Taiwania 65(3): 382–390, **2020**
DOI: 10.6165/tai.2020.65.382

Molecular identification and Morphological traits of the native and exotic *Azolla* species in Taiwan

Mei-Chen CHANG[1], Cheng-Tai HUANG[1], Ching-Chu TSAI[1], Wen-Yuan KAO[1,2,*]

1. Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei, Taiwan.
2. Department of Life Science, National Taiwan University, Taipei, Taiwan.
[*]Corresponding author's Telephone: +886-2-3366-2511; Fax: +886-2-2368-6750; e-mail: wykao@ntu.edu.tw

(Manuscript received 6 February 2020; Accepted 15 July 2020; Online published 22 July 2020)

ABSTRACT: *Azolla* is a perennial aquatic fern and often forms symbiosis with nitrogen-fixing cyanobacteria. Flora of Taiwan records only one native *Azolla* species, *A. pinnata* R. Br., in the Island. Recently, an exotic *Azolla* species has been documented with naturalized populations in Taiwan and been reported as *A. japonica* or *A. caroliniana*. However, data supporting its identity have not been presented. The purposes of this study are: (1) to test the hypothesis that the exotic *Azolla* species is *A. japonica* or *A. caroliniana*, and (2) to illustrate the morphological differences between the exotic and the native *Azolla* species.

The DNA sequences of four plastid regions, *rbcL*, *atpB*, *rps4* and *rps4-trnS*, were generated from plants of the *Azolla* species growing in different regions of Taiwan. Our phylogenetic analyses reveal that the native species belongs to *A. pinnata,* while plants of the exotic species are phylogenetically nested within a clad, including *A. microphylla. A. mexicana and A. craoliniana*. The host-symbiont specificity between *Azolla* spp. and their cyanobionts was confirmed by the phylogeny of 16S rRNA genes, which also supports the grouping of the exotic species to the aforementioned clad. Thus, the phylogenetic results suggest that the exotic *Azolla* is more closely related to *A. caroliniana* than to *A. japonica*. A table summarizing the morphological characters of their sporophytes illustrates the differences in the gross form, epidermal structures and surface of rhizome between the native and exotic *Azolla* species which can be used to differentiate the two species in Taiwan.

KEY WORDS: Aquatic fern, *Azolla*, *A. caroliniana, A. japonica*, *A. pinnata*, molecular identification, symbiotic cyanobiont.

INTRODUCTION

Azolla, a floating fern of order Salviniales, family Azollaceae, is distributed in warm-temperate, subtropical and tropical regions of Africa, Asia, and America (Lumpkin and Plucknett, 1980). The plant consists of a short and branched rhizome floating on the water, two rows of leaves growing on the rhizome, and roots elongating into the water. Each leaf is dissected into two lobes, an aerial chlorophyllous dorsal lobe and a ventral lobe contacting with water surface (Svenson, 1944; Lumpkin and Plucknett, 1980; Peters and Meeks, 1989; Wagner, 1997). In natural habitats, *Azolla* is vertically transferred and forms symbiosis with cyanobacterium *Anabaena azolla*, which lives in the cavities of the dorsal lobes and is capable of fixing atmospheric nitrogen (see a review of Peters and Meeks, 1989; Carrapiço, 2010). Because of harvesting nitrogen source by cyanosynbiont, *Azolla* is traditionally served as an important biofertilizer and often co-farmed with rice in tropics and subtropics (Lumpkin and Plucknett, 1980). In addition, the symbiotic relationship might also confer *Azolla* competitive advantages in nitrogen-poor habitats and thus contribute to its widely distribution (Carrapiço, 2010).

Seven extant species of *Azolla* have been recognized (Svenson, 1944; Saunders and Fowler, 1992, 1993; Reid *et al.*, 2006; Pereira *et al.*, 2011). Some of these species have been introduced and designated as invasive plants in some regions or countries (Ahad *et al.*, 2012; Murillo *et al.*, 2007; Gratwicke and Marshall, 2001; Szczesniak *et al.*, 2009; Shaw *et al.*, 2004; Hashemloian and Azimi, 2009; Masoodi and Khan, 2012). For example, the Invasive Species Compendium lists *A. filiculoides, A. Mexicana* and *A. pinnata* as invasive species. In addition to the native *Azolla - A. pinnata* R. Br. (Devol, 1994), one exotic *Azolla* species was also documented in Taiwan, and reported as *A. japonica* previously (Hwang, 2004) and as *A. caroliniana* (Chen, 2008; Taiwan Pteridophyte Group, 2019). However, data supporting its identity have not been presented. Features on reproductive organs, such as the glochidia surrounding the massulae and the perine of megaspore, have been used as the important diagnostics for *Azolla* species identification (Perkins *et al.*, 1985; Saunders and Fowler, 1993). Unfortunately, reproductive organs of the exotic *Azolla* in Taiwan have not been found in field or under cultivation. So far, species identification of this Taiwanese exotic *Azolla* species is still difficult solely based on morphology. More recently, DNA phylogeny in combination with morphological traits have been successfully used to reveal the species taxonomy of *Azolla* (Reid *et al.*, 2006; Metzgar *et al.*, 2007; Madeira *et al.*, 2013; Li *et al.*, 2018). Results of these reports suggest that the molecular analyses in combination with morphological characters may be helpful in identifying the exotic *Azolla* species in Taiwan. In addition, because *Azolla* species have specific symbiont cyanobacteria, identify of cyanobionts may provide additional evidence supporting the identity of their host *Azolla*. Taking the

advantage of the relationship, we thus also extracted and analyzed molecular components of the cyanobionts associated with the targeted *Azolla* species hoping that the result could be reinforced.

Accordingly, the purposes of the study were (1) to test the hypothesis that the exotic *Azolla* species is *A. japonica* (reported by Huang, 2004) or *A. caroliniana* (reported by Chen, 2008 and by Taiwan Pteridophyte Group, 2019) and (2) to compare the morphological differences between the two *Azolla* species in Taiwan. To achieve the purposes, we observed the morphological characters and generated the DNA sequences of four plastid regions from plants of the *Azolla* species growing in different regions of Taiwan.

MATERIALS AND METHODS

The native and exotic *Azolla* species were collected from a greenhouse of National Taiwan University (NTU), Taipei, and the rice field of Academic Sinica, respectively. These materials were cultivated in an artificial pond of NTU.

Leaves of the native and the exotic species were also collected from field growing plants in habitats located in different regions of Taiwan for the following genetic analyses. To avoid being asexual clones, field growing plants were selected from habitats located at geographically distant regions.

Sequencing the four plastid regions of the two Azolla spp.

Leaf samples of the exotic species were collected from the culture (the aforementioned pond in NTU) and from three different habitat regions, Ilan (24°45'07.6"N 121°36'45.7"E), Guantian (23°11'05.2"N 120°18'47.4"E), and Beitou (25°09'36.0"N 121°31'54.9"E), and those of the native species were from the culture and from 2 habitats regions, Fulong (25°00'23.7"N 121°57'18.3"E) and Shoufeng (23°53'52.8"N 121°30'35.6"E), in Taiwan. Two replicates were taken in each collecting sites. Totally, eight samples and 6 samples of the exotic and the native species, respectively, were sampled for the following analyses. Voucher specimens of the native and exotic *Azolla* species were deposited in the herbarium of National Taiwan University.

Total genomic DNA of leaves (ca. 2 cm^2) of native and exotic species was extracted with Tissue Genomic DNA extraction Mini kit (Favorgen Biotech Corporation, Pingtung, Taiwan). PCR amplifications of the four chloroplast regions, *rps4-trnS* intergenetic spacer (IGS), *rbcL*, *atpB*, and *rps4* genes were performed. The primers and PCR conditions are listed in Supplementary Table S1. PCR products were first checked on 1.5% agarose gel and purified with DNA Clean & Concentrator Kit (Zymo Research, California, USA). All sequencing reactions were performed by using the ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Sequencing 16S rRNA gene of the symbiotic cyanobacteria

Fresh leaves (ca. 2 cm^2) of cultivated plants in NTU and of the field (in Tamsui region) growing plants of the native species, and those of individuals (sampled from 7 locations of a terraced field in Beitou region) of the exotic species (see Supplementary Table S3) were surface-sterilized by immersion in 0.5% sodium dodecylsulphate for 1 min, then 70% ethanol for 5 min, and washed three times by sterile deionized-distilled water (DDW). To release the symbiotic cyanobacteria from leaf cavity, the surface-sterilized leaves were crushed in DDW with a micropestle.

The cyanobacteria suspension was then used for DNA extraction. The partial 16S rRNA gene was amplified by using the cyanobacteria specific primer (Nübel *et al*., 1997) and the universal primer (Marchesi *et al*., 1998) (see Supplementary Table S1). Purification and sequencing of PCR products were conducted as aforementioned methods. GenBank accession numbers of the sequences obtained in this study are provided in Supplementary Table S3.

Phylogenetic analysis of Azolla species

The *rbcL*, *atpB*, *rps4* and *rps4-trnS* IGS sequences of 8 *Azolla* species (*A. caroliniana, A. filiculoides, A. japonica, A. mexicana*, *A. microphylla*, *A. nilotica*, *A. pinnata* and *A. rubra*) published by Metzgar *et al*. (2007) and Ebihara *et al.*, (2010), and those of *Salvinia cucullata* by Li *et al.*, (2018) were download from the GenBank database (https://www.ncbi.nlm.nih.gov/). (Supplementary Table S2)

The sequences of the four plastid regions obtained from this study and from GenBank were aligned by using the CLUSTALW program (Thompson *et al*, 1994). In order to obtain more robust phylogeny for *Azolla* species, the sequences of four plastid regions were concatenated to reconstruct a phylogenetic tree. The congruence of the four DNA regions were examined by partition analyses before the concatenation.

A Maximum-likelihood (ML) tree based on the four-region concatenated dataset was reconstructed under the model Tamura 3-parameter model plus Gamma rate distribution (T92 + G) using the software MEGA6 (Tamura *et al*., 2013). To test the strength of the phylogeny, the bootstrap method based on 1000 replicates was performed. In addition, the concatenated gene tree was also assessed by Bayesian Inference (BI) with MrBayes version 3.2.2 (Ronquist *et al*., 2012) using the nucleotide substitution model GTR + Gamma + I. For analysis, 1,000,000 Markov Chain Monte Carlo (MCMC) generations and trees were sampled every 250 generations. Posterior probabilities were calculated by sampling 25% post-burnin trees.

Phylogenetic analysis of symbiotic cyanobacteria

To analyze the phylogenetic relationships between

the cyanobionts associated with plants of the *Azolla* species in Taiwan and those with identified *Azolla* species, the 16S rRNA sequences of reference cyanobionts which are highly similar with the cyanobionts in this study were download from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) based upon BLAST results. The Kimura's 2-parameter distance correction model plus Gamma rate distribution (T92 + G) was used to reconstruct an ML trees of these cyanobionts by software MEGA6. The topology of the tree was evaluated by bootstrapping with 1,000 replications. In addition, a BI tree was also reconstructed using the nucleotide substitution model General Time Reversible plus Gamma rate distribution and invariant site (GTR + G + I). For analysis, 1,000,000 Markov Chain Monte Carlo (MCMC) generations and trees were sampled every 250 generations. Posterior probabilities were calculated by sampling 25% post-burnin trees.

Morphological traits

For the observation of the leaf surfaces, fresh plant samples were collected (0.3 – 0.5 cm^2), mounted directly on a metal stub with double-sided adhesive tape and observed with a SEM with cryo-holder facilitates (TM-3000, HitAmhi High-Technologies Corp., Japan). Pictures were taken for the counting of epidermal, stomatal and trichome number under the magnification of 400 x. Stomatal and trichome indexes were calculated as stomatal number/ number of total epidermal cells and trichome no. / no. of total epidermal cells, respectively. The size of the stomatal pore was also estimated by Image J (1.48p, National Institutes of Health, USA).

For the observation of the rhizome and the epidermis of leaf ventral lobe, fresh plant samples of both species were first dissected under a dissecting microscope (S8AP0, Leica). Rhizomes, with some leaves removed, were observed and photo'd under the dissecting microscope while detached leaf fragments were transferred to a slide and observed under a light microscope (CX41, Olympus) coupled with a digital camera for image acquisition.

Statistical analysis

The statistical analysis was performed by Sigmaplot 12.5 (Systat Software, Inc., USA). Data of morphological traits of the two *Azolla* species were checked for the normality and homogeneity of variance first and then compared by unpaired T-test. The α level is defined as $p < 0.05$.

RESULTS

The sequences of four plastid regions, *atpB* (1200 bp), *rbcL* (1300bp), *rps4* (600 bp) and *rps4-trnS* (400 bp) were obtained from the *Azolla* samples. Totally 3000 characters were in the four-region dataset, and were used to infer relationships among the *Azolla* species in Taiwan and other known *Azolla* species. Results from the partition analyses revealed that the congruence of the four DNA regions were not significantly different (p value > 0.05). Therefore, the sequences of the four plastid regions were concatenated to reconstruct a phylogenetic tree.

The ML tree revealed that plants of exotic and native *Azolla* sampled from the culture ponds and from habitats in different regions of Taiwan were found nested into two major clades (Fig. 1), referring to sections *Azolla* and *Rhizosperma*, respectively (Reid *et al*., 2006 ; Metzgar *et al*., 2007).

All exotic *Azolla* individuals from the four sites share the same sequences and are grouped with *A. caroliniana, A. mexicana* and *A. microphylla* in one of the major clades with strong supports (ML bootstrap value = 100%). Besides, *A. rubra*, *A. japonica* and *A. filiculoides* were grouped into the other lineage of this clade.

The sequences generated from the exotic *Azolla* were almost identical with those of an *A. mexicana* sample from Columbia (99.9% similarity). The individuals of the exotic *Azolla* and this *A. mexicana* form a clad (ML bootstrap value = 86%), which is nested in a clade mixed with *A. mexicana* and *A. microphylla* (ML bootstrap value = 89%). *A. caroliniana* clade is the sister clade to the clade contating *A. mexicana*, *A. microphylla* and the exotic *Azolla*.

In another major clad, the individuals of the native *A. pinnata* sampled from three habitat regions share identical sequences with *A. pinnata* from China. All *A. pinnata* individuals form a monophyletic group.

16S rRNA gene phylogeny of cyanobionts

Cyanobionts of the exotic *Azolla* sampled from 7 locations of a terraced field in Beitou region had identical 16S rRNA gene sequences (data not presented), thus they were presented altogether as E3 in the Fig. 2. Identical 16S rRNA gene sequences were also found in the two samples of the native species from two different regions. The results imply that the same *Azolla* species might harbor the same cyanobacterial strain.

The 16S rRNA gene phylogeny shows that the cyanobionts isolated form section *Azolla* formed a distinct group from the *Rhizosperma* section (Fig. 2). The cyanobionts of the exotic species are grouped together with cyanobionts of *A. caroliniana*, whereas those of native species are closely related to cyanobionts of *A. pinnata* (Fig. 2).

Morphological traits

The two species differ in their gross forms, the native *Azolla pinnata* appears triangular and the exotic species polygonal (Fig. 3A, D). During winter, particularly on days of low air temperature and high light intensity, the exotic species turns red while *A. pinnata* in the same habitat remains relatively green (Fig. 3A, D).

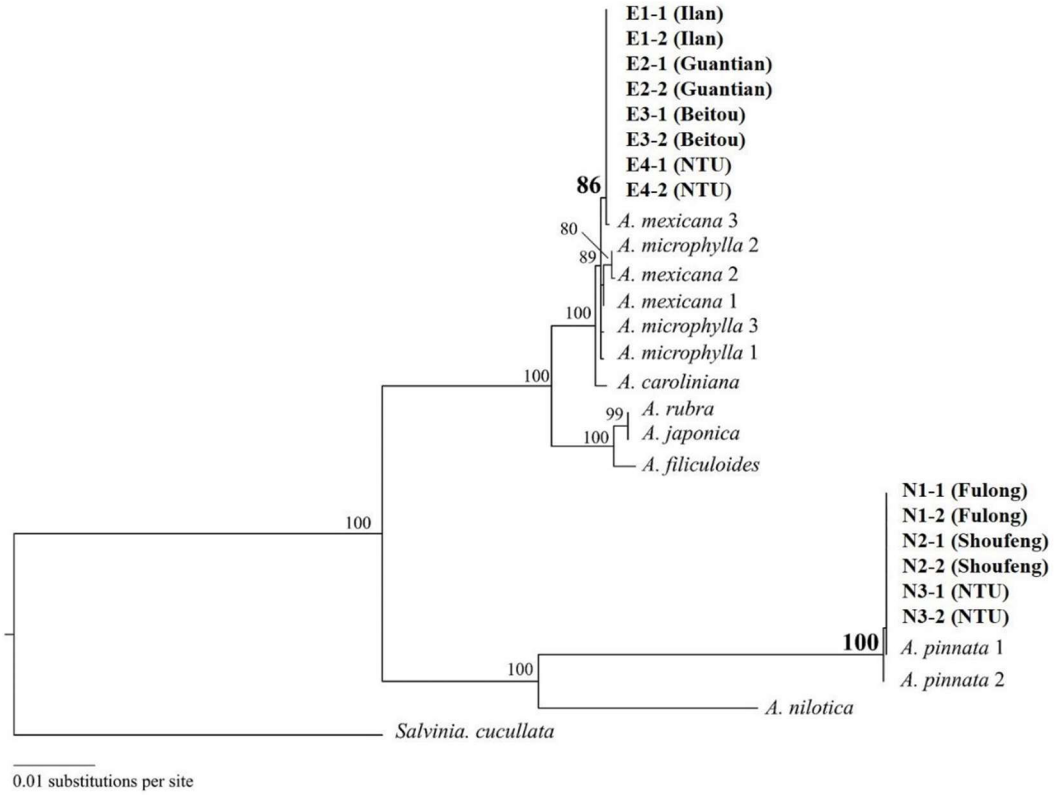

Fig. 1. Maximum likelihood phylogeny of *Azolla* species based on four plastid loci (*rbcL+atpB+rps4+rps4-trnS*). Bold letters N and E represent the native and exotic *Azolla* species, respectively, sampled from the culture ponds in National Taiwan University (NTU) and from different regions (parenthesis) of Taiwan. Two replicates for each species were sampled from each sits. The information of tip labels is in Table S2. The numbers above nodes are ML bootstrap values.

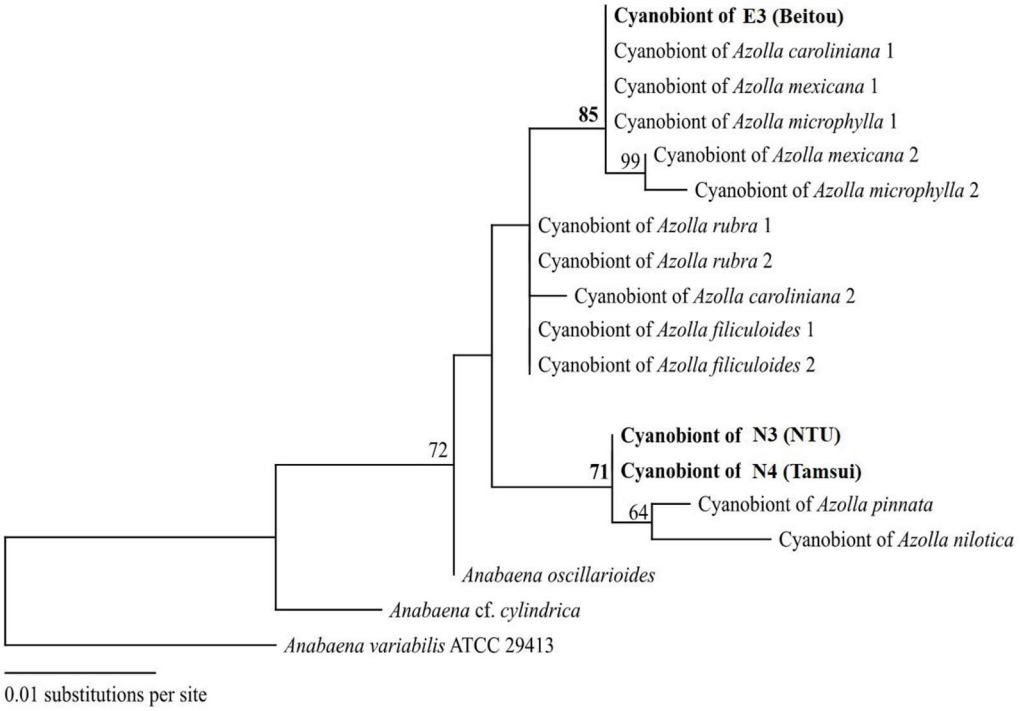

Fig. 2. Maximum likelihood (ML) phylogeny of cyanobionts of *Azolla* species based on 16S rRNA genes. Bold letters N and E represent the native and exotic *Azolla* species, respectively, sampled from the culture pond in National Taiwan University (NTU) or from different regions (parenthesis) of Taiwan. Their voucher information and GenBank accession number are provided in Table S3. The numbers above nodes are ML bootstrap values.

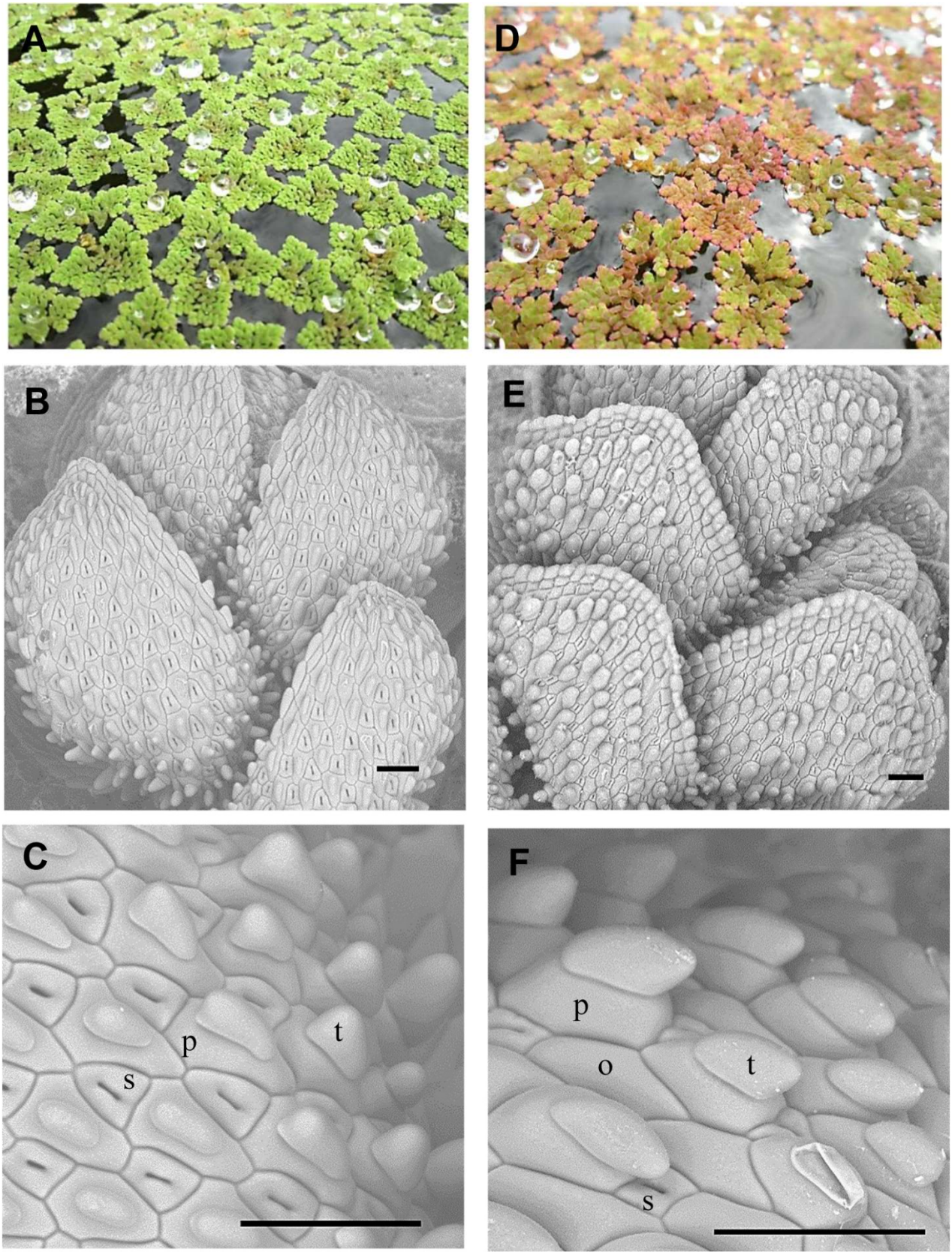

Fig. 3. The plants (**A**, **D**) and the SEM pictures (**B**, **C**, **E**, **F**) of abaxial surface of leaf dorsal lobe of the native (**A**,**B**,**C**) and exotic *Azolla* (**D**,**E**,**F**) in Taiwan. o : ordinary cell, p : pedicel cell , s: stoma, t: trichome cell. All scale bars = 100 μm.

Table 1. Morphological traits, including stomatal density (S.D.), stomatal index (S.I.), stomatal pore size, trichome density (T.D.) and trichome index (T.I.), on dorsal lobe of leaves of the native, *A. pinnata*, and the exotic *Azolla* spp. cultivated in an experimental farm of the National Taiwan University. (Mean ± S.E., n = 3)

Traits	S. D. (no. mm^{-2})	S. I. (%)	Pore size (mm)	T. D. (no. mm^{-2})	T. I. (%)
A. pinnata	194.2 ± 14.5	39.9 ± 1.7	17.7 ± 0.8^{A*}	252.8 ± 4.0^{A}	52.4 ± 1.3^{A}
Exotic *A.*	213.9 ± 13.4	35.4 ± 0.1	13.5 ± 0.7^{B}	177.7 ± 11.2^{B}	26.2 ± 0.7^{B}

*Means within columns followed by different superscripts are different at P = 0.05.

The SEM photographs reveal similarity and differences in the epidermis of leaves between the two species (Fig. 3). The apex of leaf dorsal lobe looks different between the two species, sub-round in the native species while round in the exotic species (Fig. 3B, 3E). Stomata and trichomes were observed on adaxial surfaces of leaf dorsal lobes of both species. In both species, each stoma is surrounded by a single annular guard cell forming a central pore (Fig. 3C, F) and trichomes are composed of two cells, with a pedicel cell at base and an oblong trichome cell at top (Fig 3C, F). In *A. pinnata*, the epidermis of the dorsal lobe of its leaves is composed of vertical rows of stomata and trichomes (Fig. 3C). In addition to stomata and trichome cells, ordinary epidermal cells were also observed on the epidermis of the dorsal lobe of the leaves in exotic species (Fig. 3F).

The surfaces of rhizomes and leaf ventral lobes also differ between the two species (Fig. 4). The native *Azolla* species has pubescent rhizomes (Fig. 4A) while the exotic species glabrous rhizomes (Fig. 4D). The leaf ventral lobe of *A. pinnata* is thin, appears achlorophyllous and has no stomata on both surfaces (Fig. 4B, C). In contrast, few chloroplasts and stomata were observed on the adaxial surface of leaf ventral lobe of the exotic species (Fig. 4E). Stomata, but not chloroplasts, were also observed on the abaxial surface of leaf ventral lobe of the exotic species (Fig. 4F)

Similar stomatal density and stomatal index were measured of the leaf dorsal lobes in both species, but significantly larger stomatal pore was found in *A. pinnata* than in the exotic *Azolla* species (Table 1). Significantly higher trichome density and trichome index were measured in *A. pinnata* than in the exotic species.

A comparison of the morphological characters between the native and the exotic *Azolla* species in Taiwan is presented in Table 2.

Table 2. Characteristics of the morphological traits of the native and the exotic *Azolla* species in Taiwan.

Native *Azolla* species	Exotic *Azolla* species
Deltoid sporophyte	Polygonal sporophyte
Sub-round dorsal lobe apex	Round dorsal lobe apex
Pubescent rhizome	Glabrous rhizome
Bicellular leaf dorsal lobe trichomes	Bicellular leaf dorsal lobe trichomes
Annular stomata on leaf dorsal lobe	Annular stomata on leaf dorsal lobe
Absence of stomata on leaf ventral lobe	Presence of stomata on leaf ventral lobe

DISCUSSION

Phylogenies of the *Azolla* plants and of their symbiotic cyanobionts confirm that the native species in Taiwan is *A. pinnata*, also that the exotic *Azolla* species in Taiwan is another, introduced species. Because all individuals of this exotic *Azolla* sampled from different regions of Taiwan were found genetically identical in their four plastid regions, we conclude that the exotic populations belong to the same species.

The exotic *Azolla* species in Taiwan has been documented as *A. japonica* previously (Hwang, 2004; Chen, 2008; Wu *et al*., 2010). The result that the exotic species and *A japonica* were grouped into different lineage (Fig. 1) indicates that the exotic species is phylogenetically differentiated from *A. japonica*. Thus, the molecular data does not support the classification of the exotic species as *A. japonica*. Instead, the exotic species is phylogenetically nested within the lineage mixed with *A. mexicana* and *A. microphylla*, and *A. caroliniana* is sister to this lineage. Though the sequences generated from the exotic *Azolla* were almost identical with those of an *A. mexicana* sample from Columbia. However, similar to previous studies (Reid *et al*., 2006; Metzgar *et al*., 2007), the current plastid trees are not able to resolve each of *A. maxicana* and *A. microphylla* as a monophyly.

The symbiotic cyanobacteria are not only present in the vegetative parts (dorsal lobe leaves) but also in the sexual parts (megasporocarps) of *Azolla* (Carrapiço, 2010). It is known that symbiotic cyanobacteria are vertically transmitted during sexual reproduction to subsequent generations. The co-speciation phylogenies have been demonstrated by Li *et al*. (2018). As a result, the host phylogeny is similar to the associated cyanobiont phylogeny. Results of this study also showed that the cyanobionts isolated from exotic and native *Azolla* species belong to distinct groups and the *Azolla* phylogeny fits to the cyanobiont phylogeny (Fig. 1 and Fig. 2).

The exotic species has been reported as *A. caroliniana* (Chen, 2008; Taiwan Pteridophyte group, 2019). However, phylogenetic results of this study suggest that the exotic *Azolla* might be more closely related to *A. mexicana*/*microphylla* than to *A. caroliniana* (Fig. 2). According to the description by Goff (2011), *A. caroliniana* has isodiametic trichomes randomly arranged on leaf surface while *A. mexicana* has regular arrangement of oblong trichomes on its leaf

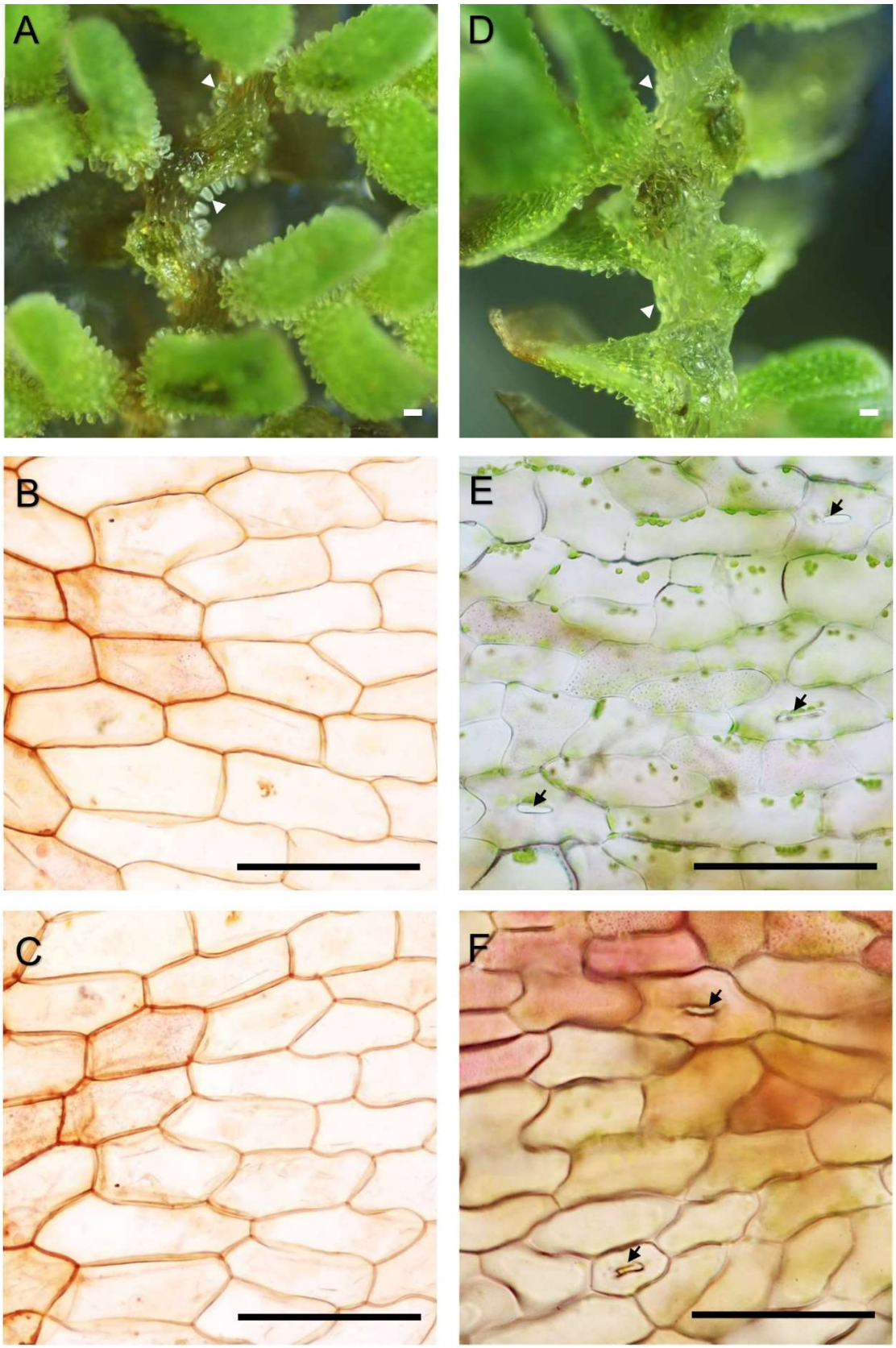

Fig. 4. The stereoscopic pictures of rhizomes (**A**, **D**), and LM pictures of adaxial (**B**, **E**) and abaxial (**C**, **F**) surfaces of leaf ventral lobes of the native (**A**, **B**, **C**) and exotic (**D**, **E**, **F**) *Azolla* in Taiwan. Arrowheads indicating the rhizomes and arrows stomata. All scale bars = 100 μm.

surface. Morphologically, the regular arrangement and the oblong shape of the trichomes observed on the leaf surface of the exotic *Azolla* (Fig. 3) are more similar to those of A. *mexicana* than those of *A. caroliniana*. Pereira *et al*. (2011) used morphological characters and RAPD markers successfully partition the *Azolla* species. According to one vegetative criterion of Pereira *et al*. (2011), stomata are present in the ventral lobe of leaves of *A. caroliniana* but not in those of *A. mexicana*. Thus, they used this character to differentiate *A. carolinaina* from *A. mexicana*. In our observation, we found stomata on the leaf ventral lobe of the exotic species (Fig. 4). In this regard, the morphological trait support the identity of the exotic species as *A. caroliniana*. Accordingly, there are conflicts in identify the exotic species as *A. caroliniana* or *A.microphylla*/*A. mexicana* using the morphological traits of the vegetative sporophytes.

In conclusion, the identification of the native species of Taiwan as *A. pinnata* is confirmed phylogenetically. The exotic individuals of *Azolla* sampled in Taiwan all belong to the same species, which is more closely related to *A. caroliniana* than to *A. japonica*. Morphologically, the native and exotic *Azolla* species differ in their gross form, leaf epidermal structures and rhizome surface, which can be used to differentiate both species.

ACKNOWLEDGMENTS

The manuscript greatly benefited from the suggestions of two anonymous reviewers. The authors thank Drs. Li-Yuang Kuo and Kuan-Ting Hish for helping with data analysis. This work was partially supported by grant from the Ministry of Science and Technology, Taiwan (NSC 102-2313-B-002-037).

LITERATURE CITED

Ahad, B., Z.A. Reshi, A.H. Ganaie and A.R. Yousuf. 2012. *Azolla cristata* in the Kashmir Himalaya. Am. Fern J. **102(3):** 224–227.

Carrapiço, F. 2010. *Azolla* as a Superorganism. Its Implication in Symbiotic Studies. In: **Seckbach, J. and M. Grube** (eds) Symbioses and Stress. Cellular Origin, Life in Extreme Habitats and Astrobiology, vol **17**. Springer, Dordrecht.

Chen, S.-H. 2008. Naturalized Plants of Eastern Taiwan. National Hualien University of Education, Hualien, Taiwan. 250pp.

Devol, C.E. 1994. Azollaceae. In: **Huang, T.-C. *et al.* (**eds.), Flora of Taiwan, 2nd ed. **1**: 541. Editorial Committee, Dept. Bot., NTU, Taipei, Taiwan.

Ebihara, A.J., H. Nitta, and M. Ito. 2010 Molecular species identification with rich floristic sampling: DNA barcoding the pteridophyte flora of Japan. PLoS One **5(12)**: e15136.

Goff, L. J. 2011. Algal symbiosis: A continuum of Interaction Strategies. Cambridge University Press.

Gratwicke, B. and B. Marshall. 2001. The impact of *Azolla filiculoides* Lam. on animal biodiversity in streams in Zimbabwe. Afr. J. Ecol. **39(2):** 216–218.

Hashemloian, B.D. and A.A. Azimi. 2009. Alien and exotic *Azolla* in northern Iran. Afr. J. Biotech. **8:** 187–190.

Hwang, S.-Y., J.-J. Peng, J.-C. Huang, and T.-W. Hsu. 2004. The Survey of Invasive Plants in Taiwan. Endemic Species Research Institute, Nantou, Taiwan. 55pp.

Li, F.-W., **P. Brouwer, L. Carretero-Paulet, S. Cheng, J. De Vries, P. M. Delaux, A. Eily, N. Koppers, L.-Y. Kuo, Z. Li, M. Simenc, I. Small, E. Wafula, S. Angarita, M.S. Barker, A. Bräutigam, C. dePamphilis, S. Gould, P.S. Hosmani, Y.-M. Huang, B. Huettel, Y. Kato, X. Liu, S. Maere, R. McDowell, L.A. Mueller, K.G.J. Nierop, S.A. Rensing, T. Robison, C.J. Rothfels, E.M. Sigel, Y. Song, P.R. Timilsena, Y.V. de Peer, H. Wang, P.K.I. Wilhelmsson, Paul G. Wolf, X. Xu, J.P. Der, H. Schluepmann, G.K.-S. Wong, and K.M. Pryer**. 2018. Fern genomes elucidate land plant evolution and cyanobacterial symbioses. Nat. Plants **4(7)**:460–472

Lumpkin, T.A. and D.L. Plucknett. 1980. *Azolla*: Botany, physiology, and use as a green manure. Econ. Bot. **34(2):** 111–153.

Madeira, P.T., T.D. Center, J.A. Coetzee, R.W. Pemberton, M.F. Purcell and M.P. Hill. 2013. Identity and origins of introduced and native *Azolla* species in Florida. Aquat. Bot. **111:** 9–15.

Marchesi, J.R., T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiom and W.G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. **64(2):** 795–799.

Masoodi, A. and F.A. Khan. 2012. A new record to the invasive alien Flora of India: *Azolla cristata*. Natl. Acad. Sci. Lett. **35(6):** 493–495.

Metzgar, J.S., H. Schneider and K.M. Pryer. 2007. Phylogeny and divergence time estimates for the fern genus *Azolla* (Salviniaceae). Int. J. Plant Sci. **168(7):** 1045–1053.

Murillo, P.G., R.F. Zamudio, S.C. Bracamonte, A.S. Martín and J.M. Espinar. 2007. The invasion of Doñana National Park (SW Spain) by the mosquito fern (*Azolla filiculoides* Lam). Limnetica **26:** 243–250.

Nübel, U., F. Garcia-Pichel and G. Muyzer. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl. Environ. Microbiol. **63(8):** 3327–3332.

Papaefthimiou, D., C. Van Hove, A. Lejeune, U. Rasmussen and A. Wilmotte. 2008. Diversity and host specificity of *Azolla* cyanobionts. J. Phycol. **44(1):** 60–70.

Pereira, A.L., M. Martins, M.M. Oliveira and F. Carrapiço. 2011. Morphological and genetic diversity of the family Azollaceae inferred from vegetative characters and RAPD markers. Plant Syst. Evol. **297(3-4):** 213–226.

Pereira, A.L. and V. Vasconcelos. 2014. Classification and phylogeny of the cyanobiont *Anabaena azollae* Strasburger: an answered question? Int. J. Syst. Evol. Microbiol. **64(6)**:1830–1840.

Perkins, S.K., G.A. Peters, T.A. Lumpkin and H.E. Calvert. 1985. Scanning electron microscopy of perine arechitecture as a axonomic tool in the genus *Azolla* Lamarck. SEM **4:** 1719–1734.

Peters, G.A. and J.C. Meeks. 1989. The *Azolla-Anabaena* symbiosis: Basic biology. Ann. Rev. Plant Physiol. Plant Mol. Biol. **40(1):** 193–210.

Reid, J.D., G.M. Plunkett and G.A. Peters. 2006. Phylogenetic relationships in the heterosporous fern genus *Azolla* (Azollaceae) based on DNA sequence data from three noncoding regions. Int. J. Plant Sci. **167(3):** 529–538.

Saunders, R.M.K. and K. Fowler. 1992. A morphological taxonomic revision of *Azolla* Lam. section *Rhizosperma*

(Mey.) Mett.(Azollaceae). Bot. J. Linn. Soc. **109(3):** 329–357.

Saunders, R.M.K. and K. Fowler. 1993. The supraspecific taxonomy and evolution of the fern genus *Azolla* (Azollaceae). Plant Syst. Evol. **184(3-4):** 175–193.

Shaw, R.H., R.H. Reeder, E. Bacon and C. Oduro, 2004. Biocontrol potential for *Azolla filiculoides* and *Hydrocotyle ranunculoides* in the UK and Europe. 13 th International Conference on Aquatic Invasine Species, pp.130. Institute of Technology, Sligo, Ireland.

Svenson, H.K. 1944. The new world species of *Azolla*. Am. Fern J. **34(3):** 69–84.

Szczesniak, E., J. Blachuta, M. Krukowski and J. Picinska-Faltynowicz. 2009. Distribution of *Azolla filiculoides* Lam.(Azollaceae) in Poland. Acta Soc. Bot. Pol. **78(3):** 241–246.

Taiwan Pteridophyte Group: **Li-Y. Kuo *et. al.*** 2019. Updating Taiwanese pteridophyte checklist: a new phylogenetic classification. Taiwania **64(4):** 367–395.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. **30(12):** 2725–2729.

Thompson, J.D., D.G. Higgins, T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22(22)**: 4673–4680.

Wagner, G.M. 1997. *Azolla*: a review of its biology and utilization. Bot. Rev. **63(1):** 1–26.

Wu, S.H., T.Y.A. Yang, Y.C. Teng, C.Y. Chang, K.C. Yang and C.F. Hsieh. 2010. Insights of the latest naturalized flora of Taiwan: change in the past eight years. Taiwania **55(2):** 139–159.

Zimmerman, W.J., T.A. Lumpkin and I. Watanabe. 1989. Classification of *Azolla* spp., section *Azolla*. Euphytica **43(3):** 223–232.

Supplementary materials are available from Journal Website.