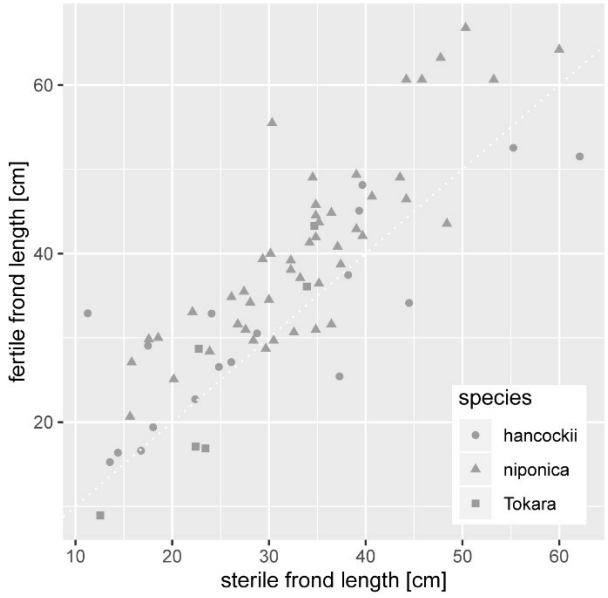

Fig. 4. A scatter plot of fertile and sterile frond length of ***Spicantopsis hancockii*** (circle), ***S. niponica*** (triangle) and the Tokara populations of "***S. hancockii***" (square).

removed. Third, the SNPs that were retained by 50% or more samples were included in the SNP dataset. Maximum likelihood phylogeny based on SNPs was inferred for all samples using RAxML 8.2.10 (Stamatakis, 2014). We used a GTRCAT model with ascertainment bias correction by Lewis method and performed 1,000 replicates of parallelized tree search bootstrapping.

For macromorphological observation, particularly of fertile and sterile frond lengths, *S. hancockii* herbarium specimens collected in Japan housed in the National Museum of Nature and Science (TNS), Kagoshima University Museum (KAG), and the Kagoshima Prefectural Museum (KAP) were examined. For morphological measurement of fertile and sterile frond lengths, 48 randomly selected specimens of *S. niponica* in TNS, 18 *S. hancockii* from Taiwan (two specimens in TNS, eight specimen images in the database of herbarium of Taiwan Forestry Research Institute (TAIF; https://taif.tfri.gov.tw/en/) and eight specimen images available via GBIF (https://www.gbif.org/) were used.

Micromorphology of sporangia was observed in two specimens of *S. hancockii* from the Tokara Islands (TNS VS-365778 and VS-832511a) using a light microscope.

RESULTS

A total of 2,495,832 raw reads (191,987±8,632 reads per sample) was obtained by MIG-seq and after quality control, 2,235,200 reads (171,939±7,150 reads per sample) were used for subsequent analysis. The sample of *S. amabilis* (GC076) was removed from the subsequent analysis due to its high rate of missing loci (98.36%), and it rendered the phylogenetic tree unrooted - however, monophyly of all the present samples of *S. hancockii* and *S. niponica* was confirmed using a plastid *rrn23-trnA* sequence dataset of 280 bp (Fig. S1) which was obtained from present MiSeq reads and the plastome sequences of *Blechnidium melanopus* (Hook.) T.Moore (GenBank accession: MT130662) and *Brainea insignis* (Hook.) J.Sm. (GenBank accession: MN623366). The results of the phylogenetic analysis using a matrix (1,342 loci, 2,324 SNPs) are shown in Fig. 3. The presence of two clusters corresponding to *S. hancockii* and *S. niponica* was robustly supported, however, the sample of *S. hancockii* from the Tokara Islands, was included in the *S. niponica* cluster. The Tokara sample of *S. hancockii* was resolved as sister to two *S. niponica* samples from Yakushima Isl.

The herbarium TNS housed three sheets of *S. hancockii* from the Tokara Islands, other than the voucher specimens used in the DNA analysis of the present study. The herbarium KAG and KAP housed four and three sheets of *S. hancockii* from the Tokara Islands, respectively (Table 2). In addition, three sheets from Osumi Islands in the herbarium KAP were also identified as *S. hancockii*. The result of morphological measurement of fronds of *S. hancockii* and *S. niponica* is shown in Fig. 4.

In our micromorphological observation of the two specimens of *S. hancockii* from the Tokara Islands, their sporangia have posterior basal cells (Fig. 5) and 18–20 arcus cells (terminology following Molino *et al.,* 2020).

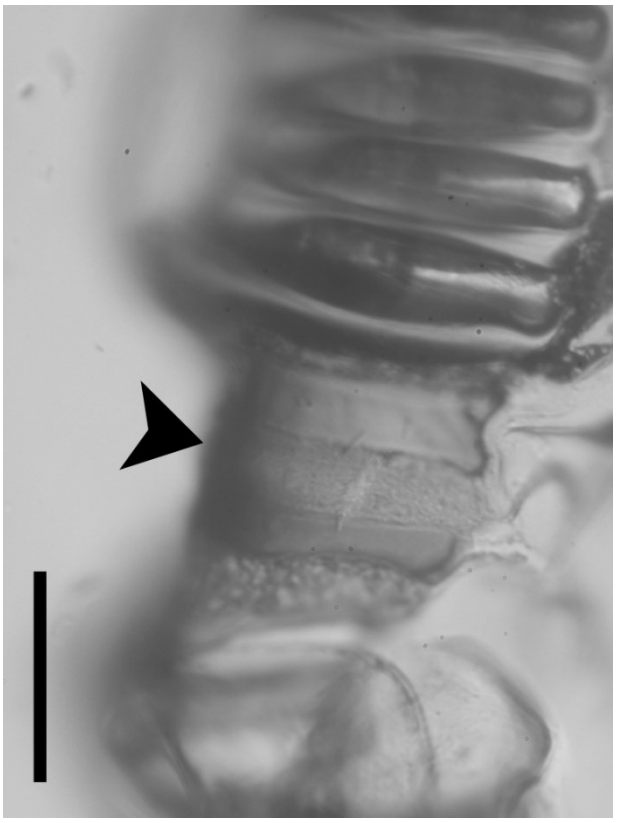

Fig. 5. Posterior basal cells (arrowhead) observed in a sporangium of "***Spicantopsis hancockii***" from the Tokara Islands (TNS VS-832511a). Scale bar = 50μm.

DISCUSSION

In our examination of specimens of *S. hancockii* from the Tokara Islands, almost a half of the specimens had fertile fronds longer than sterile ones and the remainings had sterile ones longer than fertile ones (Table 2). The voucher specimen of the Tokara sample used in our DNA analysis showed a characteristic of fertile fronds shorter than sterile ones, even though it is somewhat immature. In contrast, a specimen (KAG 084262), probably collected at the same site, has fertile fronds longer than sterile ones. This observation suggested that the comparative length of fertile fronds against sterile ones is difficult to be used as a key trait for discriminating *S. hancockii* from *S. niponica,* even though *S. hancockii* tends to have relatively shorter fertile fronds than *S. niponica* in our morphological measurement result (Fig. 4). The sporangium traits (posterior basal cells and arcus cells) observed on *S. hancockii* from the Tokara Islands perfectly match those of *S. niponica* described by Molino *et al.* (2020).

Our present genetic analysis suggested that the Tokara population of *S. hancockii* is phylogenetically included in *S. niponica*, not in *S. hancockii*. Currently, we do not have sufficient evidence to judge whether the observed genetic divergence between the two species corresponds to divergence between two independent species or to infraspecific variation in a single species. However, considering the presence of the two robust clusters and distinct micromorpholgical differences in sporangium, it is natural to distinguish the two entities at the variant or higher rank. This taxonomic treatment keeps both of the taxa within the scope of the Red List assessment (e.g., IUCN Standards and Petitions Committee, 2022). The objective of this study is to redefine the boundary of the two taxa but not to update the taxonomic treatment. Hence, we follow the classification of the two species at the species rank which has been most widely accepted in recent years.

In conclusion, it is reasonable to reidentify the populations that have been identified as *S. hancockii* in the Tokara Islands as the southernmost population of *S. niponica*. Accordingly, it is appropriate to remove *S. hancockii* from the national Red List of Japan. The elimination of *S. hancockii* from the Japanese flora has resurrected its status as an endemic species to Taiwan.

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