

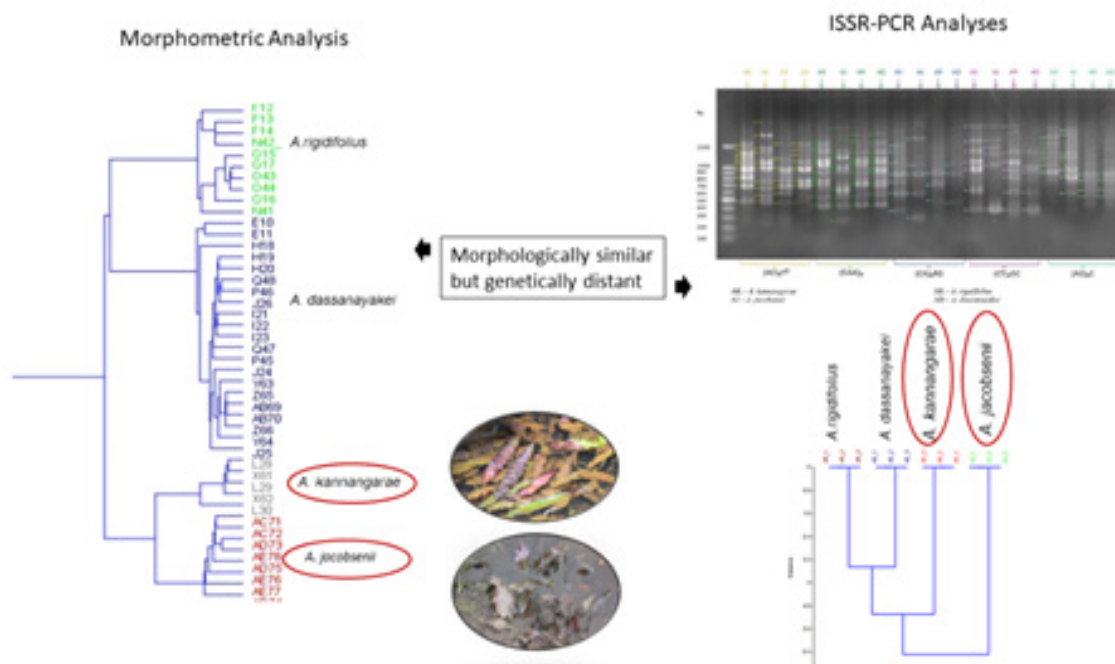
RESEARCH ARTICLE

This article is published in commemoration of the bicentenary of the Royal Botanic Gardens, Peradeniya, Sri Lanka.

Re-evaluating the Species Limits of *Aponogeton kannangarae* and *Aponogeton jacobsenii* Using ISSR Data

J. R. Wakkumbura, H. A. C. K. Ariyaratna and D. M. D. Yakandawala

Graphical Abstract



Highlights

- The recently described *A. kannangarae* morphologically closely resembles *A. jacobsenii*
- This had led to a confusion in the identification of the two taxa
- The genetic distance of these two species was determined using ISSR markers
- Even though morphologically *A. kannangarae* and *A. jacobsenii* look similar, genetically, they are distantly related

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Re-evaluating the Species Limits of *Aponogeton kannangarae* and *Aponogeton jacobsonii* Using ISSR Data

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Abstract

The genus *Aponogeton* comprises six species in Sri Lanka. Among those species, the recently described *A. kannangarae* closely resembles *A. jacobsonii*, in leaf morphology, especially colour, shape, and size. Even though differences in leaf base and follicle coat are recognized, a recent study questions the contribution of these morphological characters for recognizing it as a new species. This raises an ambiguity in the identification of *A. jacobsonii* and *A. kannangarae* as two different taxa. Since morphological data do not provide much strength to resolve ambiguities, a molecular study was conducted to gain additional evidence. The genetic distance of these two species was determined using ISSR (Inter Simple Sequence Repeats) - PCR amplification. Apart from these two species, another two morphologically similar species, *A. rigidifolius* and *A. dassanayakei* were used to obtain a clear idea about the species limits. The polymorphic bands that resulted from the ISSR- PCR amplification was coded into a data set and analyzed using the statistical software, PAST (Version 2.15). According to the cluster analysis, *A. rigidifolius* and *A. dassanayakei* are the two genetically closest species while *A. jacobsonii* has separated from *A. rigidifolius*, *A. dassanayakei* and *A. kannangarae* at a very early stage. Therefore, according to these studies, even though morphologically *A. kannangarae* and *A. jacobsonii* look similar, genetically, they are distantly related. Therefore, this molecular analysis strongly supports that *A. kannangarae* and *A. jacobsonii* are two different species.

Keywords: *Aponogeton*; ISSR- PCR amplification; morphology; molecular analysis

INTRODUCTION

Biodiversity and its conservation are a topic of concern that has gained attention both within the scientific community and the general public. The basic step towards conservation of biodiversity of a country is enumerating the number of species the country harbors. The number of species together with the relative abundance contributes to the species richness (Grassle & Maciolek, 1992). In this context, taxonomic studies play an important role in solving ambiguities related to species limits of a given taxon. The genus *Aponogeton* is such a taxon that gained the attention of taxonomists due its varied morphology (Bruggen, 1987; Chen et al., 2015; Manawaduge et al., 2016a and b and 2018; De Silva et al., 2016). The genus belongs to the family Aponogetonaceae, a monocotyledonous aquatic plant family, which consists of a single genus *Aponogeton* L. f. It contains about 60 species from Africa, Madagascar, India, and Sri Lanka through Asia and Malaysia and Australia (Chen et al., 2015; Manawaduge et al., 2016b; De Silva et al., 2016). Bruggen (1987), has revised the genus

Aponogeton in Sri Lanka and recognized four *Aponogeton* species; *A. rigidifolius* Bruggen, *A. jacobsonii* Bruggen, *A. crispus* Thunberg and *A. natans* Linnaeus of which the first two species are endemic to Sri Lanka. In addition, two recent taxonomic studies have added two new members, *A. kannangarae* De Silva (De Silva et al., 2016) and *A. dassanayakei* Manawaduge & Yakandawala (Manawaduge et al. 2016b) which are endemic to the country. However, a proceeding taxonomic study based on a morphometric analysis by Manawaduge & Yakandawala (2018), raises concerns on the morphological features that are recognized to define the new species, *A. kannangarae*. The reason for this been its close resemblance to *A. jacobsonii*, particularly, in leaf morphology, especially in colour, shape, and size. The attachment of the plumule is another subjective character that is used in separating the two species; the attachment of the plumule of *A. kannangarae* is just below the middle, whereas it is attached at the middle in *A. jacobsonii* (Figure 1, 2, and 3; Figure 1 and 3 reproduced from Manawaduge & Yakandawala [2018]). Like many other aquatic plants, *Aponogeton* shows extensive phenotypic plasticity, and

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therefore morphometry based taxonomical studies are not sufficient to understand species limits calling for support from molecular data (Bruggen, 1987 Hellquist & Jacobs, 1998).

In this context molecular tools, especially techniques such as ISSR (Inter Simple Sequence Repeat)-PCR Amplification/ Single-locus molecular markers have been successfully used as a biosystematic tool, in order to define interspecific limits within species complexes. The ISSR techniques is also useful in detecting genetic polymorphisms at species levels because it produces a large number of polymorphic fragments that can be interpreted as multiloci genetic profiles (Crawford & Mort 2004; Balasaravanan et al., 2006; Taylor & Barker, 2012; Bozchaloyi et al., 2018). Balasaravanan et al. (2006), has utilized ISSR-PCR to discriminate species and putative hybrids during selection and hybridization programmes of *Eucalyptus*. Similarly, a study on the species boundaries in *Myrcia laruotteana* complex of the family Myrtaceae, ISSR markers provided evidence to resolve the ambiguity resulting in a new taxonomic classification (Lima et al., 2015). Similarly, Son et al. (2012), has employed the ISSR technique to resolve taxonomic ambiguities in the genus *Allium*.

To understand the species boundaries of the two-endemic species of *Aponogeton*: *A. kannangarae* and *A. jacobsenii*, we have analyzed the genetic variability and structure of these two species using ISSR markers.

MATERIALS AND METHODS

The plant material was collected from the field. In addition to *A. kannangarae* and *A. jacobsenii*, two other species, *A. dassanayakei* and *A. rigidifolius*, that are closer in morphology to the former two species were also included in the study. *A. dassanayakei* and *A. rigidifolius* were identified based on morphometric analysis carried out on Manawaduge and Yakandawala (2018). Plant species; *Aponogeton kannangarae*, *A. jacobsenii*, *A. rigidifolius* and *A. dassanayakei* were collected from four locations published in Manawaduge and Yakandawala (2018). The details of the collection sites and the species collected from each locality are given in Table 1 and Figure 6. Each species was referred by an acronym for easy reference.

Samples were identified using taxonomic descriptions given in Manawaduge and Yakandawala (2018). Three plants from each sub-population that is at least 5m-10m apart were collected.

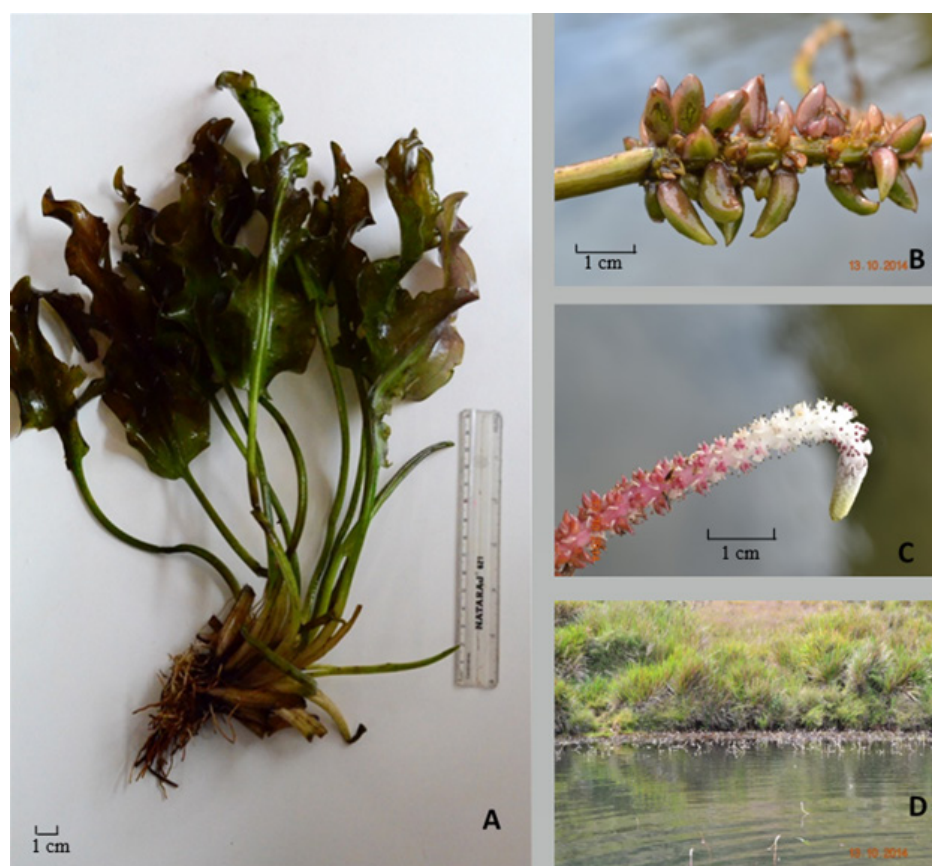


Figure 1: *Aponogeton jacobsenii*. A. Habit with purple to maroon immature leaves which turns to bright green with maturity. B. Inflorescence. C. Dark pink and white, densely flowered inflorescence with one spike up to 11 cm long. D. Habitat.: marshy pools (Manawaduge & Yakandawala, 2018)



Figure 2: *Aponogeton kannangarae*. A. Habit with purple to maroon immature leaves which turn to dark green with maturity. Note the acute bases of the leaves, which is typical of the species. B. Infructescence. C. Dark pink and white, densely flowered Inflorescence with one spike up to 18 cm long. D. Habitat



Figure 3: Leaf morphological variation recorded in different *A. kannangarae* and *A. jacobsonii* populations. A. Leaves of *A. kannangarae* populations from Sooriyakanda with a cordate base, oppose to acute base, that resembles *A. jacobsonii*. B. A leaf of *A. jacobsonii* with an acute base, oppose to cordate base, from Horton Plains resembling *A. kannangarae* (Manawaduge & Yakandawala, 2018)

Table 1: Details of the collection sites of *Aponogeton* species

Species	Acronym	Collection site, district
<i>Aponogeton kannangarae</i>	AK	Sooriyakanda Forest, Rathnapura
<i>A. jacobsenii</i>	AJ	Horton plains National Park, Nuwara Eliya
<i>A. rigidifolius</i>	AR	Mathugama, Kaluthara
<i>A. dassanayakei</i>	AD	Diyawannawa Oya, Colombo

Table 2: ISSR primers used in PCR amplification

ISSR primer	Sequence
01	(AG) ₈ YT
02	(GAA) ₆
03	(CA) ₈ RG
04	(CT) ₈ GC
05	(AG) ₈ C

Samples were grown and maintained at the Department of Botany, University of Peradeniya, Sri Lanka to provide living reference material for consultation during the course of the study. Immediately harvested fresh samples were cleaned and used for DNA extraction.

DNA was isolated from healthy fresh leafblades using CTAB method (Doyle & Doyle, 1990) with minor modifications. Ten ISSR primers were tested for amplification reactions from which 5 primers that produced better resolution and reproducible bands on the gel were selected for the ISSR-PCR amplification (Table 2). All PCR amplifications were carried out in a total 10.0 µl volume containing 5.0 µl GoTaq Green master mixture (×2), 1.0 µl ISSR primer, 0.2 µl Taq polymerase (5µ/ µl), 0.5 µl MgCl₂ (25mM), 0.5 µl BSA (0.001gml⁻¹), 1.8 µl nuclease-free water, 1.0 µl DNA template. The PCR program consisted of an initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 1 minute, annealing specific temperature for 30 s, extension at 72 °C for 2 minutes and a final extension at 72 °C for 10 minutes. The annealing temperature for (AG)₈YT was 50 °C, for (GAA)₆ was 49 °C, for both (CA)₈RG and (CT)₈GC were 51 °C, and for (AG)₈C was 47 °C. PCR products were analyzed by electrophoresis on 1 % agarose gels with 50 ml of 1× TAE buffer, under 70 V for approximately 90 minutes. The gels were stained with 2.5 µl of Ethidium Bromide, and 1500-bp ladder was used to estimate the molecular size of the fragments. In ISSR-PCR amplification, for each species, three samples were amplified using the same primer, and repeatable bands were identified. Further, two replicates were completed for each sample. Presence and absence of bands were scored as 1 and 0, and the data matrix was used in Cluster analysis (CA) using the statistical software, PAST (Version 2.15) (Hammer et al., 2001).

RESULTS

Of the primers tested (AG)₈YT, (GAA)₆, (CA)₈RG, (CT)₈GC and (AG)₈C produced multiple bands. The size of the bands that resulted from ISSR-PCR varied between 100

- 1500 bp for all the tested primers. After the amplification, 67 distinguishable and repeatable bands were observed. The total number of polymorphic bands was 59. For each ISSR primer, each species showed distinct banding patterns (Table 3 and Figure 4).

The dendrogram, resulted from the cluster analysis is given in Figure 5. According to the cluster analysis, *A. jacobsenii* deviated at a distance of 6.5 from the other three taxa indicating a distant genetic relationship. Next, *A. kannangarae* separated from *A. dassanayakei* and *A. rigidifolius*, at a distance of 4.9. Of the four *Aponogeton* species included in the analysis, *A. dassanayakei* and *A. rigidifolius* are genetically close to each other and separates at a distance of 3.4. There was no inter-population genetic variation among the three populations of each species.

DISCUSSION

The objective of the study was determining the species limits *A. kannangarae* and *A. jacobsenii* as they were morphologically similar, except for few leaf characters; colour, shape, and size and the position of the attachment of the plumule (attached just below the middle in *A. kannangarae* whereas at the middle in *A. jacobsenii*). These morphological characters used as diagnostic features were subjective. In this study, 67 distinguishable and repeatable bands were observed, and for each ISSR primer, each species showed distinct banding patterns. Each ISSR primer revealed more than 75% of polymorphisms for four *Aponogeton* species. Therefore, the genetic polymorphism observed provides evidence of unique banding patterns for each species incorporated in the study. Further, the certain degree of pattern similarities that were observed in some species, could be their evolutionary relatedness.

The cluster analysis of the data of the polymorphic bands has identified 4 separate clusters and, significant genetic distance between the clusters clearly discriminating the four *Aponogeton* species included in the study.

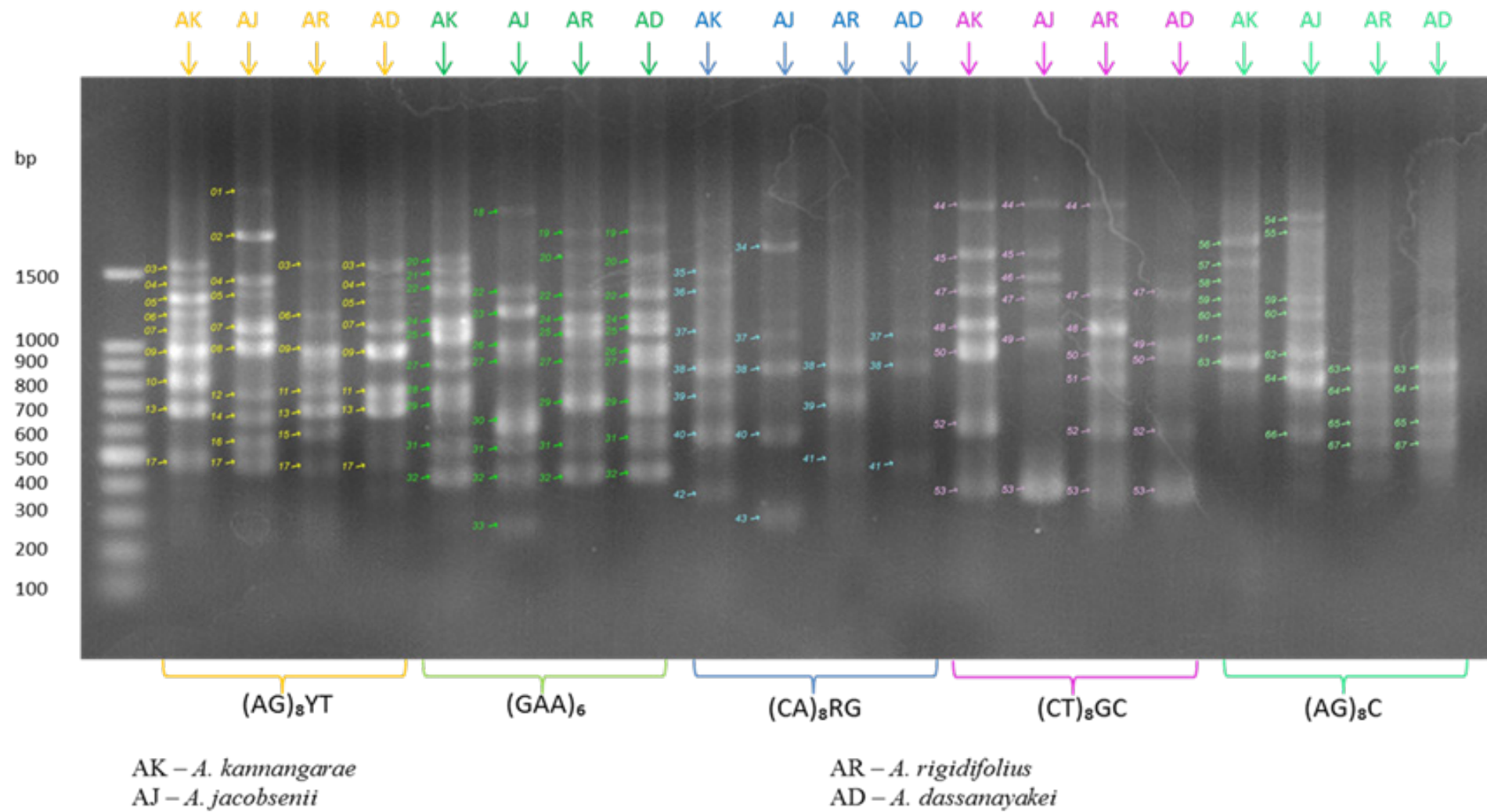
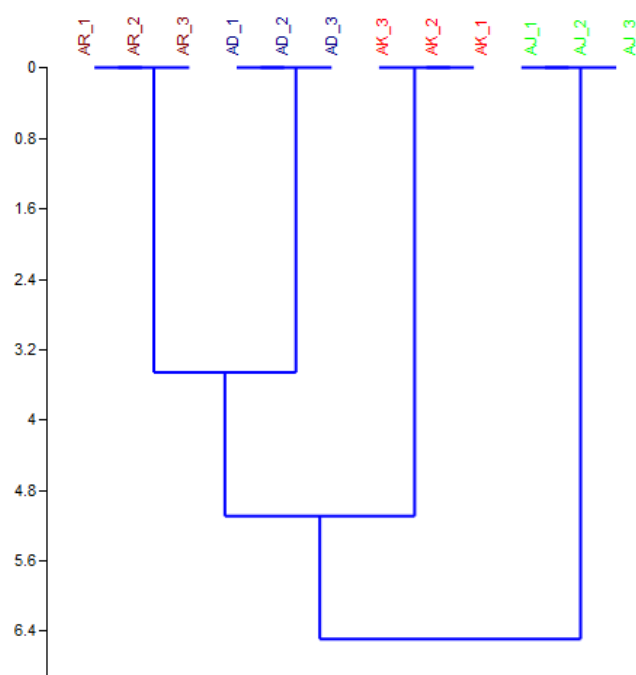


Figure 4: Population diversity for the polymorphic primers (AG)₈YT, (GAA)₆, (CA)₈RG, (CT)₈GC and (AG)₈C

Table 3: Polymorphic bands of each ISSR primers and percentage of polymorphism

Primers	Total bands	No. of monomorphic bands	No. of polymorphic bands	% of monomorphic bands	% of polymorphic band
(AG) ₈ YT	17	1	16	5.9	94.1
(GAA) ₆	16	4	12	25	75
(CA) ₈ RG	10	1	9	10	90
(CT) ₈ GC	10	2	8	20	80
(AG) ₈ C	14	0	14	0	100
Total	67	8	59	11.9	88.1

**Figure 5:** Dendrogram that resulted from the cluster analysis of the polymorphic bands for the four *Aponogeton* species; *A. dassanayakei* (AD1, AD2 and AD3); *A. jacobsenii* (AJ1, AJ2 and AJ3); *A. kannangarae* (AK 1, AK 2 and AK3) and *A. rigidifolius* (AR 1, AR 2 and AR3)

Aponogeton jacobsenii separates early from the other three species while *A. kannangarae* clusters with *A. rigidifolius* and *A. dassanayakei* indicating a genetic similarity to the latter two taxa compared to *A. jacobsenii*. The study provides molecular evidence to justify the two species, *A. jacobsenii* and *A. kannangarae* as two distinct taxa that are distantly related to each other. Considering the morphology even though *A. kannangarae* & *A. jacobsenii* expressed similar features, the study has provided genetic evidence supporting them as two separate species.

The geographical distribution of these *Aponogeton* species (Manawaduge & Yakandawala, 2018) in the country supports the results (Figure 6). *Aponogeton rigidifolius* and *A. dassanayakei* are distributed in the lowland wet zone of the country while both *A. jacobsenii* and *A. kannangarae* occur in the highlands where former is restricted Horton

plains with an altitude of 2100 meters above sea level and the later occurs in Sooriyakanda and Morningside between 1000 - 1280 meters above sea level. Common geographical distribution of species can result in expression of phenotypic plasticity and comparable morphological adaptations to thrive in similar environment conditions leading to convergent evolution (Heyduk et al., 2019). The significant morphological similarities of the two species, *A. jacobsenii* and *A. kannangarae*, despite genetic divergence can be stemming from extensive and analogous adaptations to the macro and micro environments the species share in the central highland habitat. Of the two species *A. kannangarae* occur in a lower altitude and also genetically closer to the lowland species. Expression of similar phenotypes and therefore similar morphology could resemble adaptation to similar environmental conditions (West-Eberhard, 2005).

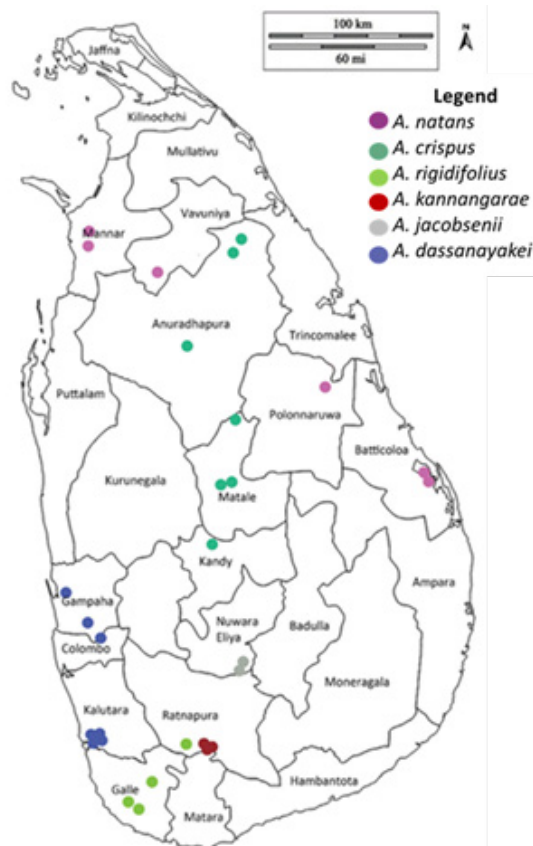


Figure 6: Map of Sri Lanka indicating geographical distribution of all six *Aponogeton* species occurring in the island (N 5°55'–9°55' / E 79°42'–81°52') (Manawaduge & Yakandawala, 2018)

However, the morphological similarities were not supported in the genetic analysis clearly indicating the possibility of convergent evolution whereby distantly related organisms independently evolve similar traits to adapt to similar necessities in similar environments. Both, *A. rigidifolius*, and *A. dassanayakei*, which are genetically closely related, occur in the wet zone and share similar morphological features.

Although morphologically similar *A. kannangarae* and *A. jacobsenii* were genetically distantly related as evident by the SSR analysis, therefore, the molecular analysis discriminates that *A. kannangarae* and *A. jacobsenii* into two distinct species. Both these species are endemic and listed under the category of Critically Endangered (CR) species during the recent national red listing process (National Red list, 2020). The species delimitation of *Aponogeton* species resolves the taxonomic ambiguities within the genus and fulfils the information gaps in local biodiversity figures leading to the biodiversity conservation process.

CONCLUSION

According to the molecular analysis, *A. kannangarae* and *A. jacobsenii* were clearly resolved as two different species. *Aponogeton rigidifolius* and *A. dassanayakei* are the most closely related species among the four

endemic *Aponogeton* species. The results of the present study will contribute to the conservation of *Aponogeton* species in Sri Lanka through clarification of their taxonomic status and identification of unique evolutionary lineages.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest

FUNDING INFORMATION

No funding is received for this work.

AUTHOR CONTRIBUTION

Conceptualization, Supervision: **D.M.D.Y., H.A.C.K.A.**; Sample collection, Experimental work, Data collection, Statistical analysis: **D.M.D.Y., H.A.C.K.A., J.R.W.**; Data interpretation: **D.M.D.Y., H.A.C.K.A., J.R.W.**; Writing the first draft: **J.R.W.**; Manuscript editing and reviewing: **D.M.D.Y., H.A.C.K.A., J.R.W.**; Project administration: **D.M.D.Y.**

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study will be made available to the public upon request.

REFERENCES

- Balasaravanan, T., Chezian, P., Kamalakannan, R., Yasodha, R., Varghese, M., Gurumurthi, K., & Ghosh, M. (2006). Identification of species-diagnostic ISSR markers for six Eucalyptus species. *Silvae Genetica*, **55**(3), 119-122.
- Bozchaloyi, E.S., Sheidai, M., Keshavarzi, M., & Noormohammadi, Z. (2018). Species relationship and population structure analysis in Geranium subg. Robertium with the use of ISSR molecular markers. *Acta Botanica Hungarica*, **60**(1-2), 47-65. doi: 10.1556/034.60.2018.1-2.4
- Bruggen, H. W. E. (1987). Aponogetonaceae. In Dassanayake, M. D. & Fosberg, F. R. (Eds.), A Revised Hand Book to the Flora of Ceylon, **4**, pp.3-16. New Delhi: Amerind Publishing.
- Chen, L., Grimm, G. W., Wang, Q., & Renner, S. S. (2015). A phylogeny and biogeographic analysis for the Cape-Pondweed family Aponogetonaceae (Alismatales). *Molecular Phylogenetics and Evolution*, **82**, 111-117. doi: 10.1016/j.ympev.2014.10.007
- Crawford, D. J., & Mort, M. E. (2004). Single-locus molecular markers for inferring relationships at lower taxonomic levels: observations and comments. *Taxon*, **53**(3), 631-635.
- De Silva, M. A., Deshaprema, K. M. S., & Manamperi, J. P. J. (2016). *Aponogeton kannangarae*, a new species of *Aponogeton* (Aponogetonaceae) from Rakwana hills, Sri Lanka. *Phytotaxa*, **272** (3), 220-224. doi:10.11646/phytotaxa.272.3.7
- Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.
- Grassle, J. F., & Maciolek, N. J. (1992). Deep-sea species richness: regional and local diversity estimates from quantitative bottom samples. *The American Naturalist*, **139**(2), 313-341.
- Hammer, O., Harper, D.A.T. & Ryan, P.D. (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, **4**, 1-9.
- Hellquist, C. B., & Jacobs, S. (1998). Aponogetonaceae of Australia, with descriptions of six new taxa. *Telopea*, **8**, 7-19. doi: 10.7751
- Heyduk, K., Moreno-Villena, J. J., Gilman, I. S., Christin, P. A., & Edwards, E. J. (2019). The genetics of convergent evolution: insights from plant photosynthesis. *Nature Reviews Genetics*, **20**(8), 485-493.
- Lima, D. F., Mauad, A. V. S., da Silva-Pereira, V., de Camargo Smidt, E., & Goldenberg, R. (2015). Species boundaries inferred from ISSR markers in the Myrcia laruotteana complex (Myrtaceae). *Plant Systematics and Evolution*, **301**(1), 353-363.
- Manawaduge, C. G., & Yakandawala, D. (2018). Morphometrics and taxonomic update to the Sri Lankan Aponogetonaceae. *Phytotaxa*, **365**(3), 201-224. doi: 10.11646/phytotaxa.365.3.1
- Manawaduge, C. G., Yakandawala, D., & Les, D. H. (2016a). A reappraisal of leaf morphology in *Aponogeton natans* (Aponogetonaceae). *Phytotaxa*, **265** (1): 095-097. doi. org/10.11646/phytotaxa.275.3.3
- Manawaduge, C. G., Yakandawala, D., & Les, D. H. (2016b). Morphometric analysis reveals a new species of *Aponogeton* (Aponogetonaceae) in Sri Lanka. *Phytotaxa*, **275** (3), 243-262. doi:10.11646/phytotaxa.275.3.3
- Son, J. H., Park, K. C., Lee, S. I., Kim, J. H., & Kim, N. S. (2012). Species relationships among Allium species by ISSR analysis. *Horticulture, Environment, and Biotechnology*, **53**(3), 256-262.
- Taylor, C. L., & Barker, N. P. (2012). Species limits in Vachellia (*Acacia*) karroo (Mimosoideae: Leguminosae): Evidence from automated ISSR DNA "fingerprinting". *South African Journal of Botany*, **83**, 36-43.
- The National Red List 2020 – Conservation Status of the Flora of Sri Lanka (2020). Sri Lanka: Biodiversity Secretariat, Ministry of Environment and the National Herbarium, Department of National Botanic Gardens.
- West-Eberhard, M. J. (2005). Developmental plasticity and the origin of species differences. *Proceedings of the National Academy of Sciences*, **102**(suppl_1), 6543-6549.