Pertanika J. Trop. Agric. Sci. 49 (2): 733 - 759 (2026)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Unravelling the Diversity of *Azolla cristata* from the Progo and Opak River Basins based on Inter-Simple Sequence Repeat Marker and Morphological Analysis

**Nafiah Khoirunnisa[1,2], Adib Fakhruddin Yusuf[2], Aji Sukma Iqbal Najibulloh[3], Abdul Razaq Chasani[4], Rina Sri Kasiamdari[4], and Budi Setiadi Daryono[2]***

*[1]Doctoral Biology Program, Faculty of Biology, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia*
*[2]Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia*
*[3]Tropical Biology Department, Faculty of Biology, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia*
*[4]Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia*

## ABSTRACT

*Azolla* is a floating aquatic plant that offers numerous advantages, including high growth rates and significant protein content, making it a valuable resource for animal feed, fertiliser, and a potential carbon capture agent. Taxonomic analysis and the diversity of *Azolla* in Indonesia remain unclear. Therefore, this study explores the diversity of *Azolla* species in the Progo and Opak river basins, using 20 morphological characters and 10 primers of inter-simple sequence repeat (ISSR). Morphological characters that strongly contribute to differences between genotypes are branching patterns, dorsal lobe shapes, presence of ventral stomata, lobe length, hyaline ratio, and number of branches. The genotypes are identified as *Azolla cristata*, which are synonyms of conspecific species *Azolla mexicana* and *Azolla microphylla*. Among 10 inter-simple sequence repeats (ISSR), UBC 814 and UBC 841 exhibit the high PIC (0.29; 0.28) and RP (19.71; 23.29), respectively. The unweighted pair group method cluster analysis grouped the accessions into two clusters based on their morphological and genetic traits. Diversity indices were moderately high across the two populations, with Nei's gene diversity (He) = 0.230 and Shannon's information index (I) = 0.342. AMOVA revealed that 93% of the overall genetic variation occurred within populations, while only 7% was attributed to differences among populations. The low differentiation suggests high gene flow between the two river basins, likely facilitated by water currents, waterfowl, or humans, effectively maintaining a single large, genetically diverse metapopulation.

*Keywords: Azolla cristata*, conspecific species, environmental data, morphological characteristic, phenetic relationship, similarity index

ARTICLE INFO

*Article history:*
Received: 25 April 2025
Accepted: 16 March 2026
Published: 17 April 2026

DOI: https://doi.org/10.47836/pjtas.49.2.15

*E-mail addresses*:
nafiahkhoirunnisa1999@mail.ugm.ac.id (Nafiah Khoirunnisa)
adib.fakhruddin.yusuf@mail.ugm.ac.id (Adib Fakhruddin Yusuf)
ajisukmaiqbalnajibulloh@mail.ugm.ac.id (Aji Sukma Iqbal Najibulloh)
ar.chasani@ugm.ac.id (Abdul Razaq Chasani)
rkasiamdari@ugm.ac.id (Rina Sri Kasiamdari)
bs_daryono@mail.ugm.ac.id (Budi Setiadi Daryono)
* Corresponding author

e-ISSN: 2231-8542

## INTRODUCTION

*Azolla* is an aquatic fern primarily indigenous to warm climates, including tropical and subtropical countries such as Africa, Asia, and the America (Bocchi & Malgioglio, 2010). *Azolla* is referred to as the "green gold mine" due to its numerous advantages (Wagner, 1997). *Azolla* has a very high growth rate of only 2-5 days in multiplication, making it potentially used as a carbon capture agent (Hamdan & Houri, 2022). Moreover, the high protein content in *A. pinnata, A. filiculoides,* and *A. mexicana* makes this aquatic fern a valuable supplement for animal feed, including for chickens, ducks, and fish (Nasir et al., 2022b; Sarah & Ratnam, 2020). Several studies have shown the effectiveness of *A. filiculoides* and *A. pinnata* as a biofertiliser, phytoremediation, and wastewater treatment (Amare et al., 2018; Brouwer et al., 2018; Kadir et al., 2020). The numerous benefits of *Azolla* require adequate conservation efforts to ensure that each type of *Azolla* may be used optimally for its intended function. Hence, the taxonomy of *Azolla* remains controversial to this day, especially within the *Azolla* section, due to species-level similarities and varying characteristics across different geographical and ecological environments (Pereira et al., 2001).

Genus *Azolla* has 7 species that are divided into 2 sections: *Azolla* and *Rhizosperma*. Section *Azolla* consists of *A. microphylla* Kaulf., *A. caroliniana* Willd., *A. mexicana* Schlecht. & Cham. ex K. Presl, *A. rubra* R. Br., and *A. filiculoides* Lam. The section *Rhizosperma* comprises *A. pinnata* R.Br., and *A. nilotica* Decne. ex-Mett (Pereira et al., 2011). However, Evrard & Van Hove (2004), considered *A. mexicana* and *A. microphylla* as a single species based on certain morphological features. Metzgar et al. (2007) and Reid et al. (2006) also classified them as a single evolutionary lineage. The *Azolla* taxonomic controversy over the years is largely due to frequent misinterpretation of morphological characters. Some reproductive characters of *Azolla* spp., such as the morphology and septum number of glochidia around the massulae of microsporocarps, along with the surface characteristics and stratification of the megasporocarp perine (Svenson 1944; Saunders & Fowler, 1992; Perkins et al., 1985), are considered more reliable. However, this reproductive character is difficult to find due to unfavourable environmental conditions (Kumar et al., 2021). Therefore, Pereira et al. (2011) and Kumar et al. (2021) in their research used vegetative characters to distinguish between *Azolla* species.

In Indonesia, the diversity and distribution of *Azolla* is barely studied, with only three species having been identified, namely *A. filiculoides*, *A. pinnata*, and *A. microphylla* (Caruso et al., 2023; Utomo et al., 2019; Wiryati, 2021). This is mainly due to the anthropogenic activities and the wide range of phenotypic variations of *Azolla* that are often misinterpreted by people. *Azolla* displays phenotypic variability in colour, size, and other traits. However, these variances may not directly correlate with alterations in their genotypes but instead indicate environmental factors. For example, heat can induce stress,

typically resulting in pigment shifts between green to reddish-brown due to the synthesis of anthocyanins and a decrease in chlorophyll (Cannavò et al., 2023; Kösesakal, 2014). Moreover, the lack of identification and genetic diversity information arises because studies of *Azolla* in Indonesia primarily focus on the utilisation of *Azolla* in agriculture, such as biofertilization (Jama et al., 2023; Meiriani & Tarigan, 2024), alternative feed for livestock (Susilo et al., 2024), and phytoremediation agents (Lestari et al., 2024). The lack of information on genetic diversity hinders the development of effective conservation strategies.

Therefore, the aim of this study is to describe the diversity of *Azolla* across two populations in the Progo and Opak river basins in Central Java and D.I Yogyakarta using morphological and inter-simple sequence repeat (ISSR). Moreover, we also integrate the ecological data of *Azolla* sampling locations to elaborate on the environmental factors that might affect *Azolla* diversity. ISSR is a simple-to-use marker that has a high level of polymorphism and reproducibility, and is a common molecular marker to analyse genetic diversity in plants (Alaaddin et al., 2022; Dong et al., 2007; Nasir et al., 2022a).

However, research on genetic diversity analysis and the *Azolla* population using ISSR in Indonesia is still very limited. Although genetic diversity analysis using ISSR has been used to analyse other aquatic ferns like *Ceratopteris pteridoides*, the use of ISSR on *Azolla* is limited to verifying homology and distinction between crossbred *Azolla* in China (Dong et al., 2007; Nasir et al., 2022a; Zhong et al., 2011). Other molecular markers used for the analysis of identification and genetic diversity in *Azolla* are RAPD, *trnL-trnF*, ITS1, *rbcL*, *atpB*, *trnG-trnR*, and *rps4-trnS* (Madeira et al., 2016, 2019; Metzgar et al., 2007; Pereira et al., 2011). Specifically, the genetic analysis that has been conducted in Indonesia is identification using *rbcL*, and genetic variation using *trnL-trnF* (Mantang et al., 2018; Najibulloh et al., 2025). The application of ISSR markers in this study, therefore, delivers additional information about the entire genome by focusing on many unidentified nuclear loci. This enables a more detailed examination of genetic variation both within and among populations (Hadipour et al., 2020). Chloroplast and ribosomal markers are often stable and more effective for species identification (Madeira et al., 2019). Conversely, using ISSR enhances the precision of polymorphism assessment in *Azolla cristata*, particularly regarding the connectivity of populations between the Progo and Opak river basins.

## MATERIALS AND METHODS

### Sampling Area and Plant Material

Field sampling was conducted for 6 months, from June to November 2024. Because *Azolla* is an aquatic plant, purposive sampling was carried out in rice fields and ponds, covering both wild and cultivated species along the Progo and Opak river basins in Central Java and D.I Yogyakarta. The number of sampling locations is proportional to the length of each river system.

Ten samples were collected from the Progo river basin (approximately 138 km), while four samples were collected from the Opak river basin (approximately 65 km). Sampling locations were selected to represent upstream-downstream river gradients and areas where *Azolla* is commonly found, including river-connected agricultural habitats (e.g., rice fields receiving irrigation directly from the river system). The sample size reflects both the spatial distribution and natural availability of *Azolla* along the river basin and ecological scarcity of populations during the dry season (June-November 2024), when reduced river flow leads to fragmented and ephemeral populations. Therefore, this sampling strategy was designed to capture representative genetic variation across river basins rather than to estimate population size or density. This purposive sampling technique was selected for its practicality, offering an efficient and cost-effective method to identify the target population for the study (Lydia et al., 2023; Rebai, 2023). The map of 14 collections of *Azolla* was made with QGIS (Figure 1). Environmental conditions such as light intensity, total dissolved solids (TDS), electrical conductivity (EC), water temperature, humidity (H), and shade condition were recorded as detailed in Table 1.

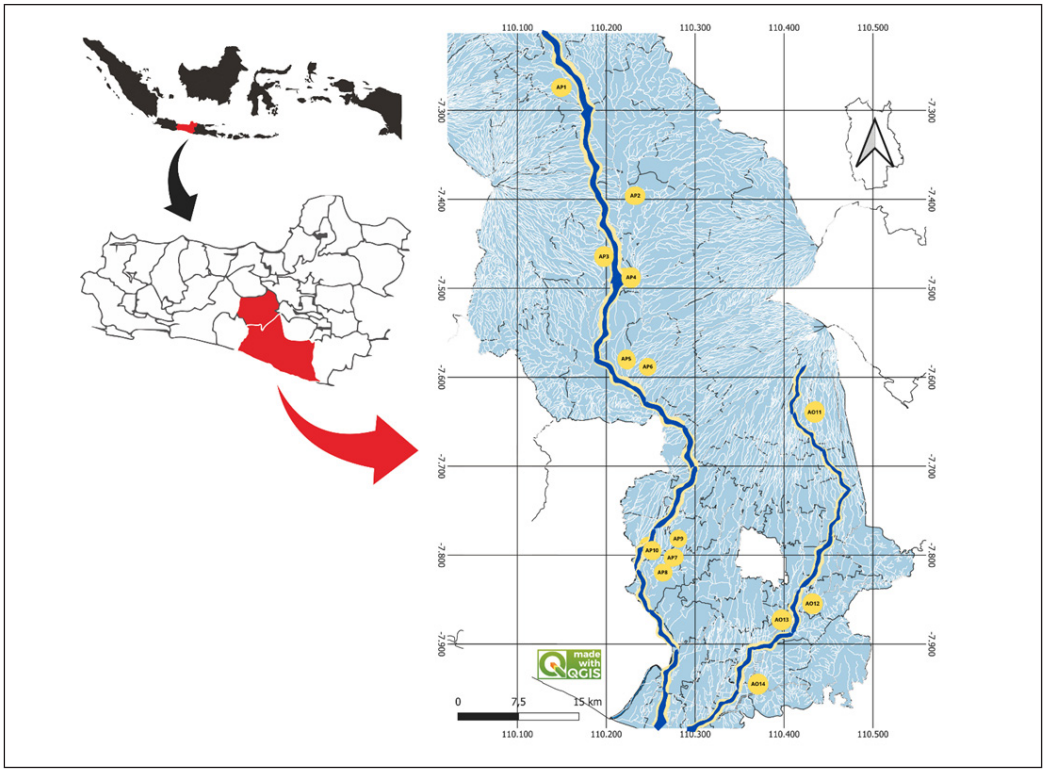



*Figure 1.* Sampling location of *Azolla cristata* collected from the Progo and Opak river basins, Yogyakarta, Indonesia. Sampling sites AP1-AP10 represent locations along the Progo river basin, while AO11-AO14 represent locations from the Opak river basin

Table 1
*Ecological condition of* Azolla *collection sites*

| Acc. | WT | TDS | EC | LI | H | SC | Latitude, Longitude |
|---|---|---|---|---|---|---|---|
| AP1 | 29.10$^{ab}$ | 108.0$^{b}$ | 216.0$^{abc}$ | 49.87$^{b}$ | 52.7$^{a}$ | Shaded | 7°16'29.40"S, 110° 8'55.50"E |
| AP2 | 31.83$^{b}$ | 71.7$^{a}$ | 143.3$^{a}$ | 83.37$^{ef}$ | 53$^{a}$ | Unshaded | 7°23'48.23"S, 110°13'49.13"E |
| AP3 | 37.97$^{c}$ | 177.0$^{e}$ | 356.7$^{de}$ | 96.73$^{g}$ | 54$^{a}$ | Unshaded | 7°27'50.55"S, 110°12'25.20"E |
| AP4* | 31.83$^{b}$ | 79.3$^{a}$ | 169.3$^{ab}$ | 58.33$^{bc}$ | 55.7$^{a}$ | Shaded | 7°29'15.02"S, 110°13'13.61"E |
| AP5 | 26.57$^{a}$ | 127.3$^{c}$ | 255.3$^{bcd}$ | 57.67$^{bc}$ | 56$^{a}$ | Unshaded | 7°35'0.09"S, 110°13'35.49"E |
| AP6 | 30.20$^{ab}$ | 166.0$^{e}$ | 321.3$^{de}$ | 51.10$^{b}$ | 60.3$^{ab}$ | Unshaded | 7°35'14.56"S, 110°14'28.89"E |
| AP7 | 29.83$^{ab}$ | 174.0$^{e}$ | 366.3$^{e}$ | 59.93$^{bc}$ | 61$^{abc}$ | Shaded | 7°48'54.57"S, 110°16'4.08"E |
| AP8 | 30.63$^{ab}$ | 183.0$^{f}$ | 368.0$^{e}$ | 27.47$^{a}$ | 67.3$^{bcd}$ | Shaded | 7°48'3.97"S, 110°16'29.18"E |
| AP9 | 31.07$^{ab}$ | 150.0$^{c}$ | 298.7$^{cde}$ | 76.80$^{de}$ | 70$^{cde}$ | Shaded | 7°47'6.36"S, 110°16'44.90"E |
| AP10 | 31.27$^{ab}$ | 164.3$^{e}$ | 348.7$^{de}$ | 47.87$^{b}$ | 70.3$^{cde}$ | Shaded | 7°47'22.10"S, 110°14'48.90"E |
| AO11 | 30.10$^{ab}$ | 100.0$^{b}$ | 197.3$^{abc}$ | 52.00$^{b}$ | 71$^{de}$ | Unshaded | 7°38'20.07"S, 110°26'8.83"E |
| AO12 | 29.83$^{ab}$ | 182.7$^{f}$ | 357.3$^{de}$ | 95.00$^{fg}$ | 72.3$^{de}$ | Unshaded | 7°51'27.30"S, 110°25'57.22"E |
| AO13* | 30.27$^{ab}$ | 351.0$^{g}$ | 576.0$^{f}$ | 69.70$^{cd}$ | 75.7$^{de}$ | Shaded | 7°52'23.85"S, 110°23'51.84"E |
| AO14 | 30.32$^{ab}$ | 176.3$^{e}$ | 375.0$^{e}$ | 66.53$^{cd}$ | 79$^{e}$ | Shaded | 7°56'45.96"S, 110°22'15.47"E |

*Note.* Acc. accession; AP, Progo river basin; AO, Opak river basin; *cultivated; WT: water temperature (°C); TDS: total dissolved solids (ppm); EC: electrical conductivity (µs/cm), AH: air humidity (% rh); LI: light intensity (Klux); H: Humidity (% rh); SC: Shade condition. Lowercase letters represent statistically distinct subsets at the 0.05 significance level

## Morphological Characterisation and Analysis

Morphological identification of *Azolla* species was conducted based on vegetative traits described by Goff (2011), Kumar et al. (2021), Pereira et al. (2011), Svenson (1944), Saunders & Flowers (1992), and along with additional characters supplemented by DMRT-based annotations (Table 2). Observations were performed using an Olympus SZ61 stereomicroscope, a light microscope, and an Optilab® Advance Plus. Quantitative traits were statistically evaluated using one-way ANOVA (Yusuf et al., 2023), followed by Duncan's multiple range test. To assess morphological clustering and trait variation, a dendrogram was constructed using the unweighted pair group technique with arithmetic mean (UPGMA), and principal component analysis (PCA) was executed in Minitab.

## Genomic DNA Extraction

Silica gel-dried samples were crushed into a fine powder using liquid nitrogen, and 1 g of polyvinylpyrrolidone (PVP) was added to improve the amount and quality of DNA. The Geneaid DNA isolation kit was used with slight modification. The concentration and purity of the extracted DNA were assessed using a NanoDrop Lite Plus spectrophotometer (ThermoScientific, USA).

Table 2
*Morphological parameters in this study*

| No. | Description | References |
|---|---|---|
| 1 | Sporophyte shape: (0) triangular, (1) starshaped | (Kumar et al., 2021) |
| 2 | Imbrication of lobe; (0) slightly imbricate, (1) moderately imbricated, and (2) highly imbricated | (Kumar et al., 2021) |
| 3 | Ventral lobe colour: (0) green, (1) pink | (Kumar et al., 2021) |
| 4 | Angle of dorsal and ventral lobes: (0) obtuse, (1) acute | (Kumar et al., 2021) |
| 5 | Dorsal lobe shape: (0) rounded, (1) sub-round | (Pereira et al., 2011) |
| 6 | Rhizome indumentum: (0) glabrous, (1) pubescent | (Pereira et al., 2011) |
| 7 | Trichome lobe structure: (0) unicellular, (1) bicellular | (Pereira et al., 2011) |
| 8 | Hyalin layers: (0) 3-4 layers, (1) 2-6 layers | (Pereira et al., 2011) |
| 9 | Ventral lobe stomata: (0) absent, (1) present | (Pereira et al., 2011) |
| 10 | Branching pattern: (0) dichotomous, (1) pinnate | (Svenson, 1944) |
| 12 | Trichome lobes arrangement: (0) irregular, (1) in-a-row | (Goff, 2011) |
| | Hyalin ratio: multistate | (Saunders & Flowers, 1992) |
| 13 | Dorsal lobe length: multistate | (Saunders & Flowers, 1992) |
| 14 | Dorsal lobe width: multistate | (Saunders & Flowers, 1992) |
| 15 | Ventral lobe length: multistate | (Saunders & Flowers, 1992) |
| 16 | Ventral lobe width: multistate | (Saunders & Flowers, 1992) |

Table 3
*Sequence and annealing temperature of ISSR primers used in the study*

| No. | Primers | Microsatellites Motifs | Annealing Temperature, ºC |
|---|---|---|---|
| 1 | UBC-814 | $(AT)_8YC$ | 45.5 |
| 2 | UBC-815 | $(GA)_8YT$ | 50 |
| 3 | UBC-826 | $(AC)_8C$ | 55 |
| 4 | UBC-834 | $(AG)_8YT$ | 49.3 |
| 5 | UBC-841 | $(GA)_8YC$ | 51.2 |
| 6 | UBC-845 | $(CT)_8RG$ | 50.1 |
| 7 | UBC-856 | $(AC)_8YA$ | 51.5 |
| 8 | UBC-860 | $(TG)_8RA$ | 51.5 |
| 9 | UBC-873 | $(GACA)_4$ | 50.1 |
| 10 | UBC-880 | $(GGAGA)_3$ | 50.1 |

### PCR-ISSR Amplification

This study utilised ten ISSR primers: UBC-814, UBC-815, UBC-826, UBC-834, UBC-841, UBC-845, UBC-856, UBC-860, UBC-873, and UBC-880 (Table 3). PCR amplification was conducted utilising a Bio-Rad T100 thermal cycler (Bio-Rad, USA) under the following protocols: initial denaturation at 94 °C for 4 minutes, followed by 40 cycles that included 30 seconds of denaturation at 94 °C, annealing at primer-specific temperatures for 60 seconds (Table 3), and extension at 72 °C for 90 seconds. A final extension was performed at 72 °C for 7 minutes. Amplification products were separated by electrophoresis and observed using a GelDoc-UV light transilluminator.

### Molecular Data Analysis

Reproducible ISSR bands were recorded as binary data: presence (1) or absence (0) for all samples, resulting in the creation of a binary matrix. The number of scored bands (NA), the number of polymorphic bands (NP), and the polymorphic rate (PR) were recorded. Marker informativeness was assessed by calculating the polymorphic information content (PIC) and resolving power (RP) according to Prevost & Wilkinson (1999). PIC for each primer was determined as shown in Equation (1):

$$PICi=2fi\ (1-fi) \quad [1]$$

Where *fi* is the frequency of band presence at a given locus (Kumar et al., 2014; Roldán-Ruiz et al., 2000). RP was calculated using Equation (2):

$$I_b=1-2\times(0.5-Pi) \quad [2]$$

where Pi represents the proportion of individuals exhibiting a band at a certain locus. Genetic diversity measurements, such as Nei's gene diversity (He), Shannon's information index (I), effective alleles count (Ne), polymorphism percentage (PBB), and molecular variance analysis (AMOVA), were calculated with GenAlEx 6.5 (Freeland et al., 2011; Weising et al., 2005). Phenetic links across samples were illustrated using a UPGMA dendrogram derived from Neighbour Joining distance in MVSP software. Clustering was further validated by Principal Coordinates Analysis (PCoA) utilising GeneAlex.

## RESULTS AND DISCUSSION

### Descriptive Statistics and Morphological Traits Among the Accessions

The vegetative characteristics used to describe *Azolla* revealed 6 monomorphic traits and 10 polymorphic traits. Although monomorphic characters did not show significant

differences between accessions, they still contributed to the identification of *Azolla* spp. The polymorphic features are eventually utilised for clustering and principal component analysis.

### *Characteristics of the Sporophyte*

All fourteen *Azolla* samples exhibited star-shaped sporophytes with moderate to high levels of leaf imbrication, as illustrated in Figure 2. These morphological characteristics clearly indicate that all accessions belong to the section *Azolla* (Kumar et al., 2021; Madeira et al., 2013). The star-shaped morphology is characterised by overlapping leaf arrangements and closely spaced branches, resulting in angular structures resembling a star, typical of the *Azolla* section (Kumar et al., 2021). Pereira et al. (2011) reported that members of the section *Azolla* exhibit a polygonal frond outline, whereas deltoid shapes are characteristic of the section *Rhizosperma*. The present study provides further clarification by demonstrating that the *Azolla* section also includes forms with pinnately arranged branches, deltoid outlines, and high levels of imbrication (Figure 2). These findings are consistent with earlier observations by Svenson (1944) and Gleason & Cronquist (1991), who reported that *A. microphylla* and *A. mexicana*, both members of the *Azolla* section, develop pinnate structures at full maturity.

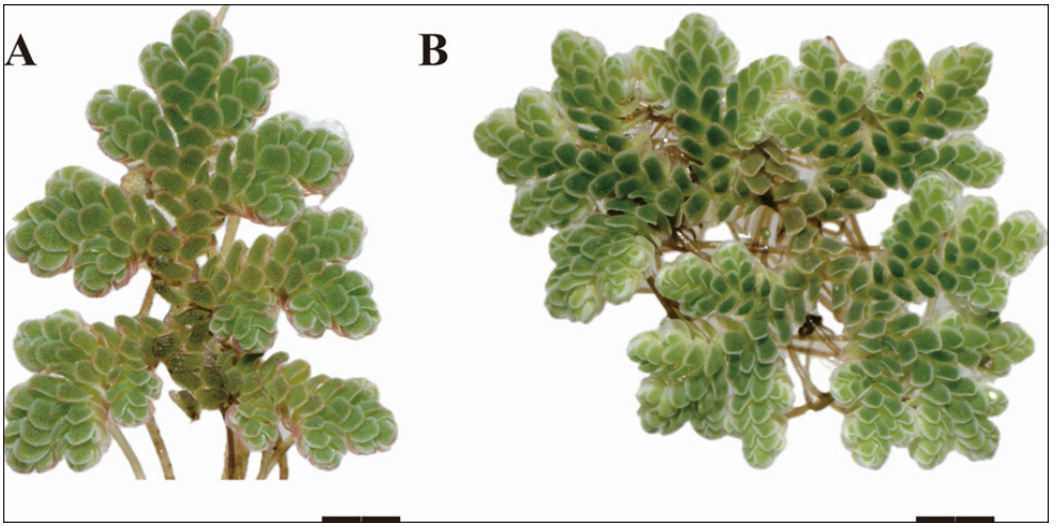



*Figure 2.* Star-shaped sporophyte with pinnate (A); and isotomous (B) branching. Scale bar = 2mm

### *Characteristics of Rhizome and Lobe*

Rhizome indumentum of glabrous branches (Figure 3A) supported the conclusion that all accessions belong to the *Azolla* section (Pereira et al., 2011). The observed angle between the dorsal and ventral lobes indicates that all samples have an acute angle with the range between 64.5° and 87.6° (Figure 3B and 3C), reinforcing the identification of all accessions into the *Azolla* section (Kumar et al., 2021). This study also revealed that *Azolla* samples are predominantly rounded dorsal lobes, while subround lobes were only identified in the AP4 and AO13 accessions. Pereira et al. (2011) stated that the rounded shapes are possessed by the section *Azolla*, whereas subround are only found in section *Rhizosperma*. However, findings by Kumar et al. (2021) and Svenson (1944) offer a different perspective, noting that *A. mexicana, A. microphylla, A. rubra,* and *A. filiculoides* from the *Azolla* section have slightly acuminated, similar to subround dorsal lobes. Therefore, our findings suggest that dorsal lobe shape is not a reliable trait for distinguishing *Azolla* species. This is likely because the accessions with subrounded dorsal lobes (AP4 and AO13) were collected from cultivated ponds, indicating that environmental conditions may influence this morphological feature. This observation is consistent with the findings of Dunham and Fowler (1987), who concluded that dorsal lobe shape is an unreliable character for species identification due to its variability with developmental stage and environmental factors.

The environment also influences the colour of the ventral lobe. Six of the 14 accessions exhibited a pink ventral lobe, while the remaining samples displayed green colouration. The results showed that unshaded accessions had pink ventral lobes, indicating that sunlight exposure occurred directly on *Azolla* accessions (Table 1). This finding is supported by Sadeghi et al. (2013), who stated that *Azolla* typically maintains a green colour under shaded conditions, while exposure to direct light causes the fronds to turn reddish. In some cases, the pink colour of the ventral lobe can be caused by low nutrients in the *Azolla* media; for instance, AP2, which is in a bright environment with a light intensity of 83.37 klux and low nutrients, has a TDS value of 71.7 ppm and an EC of 143.3 µS/cm, which together indicate low nutrient content in the medium. Total Dissolved Solids (TDS) indicate the concentration of inorganic and organic compounds dissolved in water, while Electrical Conductivity (EC) reflects the ability of water to conduct electricity due to the presence of these dissolved ions. Lower values of both TDS and EC typically correspond to nutrient-poor conditions. *Azolla* tends to be green when in a nutrient-rich environment with sufficient light intensity. A previous study from Cannavò et al. (2023) showed that higher intensity starting from 700 µmol $m^{-2}$ $s^{-1}$ (around 40 kLux) reduces the content of chlorophyll in *Azolla* and makes the lobe of *Azolla* become reddish. Da Silva et al. (2022) also added that *Azolla* could turn red because of the lack of nitrogen, coupled with light intensity. The colour change of the lobes occurred as a result of stress mechanisms caused

by nutrient deficiency or high light intensity, which led to the production of flavonoids such as anthocyanin and phlobaphene (Cannavò et al., 2023; Janes, 1998).

All samples in this study had a bicellular trichome structure (Figure 3E), suggesting they belong to the species *A. caroliniana*, *A. mexicana*, or *A. microphylla* (Pereira et al., 2011). Moreover, all accessions examined in this study displayed a regular (in-a-row) trichome arrangement on the dorsal lobes (Figure 3D), further narrowing the potential identification to *A. mexicana* or *A. microphylla*. This is based on the observation that *A. caroliniana* and *A. filiculoides* typically exhibit irregular trichome distribution on the dorsal lobes (Goff, 2011). This research highlights that the structure of bicellular trichome and the regular or in-a-row arrangement of bicellular trichome in lobus dorsal successfully used to identify *A. mexicana* and *A. microphylla* among species in the *Azolla* section (Chang et al., 2020; Goff, 2011; Pereira et al., 2011).

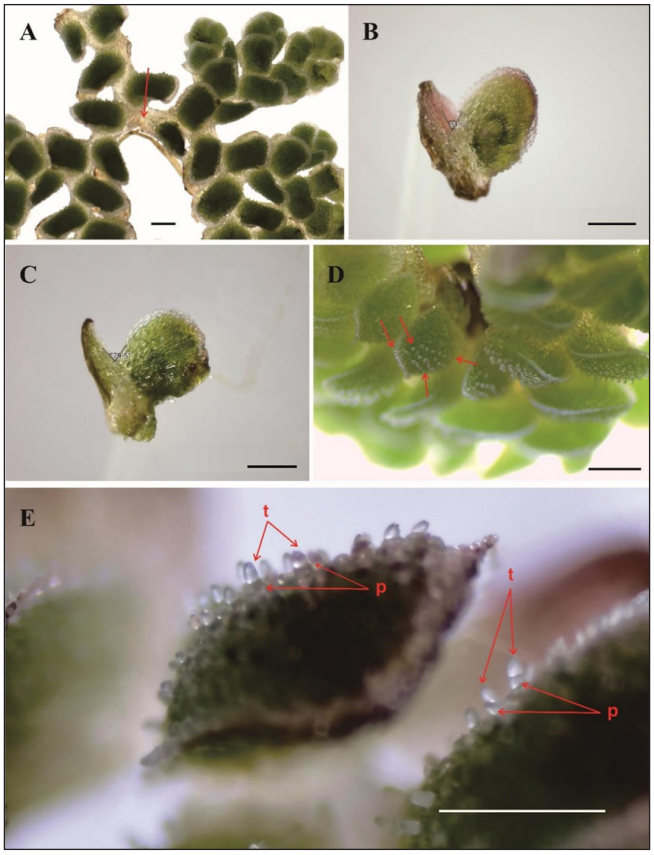



*Figure 3.* Characteristics of lobes, trichomes and stomata of *Azolla*. Glabrous rhizome (A) acute angle with pink (B) and green (C) ventral lobe. Dorsal lobe with a row arrangement of bicellular trichomes (D) bicellular trichome composed of trichome (t) and pedicle (p) cell (E)

Saunders and Fowler (1992) previously used multivariate statistical analysis on measurements such as the length and width of the dorsal and ventral lobes, as well as the ratio of hyaline layer (Figure 4A), to differentiate *A. nilotica* and *A. pinnata* in the section *Rhizosperma*. This study examined the same measurements using one-way ANOVA for section *Azolla*. The length of the dorsal lobe showed a significant difference ($F = 4.73$; $P < 0.05$), with a range of 0.83-1.32 mm. This result supports the assumption that the sample is likely *A. mexicana* or *A. microphylla* because the lobe length is more than 0.7 mm. Svenson (1944) mentioned that only *A. caroliniana* has a lobe length of about 0.5 mm. The length of the ventral lobe was also noticeably different ($F = 3.96$; $P < 0.05$), measuring between 0.70 and 1.26 mm. In contrast, the width of the dorsal lobe ($F = 0.62$; $P > 0.05$) and the width of the ventral lobe ($F = 1.03$; $P > 0.05$) did not show significant differences. The hyaline layer ratio showed a significant difference ($F = 3.83$; $P < 0.05$) with values ranging from 4.26 mm to 12.1 mm and an average of 8.9 mm.

Accessions AP1, AP4, AP7, AP8, AP9, AP10, AO11, AO12, and AO14 exhibited the presence of stomata on the ventral lobes, whereas other accessions showed few or no stomata in this region (Figure 4B). This characteristic, however, remains inconsistent for defining members of the section *Azolla*. For instance, Pereira et al. (2011) indicated that stomata were exclusively observed in *A. caroliniana*, suggesting limited taxonomic value. However, Chang et al. (2020) and Lumpkin & Plucknett (1980) found contrary results. Chang et al. (2020) mentioned that samples morphologically resembling *A. caroliniana* due to the presence of stomata were actually genetically closer to *A. mexicana.* Moreover, Lumpkin & Plucknett (1980) also reported that *A. filiculoides* has stomata in the ventral lobe. This ambiguity implies that the stomata characteristic alone is not sufficient for species delimitation. Busby & Gunning (1984) supported this idea, stated that the presence of stomata is variable and could appear on both the ventral and dorsal lobes of *Azolla*. The stomata formation depends on specific patterns of unequal cell divisions during the development of *Azolla*.

Considering the ongoing debate surrounding the classification of *Azolla*, this research highlights that all collected samples belong to the conspecific species *Azolla mexicana* or *Azolla microphylla*, synonymous with *Azolla cristata*. This conclusion is supported by the presence of bicellular trichomes, the regular arrangement (in a row) of trichomes on the dorsal lobe, and the length of the dorsal lobe (Ahad et al., 2012; Goff, 2011; Pereira et al., 2011; Saunders & Fowler, 1992). However, this morphological identification should be further validated through molecular characterisation, which is discussed in the following section.

## Principal Component and Cluster Analysis Derived from Morphological Traits

Cluster analysis based on morphological characters showed the formation of two main clusters (Figure 5A). Cluster I consisted of two accessions from cultivated samples,

AP4* and AO13*. While the other wild accessions (AP1, AP2, AP3, AP5, AP6, AP7, AP8, AP9, AP10, AO11, AO12, and AO14) were clustered in Cluster II. These two clusters are separated because of morphological differences in the size of quantitative characters, such as the length and width of the ventral and dorsal lobes. Saunders and Fowler (1992) demonstrated the use of these quantitative characters to observe morphological variation. Interestingly, two accessions from the Progo and Opak river basins ("AP" and "AO") merged into one cluster, and only AP4 and AO13 from cultivated ponds separated. Interestingly, two accessions from the Progo and Opak river basins ("AP" and "AO") merged into one cluster, and only AP4 and AO13 from cultivated ponds remained distinct. These results were confirmed with *Principal Component Analysis,* where AP4 and AO13 also separated from others (Figure 5B). In cultivated conditions, the use of organic fertilisers to *Azolla* supplies essential nutrients such as nitrogen (N) and phosphorus (P), which

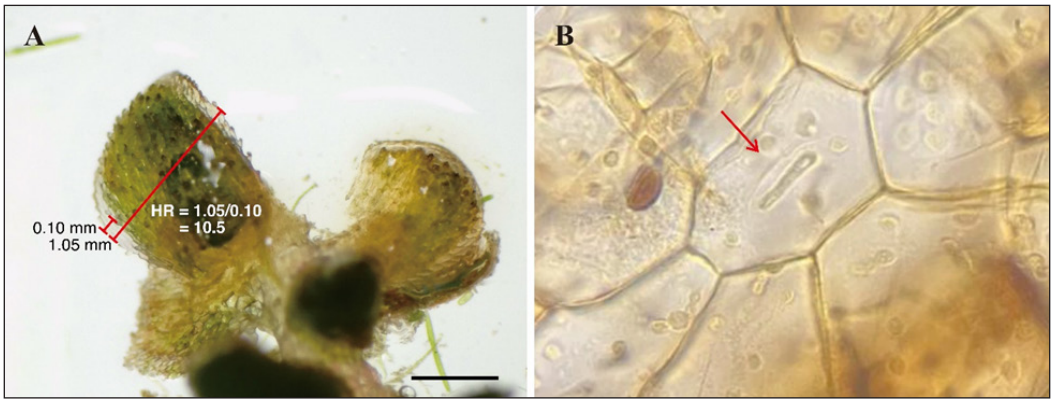



*Figure 4.* Ratio of hyalin layer (A) and presence of stomata (B)

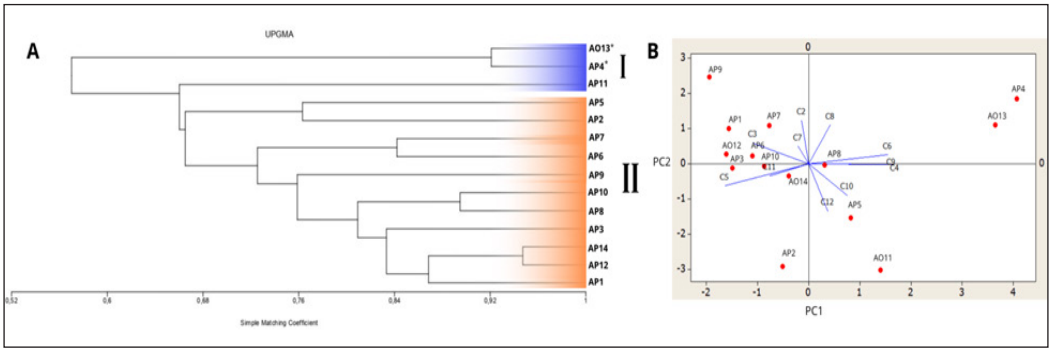



*Figure 5.* Unweighted pair group method dendrogram among 14 accessions of *Azolla* using morphological markers (A) and principal component analysis of *Azolla* accessions (B)

increase the nutrient content in the plant. This results in enhanced production and growth of *Azolla*, accompanied by an increase in protein content. Moreover, this could affect the geographical distribution, which is different from other wild types of *Azolla* (Azab & Soror, 2020). PCA successfully simplified 18 characters into 11 polymorphic characters, with the highest contributions being C2-branching shape (0.484), C4-branching number (0.459), C5-dorsal lobe shape (0.456), C7-ventral stomatal presence (0.488), C11-hyaline ratio (0.521), and C12-ventral lobe length (0.525) (Figure 5B).

**ISSR Analysis**

Primer optimisation was conducted by screening 20 ISSR primers synthesised by the University of British Columbia, and 10 of the most optimal primers were obtained. These primers succeed to amplifying 14 *Azolla* accessions, yielding 158 DNA bands and 135 polymorphic bands with sizes ranging from 101 to 1983 bp. The polymorphic band range is 73.3% to 94.12%, with the PIC value averaging 0.23, and the RP value being 18.53 (Table 4). The PCR-ISSR results indicated that the UBC 841 primer generated the highest level of polymorphism at 95.24%, while the UBC 834 primer produced the lowest at 73.3%. Yusuf et al. (2022) claimed that a polymorphic band percentage of 50% is considered high, indicating that the ten primers used in genetic variation analysis are effective. The PIC evaluates the quality and genetic information of primers; PIC values span from 0.0 to 0.5, since they serve as dominant markers. A greater PIC value indicates increased genetic diversity and greater primer effectiveness (Jamil et al., 2022). In addition to PIC, the resolving power (RP) parameter is utilised to evaluate the capacity of primers to distinguish between genotypes (Chesnokov & Artemyeva, 2015; Gilbert et al., 1999; Prevost & Wilkinson, 1999). The primers UBC 814 and UBC 841 are considered the most effective because of their high PR% (95.12; 94.24), RP (19.71; 23.29), and PIC (95.12; 94.24) values, respectively.

The ISSR-based dendrogram analysis classifies the *Azolla* collection into two primary clusters. Cluster I comprises only AO13, while AP1, AP2, AP3, AP4, AP5, AP6, AP7, AP8, AP9, AP10, AO11, AO12, and AO14 categorized inside cluster II (Figure 6). The dendrogram indicates that the two river basins are closely related, as there is no cluster separation based on their location. The only one that separated from the dendrogram was AO13, which came from the cultivated ponds and distinguished itself from cluster II by a similarity value of merely 38%. The highest level of similarity was observed between the accessions of AP6 and AP7 (93%), AP4 and AP5 (90%). According to Jaccard's coefficient, the greatest genetic distance was observed between AO13 and AO11 accessions with a value of 0.685 (Figure 7A). This was followed by genetic distance between AO13 and AO12, AO13 and AP3, AO13 and AP2, with values 0.627, 0.623, and 0.619, respectively.

Table 4
*Measured indices of ISSR primers used in* Azolla *accessions*

| Primer | NA | NP | PR (%) | FS | PIC | RP |
|---|---|---|---|---|---|---|
| UBC 814 | 17 | 16 | 94.12 | 410-1695 | 0.29 | 19.71 |
| UBC 815 | 11 | 10 | 90.90 | 493-1881 | 0.21 | 10.86 |
| UBC 826 | 13 | 11 | 84.61 | 282-1209 | 0.25 | 13.57 |
| UBC 834 | 15 | 11 | 73.30 | 101-1340 | 0.19 | 21.28 |
| UBC 841 | 21 | 20 | 95.24 | 235-1373 | 0.28 | 23.29 |
| UBC-845 | 15 | 13 | 86.67 | 305-1742 | 0.25 | 15.85 |
| UBC-856 | 17 | 13 | 76.47 | 236-1983 | 0.20 | 20.49 |
| UBC-860 | 15 | 13 | 86.67 | 221-1651 | 0.22 | 16.86 |
| UBC-873 | 17 | 14 | 82.35 | 262-1835 | 0.28 | 19.71 |
| UBC-880 | 17 | 14 | 82.35 | 255-1628 | 0.17 | 21 |
| Total | 158 | 135 | | | | |
| Mean | | 13.5 | 85.27 | | 0.23 | 18.53 |

*Note.* NA (number of scored bands); NP (number of polymorphic bands); PR (polymorphic rate); FS (fragment size); PIC (polymorphic information content); and RP (resolving power)

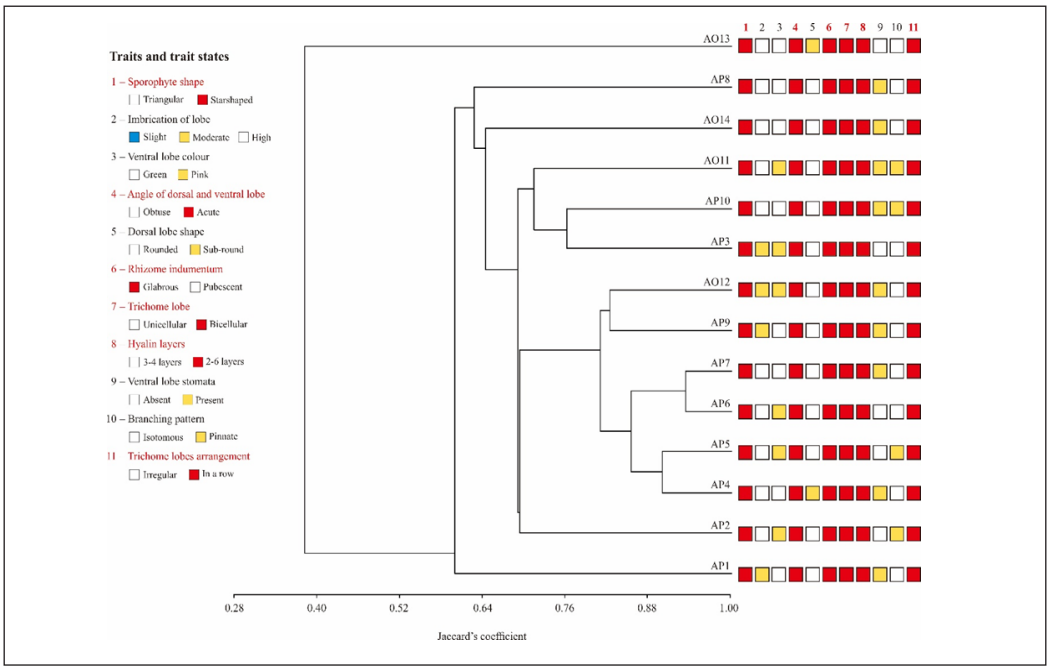
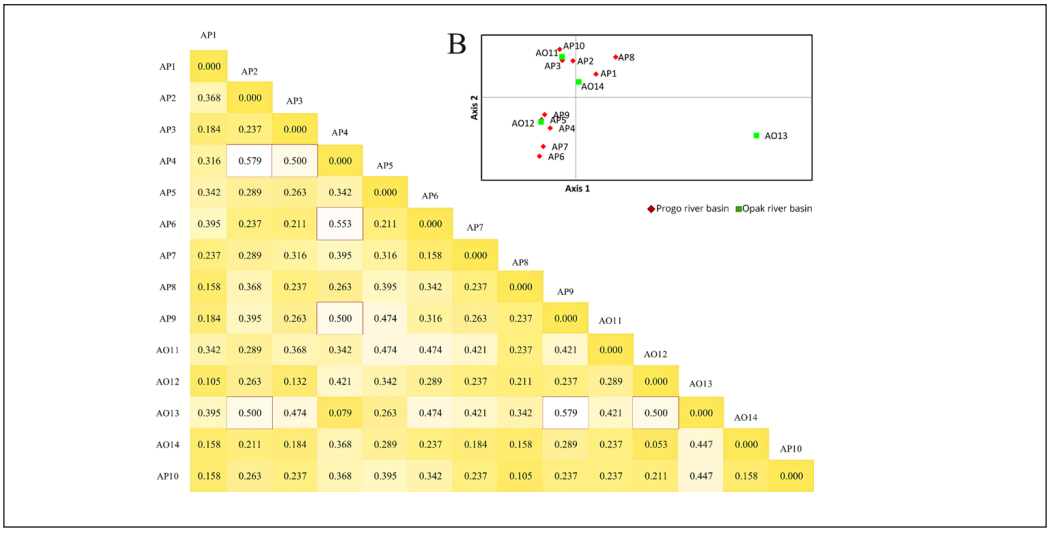



*Figure 6.* Molecular phenetic tree based on ISSR primers and relatedness to morphological traits. Trait states written in red are the key morphological characters in the populations




| | AP1 | AP2 | AP3 | AP4 | AP5 | AP6 | AP7 | AP8 | AP9 | AO11 | AO12 | AO13 | AO14 | AP10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AP1 | 0.000 | | | | | | | | | | | | | |
| AP2 | 0.368 | 0.000 | | | | | | | | | | | | |
| AP3 | 0.184 | 0.237 | 0.000 | | | | | | | | | | | |
| AP4 | 0.316 | 0.579 | 0.500 | 0.000 | | | | | | | | | | |
| AP5 | 0.342 | 0.289 | 0.263 | 0.342 | 0.000 | | | | | | | | | |
| AP6 | 0.395 | 0.237 | 0.211 | 0.553 | 0.211 | 0.000 | | | | | | | | |
| AP7 | 0.237 | 0.289 | 0.316 | 0.395 | 0.316 | 0.158 | 0.000 | | | | | | | |
| AP8 | 0.158 | 0.368 | 0.237 | 0.263 | 0.395 | 0.342 | 0.237 | 0.000 | | | | | | |
| AP9 | 0.184 | 0.395 | 0.263 | 0.500 | 0.474 | 0.316 | 0.263 | 0.237 | 0.000 | | | | | |
| AO11 | 0.342 | 0.289 | 0.368 | 0.342 | 0.474 | 0.474 | 0.421 | 0.237 | 0.421 | 0.000 | | | | |
| AO12 | 0.105 | 0.263 | 0.132 | 0.421 | 0.342 | 0.289 | 0.237 | 0.211 | 0.237 | 0.289 | 0.000 | | | |
| AO13 | 0.395 | 0.500 | 0.474 | 0.079 | 0.263 | 0.474 | 0.421 | 0.342 | 0.579 | 0.421 | 0.500 | 0.000 | | |
| AO14 | 0.158 | 0.211 | 0.184 | 0.368 | 0.289 | 0.237 | 0.184 | 0.158 | 0.289 | 0.237 | 0.053 | 0.447 | 0.000 | |
| AP10 | 0.158 | 0.263 | 0.237 | 0.368 | 0.395 | 0.342 | 0.237 | 0.105 | 0.237 | 0.237 | 0.211 | 0.447 | 0.158 | 0.000 |

*Figure 7.* Pairwise genetic distance (A) and principal coordinate analysis of 14 Azolla accessions produced by ISSR analysis (B)

Validation by PCoA further supports the genetic distinctions of AO13 compared to other clusters (Figure 7B). The differences of AO13 compared to other samples are supported by the numerous specific bands that appear in the accession across almost all primers, for example, UBC 841 at 1296 bp, 1109 bp, and 336 bp, and UBC 814 at 1132 bp and 817 bp (Figure 8).

AO13, which was morphologically identified as *A. cristata*, consistently clustered separately from other accessions in both morphological and molecular analyses. This distinction may be attributed to phenotypic variation arising from its origin in a cultivated pond environment. AO13 is noticeably different from other accessions because its TDS and EC values are much higher, specifically TDS at 351 mg/L and EC at 576 µS/cm, which are double those of other sample locations (Table 1). Total Dissolved Solids (TDS) indicate the concentration of inorganic and organic compounds dissolved in water, while Electrical Conductivity (EC) reflects the ability of water to conduct electricity due to the presence of these dissolved ions. The higher the TDS and EC values, the more dissolved substances, including nutrients, are present in the water. Despite elevated TDS and EC levels negatively impacting aquatic plants, *Azolla*, an aquatic fern, is often used as a phytoremediation agent, effectively absorbing various heavy metals. The water fern may thrive in nitrogen-deficient circumstances by assimilating atmospheric nitrogen, allowing it to develop without a dedicated nitrogen nutrient supply. As a result, it may thrive in stressful environments, such as polluted water bodies, and accumulate various substances in its vegetative sections (Arora et al., 2006; Umali et al., 2006). Moreover, studies from Kumar et al. (2020) showed

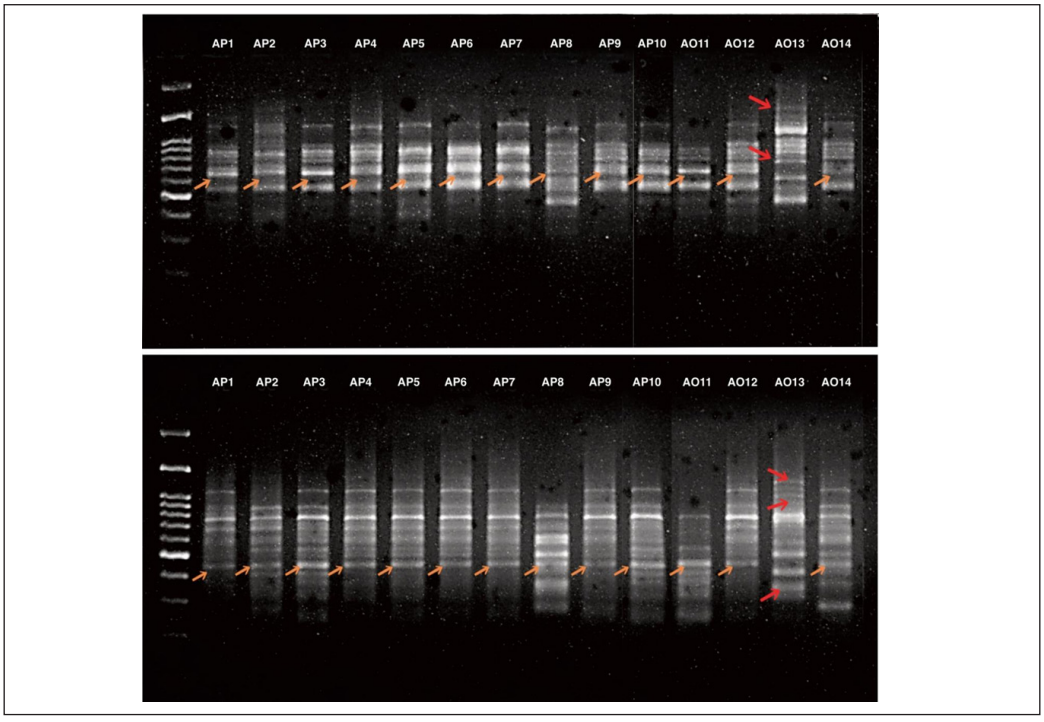



*Figure 8.* Gel electrophoresis profile of amplified products using primers UBC-814 (top) and UBC-841 (bottom). Red arrows indicate additional bands specific to accession AO13; while orange arrows show unique bands present in other accessions

that *Azolla* can survive in a high TDS environment with a range of 300-900 mg/L and an EC with a range of 500-1300 µS/cm, and even manages to reduce the value of pollutants by decreasing TDS and EC values periodically until day 28. AO13 was separated from other samples, possibly because the prolonged exposure to varying levels of organic and inorganic compounds, such as salts, heavy metals, and other dissolved ions, can lead to intraspecific genetic variation. This is supported by Gupta & Sarin (2009), who found that aquatic macrophytes, where environmental stressors, including nutrient loading, salinity, and heavy metal contamination, could create a change of genetic structure and variation, while analysing it using microsatellite molecular markers.

Through morphology, we agree with Pereira et al. (2011) and Kumar et al. (2021) that some characters could differ between sections *Azolla* and *Rhizosperma* through imbrication, sporophyte shape, rhizome indumentum, angle of lobe, and hyaline layers. In the present study, we confirm that bicellular trichomes, the regular (in-a-row) arrangement of trichomes on the dorsal lobe, and the length of the dorsal lobe could distinguish *A. mexicana* and *A. microphylla* from other species in the *Azolla* section (Goff, 2011; Pereira et al., 2011; Saunders & Fowler, 1992). Initially, we assumed that *A. mexicana* possessed a ventral stomatal lobe while *A. microphylla* lacked one (Chang et al., 2020; Pereira et al., 2011).

However, the ISSR data disclosed an unexpected finding (Figure 6). Accessions that were absent of ventral stomata (AP 2, AP3, AP5, and AP6) joined into one cluster with accessions with the presence of ventral stomata, indicating that the presence of stomata is insufficient for identifying species of *Azolla.* This final result also confirms the findings of Evrard & Van Hove (2004) about detailed microscopic evidence from various cultures and specimens of *Azolla*, showing that *A. microphylla* and *A. mexicana* are morphologically indistinguishable and should be treated as a single species. Previous studies about *Azolla* molecular taxonomy from Madeira et al. (2013), Metzgar et al. (2007), and Reid et al. (2006) supported this theory that *A. microphylla* and *A. mexicana*, based on established nomenclature precedent, are now referred to as *A. cristata* Kaulf.

**Environmental Factors**

The previous result and discussion suggested that specific environmental factors, like high levels of electrical conductivity (EC) and total dissolved solids (TDS), can affect the quantitative lobe size and intraspecific variation of *Azolla* based on morphological and molecular characterisations (Azab & Soror, 2020; Gupta & Sarin, 2009; Saunders & Fowler, 1992). Moreover, low nutrient content (shown by low TDS and EC) and direct light exposure may have an impact on the colour of *Azolla* leaves (Da Silva et al., 2022; Kumar et al., 2020; Sadeghi et al., 2013).

Moreover, this study discovered that elevation may have an impact on *Azolla's* abundance, even though it did not seem to have an impact on its morphological or molecular characteristics. Only one *Azolla* sample was found at each of the two sampling locations with the highest elevations, AP1 and AO11, which were 648 m and 556 m above sea level (a.s.l.), respectively. These sites were also quite far from the closest *Azolla* population (Figure 1). On the other hand, *Azolla* abundance increased in sites that were geographically close at lower elevations. For example, the samples of AP7, AP8, AP9, and AP10, which are located near one another, were all classified as low-altitude areas with elevations ranging from only 70 to 90 meters a.s.l (Figure 1). The study by Lydia et al. (2023) supports this conclusion by indicating that high altitude adversely affected the dispersal of *Azolla*. Reduced water levels could be the cause of this phenomenon, as *Azolla* thrives on stagnant or relatively slow-moving water. This study examines high-altitude zones (AP1 and AO11) located in mountainous regions, which have rapid downhill water flow. These hydrological conditions possibly limit the growth and survival of *Azolla* in these areas.

Based on the environmental data (Table 1), water temperature ranged from 26.57 °C to 37.97 °C. The suitable water temperature range for *Azolla* survival is approximately 27-35 °C, with the optimal condition around 28 °C (Korsa et al., 2024). The humidity levels at the *Azolla* sampling sites in this study were also supportive of its growth, ranging from 52.7% to 79%. Sadeghi et al. (2013) further supported that the average relative humidity suitable for the growth of *Azolla* spp. ranges around 55% - 83%.

### *Genetic Diversity*

Despite the small sample size, the samples can represent the genetic variation in the two river basins because they were collected to capture the upstream-to-downstream area where *Azolla* was present at the time of sampling. A study by Najibulloh et al. (2025) also used comparable population genetic studies of *Azolla* in the Special Region of Yogyakarta and examined genetic variation and population structure using 11 samples from wild and developed habitats. This previous study successfully identified haplotype patterns and genetic divergence within the region using *trnL-trnF* molecular markers. This finding indicates that, despite limited sampling due to the dry peak season in Indonesia (June-November 2024), molecular markers can effectively detect genetic variation in *Azolla*. Reitsema et al. (2020) and Rivaes et al. (2022) stated that aquatic macrophytes depend totally on their habitat's water supply and are clearly vulnerable to hydrological changes caused by global climate change. Diminished precipitation and reduced water availability in habitats negatively impact the living conditions of macrophytes; conversely, a wetter climate may expand their potential habitats. In river ecosystems, changes in flow patterns have a big impact on macrophytes. Fernández-Zamudio et al. (2010) found that spores of the invasive *A. filiculoides* in a Mediterranean temporary wetland had lower but still significant germination rates after being dried out compared to controls that were always wet. This shows that some spores can survive dry periods and grow new plants when water returns.

The genetic diversity analysis revealed a moderate level of genetic variation within both *Azolla* populations, as indicated by the expected Nei's gene diversity (He = 0.230). The Opak population exhibited higher values of observed alleles (Na = 1.627), effective alleles (Ne = 1.470), Nei's gene diversity (He = 0.266), Shannon's information index (I = 0.392), and percentage of polymorphic bands (PBB = 69.62%), indicating that Opak has higher genetic diversity than the Progo River basin (Table 5). Results from the AMOVA indicated that the majority of genetic variation was distributed within populations (93%) rather than among populations (7%) (Table 6). However, the PhiPT value of 0.067 was not statistically significant ($P > 0.05$), indicating that no statistically significant genetic differentiation was detected between *Azolla* populations from the Progo and Opak river basins. The higher genetic diversity observed in the Opak river basin compared to the Progo river basin is primarily attributed to the genetic difference of sample AO13 compared to other samples from the Opak river basin, which increased the overall variation within the Opak population. This is consistent with clustering analysis that showed the most accessions from both river basins grouped together, except AO13 genetically distinct. However, this differentiation occurred at the individual level and did not result in significant population-level genetic structuring. According to Peakall and Smouse (2012), PhiPT values lower than 0.05 generally indicate negligible or weak genetic differentiation, while higher values reflect stronger population structure. In this study, the PhiPT value of 0.067 with a non-significant

permutation test (P = 0.108 > 0.05) suggests non-significant genetic differentiation between the two river populations. In this study, the PhiPT value of 0.067 with a non-significant permutation test (P = 0.108 > 0.05) suggests that *Azolla* populations from both river systems do not exhibit distinct genetic structure and can be considered part of a single panmictic population. This pattern suggests extensive gene flow or shared ancestry between accessions from the two river basins. This finding is consistent with Al Salameen et al. (2020), who reported that low PhiPT values indicate populations that are not yet genetically structured into distinct groups. Therefore, the ISSR-based results imply high gene flow or shared ancestry among *Azolla* accessions from both rivers, supporting the idea that environmental and geographic factors may influence local genotypic variation (Ahmad et al., 2019).

The high percentage of genetic variation within the population (93%) and the lack of significant population structure are ecologically expected for aquatic plants that possess strong dispersal abilities. *Azolla* is a member of the Salviniaceae family that uses spores as a propagule. According to Reynolds et al. (2015), a propagule is a structure that serves as an agent for reproduction and propagation, including seeds, eggs, spores, statoblasts, and cysts. The propagule found in water ferns, such as *Azolla*, may serve as a potential vector for passive dispersal through various means, including water currents (hydrochory),

Table 5
*Genetic variability estimated among two populations of* Azolla

| Population | Na | Ne | I | He | PBB (%) |
|---|---|---|---|---|---|
| Progo River Basin | 1.475 ± 0.056 | 1.335 ± 0.031 | 0.292 ± 0.023 | 0.194 ± 0.016 | 59.49 |
| Opak River Basin | 1.627 ± 0.049 | 1.470 ± 0.032 | 0.392 ± 0.022 | 0.266 ± 0.016 | 69.62 |
| Total | 1.551 ± 0.037 | 1.403 ± 0,022 | 0.342 ± 0,016 | 0.230 ± 0,016 | 64.56 |

*Note.* Na (number of alleles); Ne (number of effective alleles); I (Shannon's diversity index); He (Nei's gene diversity); and PBB (polymorphic loci)

Table 6
*Analysis of molecular variance (AMOVA) results from 14* Azolla *accessions*

| ISSR | df | SS | MS | Est. Var. | Value % | PhiPt | P value |
|---|---|---|---|---|---|---|---|
| Among populations | 1 | 27.664 | 27.664 | 1.413 | 7 | 0.067 | > 0.05 |
| Within populations | 12 | 235.05 | 19.588 | 19.588 | 93 | | |

*Note.* df: degree of freedom; SS: sum of squared observations; Est.Var: estimated variance; Value%: percentage of variation; PhiPt: proportion of total genetic variance distributed among populations. P (rand ≥ data): probability based on a permutation test indicating the statistical significance of PhiPT

animals (zoochory), wind (anemochory), and human activities. Such dispersal mechanisms can facilitate the spread of these ferns to new habitats and enhance their ecological success (Casper et al., 2012; Trakhtenbrot et al., 2005), which has potential consequences for gene flow across regions (Ronce, 2007). Previous research has found that *Azolla* sp. fossils are often found in freshwater coastal environments (lagoon or similar) and wetland environments across a wide geographical range. Climate conditions and water flow (hydrochory) facilitate the easy transportation of *Azolla* propagules. Some data showed that *Azolla* fossils found still have microspore massulae attached to the megaspores (De Benedetti et al., 2018). Besides hydrochory, research by Coughlan et al. (2016) showed that *Azolla* was seen to be spread ex-situ with high frequency by mallard ducks (*Anas platyrhynchos*). Bird-mediated dispersal or epizoochory often occurs and significantly contributes to the colonisation, range expansion, and biological invasion. Empirical evidence shows similar mechanisms: waterbirds have been documented dispersing aquatic plant propagules between habitats (Figuerola & Green, 2002; Jones et al., 2020). In contrast, wind had no effect on *Azolla* dispersal (Coughlan et al., 2016).

The Progo and Opak river basins are geographically near and connected hydrologically, offering no significant ecological barrier between them. Engloner et al. (2024) stated that the genetic connectivity between macrophytes increases significantly in canal-connected areas. No significant barrier implies the absence of artificial barriers such as dams that might restrict macrophyte spread and endanger its distribution and persistence (Jones et al., 2020). Nazareno et al. (2021) also supports these statements that the ability of propagules to travel through water likely promotes high genetic connectivity among aquatic plant populations, with limited geographical barriers on their distribution. Besides passive dispersal, *Azolla* is deliberately transferred by humans (anthropogenic), especially for agricultural purposes. *Azolla* is commonly utilised for biofertiliser, compost, livestock feed, and mulch. This usage results in *Azolla* being repeatedly harvested, transported, and washed, which creates numerous opportunities for fragments to reach canals, drainage ditches, or temporary ponds near fields (Korsa et al., 2024; Thepsilvisut et al., 2024).

## CONCLUSION

The recent study identified that the *Azolla* accessions in the Progo and Opak river basins are *A. cristata*, which is identical with the conspecific species *A. microphylla* and *A. mexicana,* based on their morphological traits. The use of ISSR DNA fingerprinting markers successfully aided in the patterns of genetic similarity and diversity that support morphological distinctions (Animasaun et al., 2018). The high PIC and RP values seen from the ISSR primers show that they work effectively for *Azolla*. Due to the multidirectional distribution of *Azolla* as a water plant, the genetic diversity remains at a moderate level with low and non-significant genetic differentiation among populations in the Progo and

Opak river basins, suggesting that these populations form one panmictic (randomly mating) population. This pattern is consistent with the aquatic nature of *Azolla* and its potential for gene flow through hydrological connectivity, passive dispersal mechanisms, and human-mediated (anthropogenic) means. This study suggests that further research using additional molecular markers or integrative taxonomic approaches is needed to confirm species boundaries within *Azolla* populations and to clarify population connectivity. This will support sustainable long-term conservation strategies.

## ACKNOWLEDGEMENT

This research was financed by the Indonesian Ministry of Education, Culture, Research, and Technology under the Pendidikan Magister Menuju Doktor untuk Sarjana Unggul (PMDSU) program, under contract numbers 048/E5/PG.02.00.PL/2024 and 2784/UN1/DITLIT/PT.01.03/2024.

## REFERENCES

Ahad, B., Reshi, Z. A., Ganaie, A. H., & Yousuf, A. R. (2012). *Azolla cristata* in the Kashmir Himalaya. *American Fern Journal*, *102*(3), 224-227. https://doi.org/10.1640/0002-8444-102.3.224

Ahmad, R., Malik, W., & Anjum, M. A. (2019). Genetic diversity and selection of suitable molecular markers for characterisation of indigenous *Zizyphus* germplasm. *Erwerbs-Obstbau*, *61*(4), 345-353. https://doi.org/10.1007/s10341-019-00438-0

Al Salameen, F., Habibi, N., Al Amad, S., Kumar, V., Dashti, J., Talebi, L., & Al Doaij, B. (2020). Genetic diversity analysis of *Rhanterium eppaposum* Oliv. by ISSRs reveals a weak population structure. *Current Plant Biology*, *21*, Article 100138. https://doi.org/10.1016/j.cpb.2020.100138

Alaaddin Ahmed, A., Anwar Qadir, S., & Tahir, N. A.-R. (2022). CDDP and ISSR markers-assisted diversity and structure analysis in Iraqi Mazu ( *Quercus infectoria* Oliv.) accessions. *All Life*, *15*(1), 247-261. https://doi.org/10.1080/26895293.2022.2042401

Amare, E., Kebede, F., & Mulat, W. (2018). Wastewater treatment by *Lemna minor* and *Azolla filiculoides* in tropical semi-arid regions of Ethiopia. *Ecological Engineering*, *120*, 464-473. https://doi.org/10.1016/j.ecoleng.2018.07.005

Animasaun, D. A., Oyedeji, S., Ingalhalli, R. S., & Krishnamurthy, R. (2018). Polymorphism and genetic diversity assessment of some ornamental ferns by microsatellite (ISSR) markers. *Journal of Applied Horticulture*, *20*(03), 237-241. https://doi.org/10.37855/jah.2018.v20i03.41

Arora, A., Saxena, S., & Sharma, D. K. (2006). Tolerance and phytoaccumulation of chromium by three *Azolla* species. *World Journal of Microbiology and Biotechnology*, *22*(2), 97-100. https://doi.org/10.1007/s11274-005-9000-9

Azab, E., & Soror, A. S. (2020). Physiological behaviour of the aquatic plant *Azolla* sp. in response to organic and inorganic fertilisers. *Plants*, *9*(7), Article 924. https://doi.org/10.3390/plants9070924

Bocchi, S., & Malgioglio, A. (2010). *Azolla-anabaena* as a biofertiliser for rice paddy fields in the Po Valley, a temperate rice area in Northern Italy. *International Journal of Agronomy*, *2010*, 1-5. https://doi.org/10.1155/2010/152158

Brouwer, P., Schluepmann, H., Nierop, K. G., Elderson, J., Bijl, P. K., Van Der Meer, I., De Visser, W., Reichart, G., Smeekens, S., & Van Der Werf, A. (2018). Growing *Azolla* to produce sustainable protein feed: The effect of differing species and $CO_2$ concentrations on biomass productivity and chemical composition. *Journal of the Science of Food and Agriculture*, *98*(12), 4759-4768. https://doi.org/10.1002/jsfa.9016

Busby, C. H., & Gunning, B. E. S. (1984). Microtubules and morphogenesis in stomata of the water fern *Azolla*: An unusual mode of guard cell and pore development. *Protoplasma*, *122*(1-2), 108-119. https://doi.org/10.1007/BF01279443

Cannavò, S., Bertoldi, A., Valeri, M. C., Damiani, F., Reale, L., Brilli, F., & Paolocci, F. (2023). Impact of high light intensity and low temperature on the growth and phenylpropanoid profile of *Azolla filiculoides*. *International Journal of Molecular Sciences*, *24*(10), Article 8554. https://doi.org/10.3390/ijms24108554

Caruso, D., Pouil, S., Samsudin, R., Sihabuddin, A., & Slembrouck, J. (2023). Toward ecological intensification through the co-production of the floating macrophyte *Azolla filiculoides* and the giant gourami *Osphronemus goramy* Lacepède (1801) in Indonesian ponds. *Journal of Applied Aquaculture*, *35*(1), 27-44. https://doi.org/10.1080/10454438.2021.1932667

Casper, H. A., van Leeuwen, van der Velde, G., van Groenendael, J. M., & Klaassen, M. (2012). Gut travellers: Internal dispersal of aquatic organisms by waterfowl. *Journal of Biogeography* 39*(11),* 2031-2040. https://doi.org/10.1111/jbi.12004

Chang, M.-C, Huang, C.-T, Tsai, C.-C, & Kao, W.-Y. (2020). Molecular identification and morphological traits of the native and exotic *Azolla* species in Taiwan. *Taiwania*, *65*(3), 382-390. https://doi.org/10.6165/tai.2020.65.382

Chesnokov, Y. V., & Artemyeva, A. M. (2015). Evaluation of the measure of polymorphism. *Sel'skokhozyaistvennaya Biologiya*, *50*(5), 571-578. https://doi.org/10.15389/agrobiology.2015.5.571eng

Coughlan, N. E., Kelly, T. C., & Jansen, M. A. K. (2016). "Step by step": High frequency short-distance epizoochorous dispersal of aquatic macrophytes. *Biological Invasions*, *19*(2), 625-634. https://doi.org/10.1007/s10530-016-1293-0

Da Silva, M. E. J., Mathe, L. O. J., Van Rooyen, I. L., Brink, H. G., & Nicol, W. (2022). Optimal growth conditions for *Azolla pinnata* R. Brown: Impacts of light intensity, nitrogen addition, pH control, and humidity. *Plants*, *11*(8), Article 1048. https://doi.org/10.3390/plants11081048

De Benedetti, F., Zamaloa, M. D. C., Gandolfo, M. A., & Cúneo, N. R. (2018). Heterosporous ferns from Patagonia: The case of *Azolla*. In *Transformative Paleobotany* (pp. 361-373). Academic Press. https://doi.org/10.1016/B978-0-12-813012-4.00015-2

Dong, Y.-H., Chen, J.-M., Gituru, R. W., & Wang, Q.-F. (2007). Gene flow in populations of the endangered aquatic fern *Ceratopteris pteridoides* in China as revealed by ISSR markers. *Aquatic Botany*, *87*(1), 69-74. https://doi.org/10.1016/j.aquabot.2007.03.006

Dunham, D. G. & Fowler, K. (1987). Taxonomy and species recognition in *Azolla* Lam. In International Rice Research Institute (Ed.), *Azolla utilization* (pp. 7-16). International Rice Research Institute.

Engloner, A. I., Németh, K., & Bereczki, J. (2024). The genetic diversity of the macrophyte *Ceratophyllum demersum* in backwaters reflects differences in the hydrological connectivity and water flow rate of habitats. *Plants*, *13*(16), Article 2220. https://doi.org/10.3390/plants13162220

Evrard, C., & Van Hove, C. (2004). Taxonomy of the American *Azolla* species (Azollaceae): A Critical Review, *74*(2), 301-318. http://www.jstor.org/stable/3668500

Fernández-Zamudio, R., García-Murillo, P., & Cirujano, S. (2010). Germination characteristics and sporeling success of *Azolla filiculoides* Lamarck, an aquatic invasive fern, in a Mediterranean temporary wetland. *Aquatic Botany*, *93*(2), 89-92. https://doi.org/10.1016/j.aquabot.2010.03.011

Figuerola, J., & Green, A. J. (2002). Dispersal of aquatic organisms by waterbirds: A review of past research and priorities for future studies. *Freshwater Biology*, *47*(3), 483-494. https://doi.org/10.1046/j.1365-2427.2002.00829.x

Freeland, J. R., Kirk, H., & Petersen, S. (2011). Molecular Ecology (1st ed.). Wiley-Blackwell. https://doi.org/10.1002/9780470979365

Gilbert, J. E., Lewis, R. V., Wilkinson, M. J., & Caligari, P. D. S. (1999). Developing an appropriate strategy to assess genetic variability in plant germplasm collections: *Theoretical and Applied Genetics*, *98*(6-7), 1125-1131. https://doi.org/10.1007/s001220051176

Gleason, H. A., & Cronquist, A. (1991). *Manual of vascular plants of northeastern United States and adjacent Canada* (2nd ed.). New York Botanical Garden. https://doi.org/10.21135/893273651.001

Goff, L. J. (2011). *Algal symbiosis: A continuum of interaction strategies*. Cambridge University Press.

Gupta, M., & Sarin, N. B. (2009). Heavy metal induced DNA changes in aquatic macrophytes: Random amplified polymorphic DNA analysis and identification of sequence characterised amplified region marker. *Journal of Environmental Sciences*, *21*(5), 686-690. https://doi.org/10.1016/S1001-0742(08)62324-4

Hadipour, M., Kazemitabar, S. K., Yaghini, H., & Dayani, S. (2020). Genetic diversity and species differentiation of medicinal plant Persian Poppy (*Papaver bracteatum* L.) using AFLP and ISSR markers. *Ecological Genetics and Genomics*, 16, Article 100058. https://doi.org/10.1016/j.egg.2020.100058

Hamdan, H. Z., & Houri, A. F. (2022). $CO_2$ sequestration by propagation of the fast-growing *Azolla* spp. *Environmental Science and Pollution Research*, *29*(12), 16912-16924. https://doi.org/10.1007/s11356-021-16986-6

Janes, R. (1998). Growth and survival of *Azolla filiculoides* in Britain I. Vegetative production. *New Phytologist*, *138*(2), 367-375. https://doi.org/10.1046/j.1469-8137.1998.00114.x

Jama, A., Widiastuti, D., Gafur, S., & Davis, J. (2023). *Azolla* biofertiliser is an effective replacement for urea fertiliser in vegetable crops. *Sustainability*. https://doi.org/10.3390/su15076045

Jamil, A., Razzaq, K., Rajwana, I. A., Naz, A., Akhtar, G., Ullah, S., Faried, H. N., Hussain, S. B., Li, Y., Amin, M., Sher, M. A., Altaf, M. T., Hassan, H., Ahmed, M. A. A., Alfarraj, S., & Ansari, M. J. (2022). Characterisation of indigenous phalsa (*Grewia subinequalis*) genotypes using morphological traits and ISSR markers. *Journal of King Saud University - Science*, 34(7), Article 102237. https://doi.org/10.1016/j.jksus.2022.102237

Jones, P. E., Consuegra, S., Börger, L., Jones, J., & Garcia De Leaniz, C. (2020). Impacts of artificial barriers on the connectivity and dispersal of vascular macrophytes in rivers: A critical review. *Freshwater Biology*, *65*(6), 1165-1180. https://doi.org/10.1111/fwb.13493

Kadir, A. A., Abdullah, S. R. S., Othman, B. A., Hasan, H. A., Othman, A. R., Imron, M. F., Ismail, N. I., & Kurniawan, S. B. (2020). Dual function of *Lemna minor* and *Azolla pinnata* as phytoremediator for Palm oil mill effluent and as feedstock. *Chemosphere*, *259*, Article 127468. https://doi.org/10.1016/j.chemosphere.2020.127468

Korsa, G., Alemu, D., & Ayele, A. (2024). *Azolla* plant production and their potential applications. *International Journal of Agronomy*, *2024*, 1-12. https://doi.org/10.1155/2024/1716440

Kösesakal, T. (2014). Effects of seasonal changes on pigment composition of *Azolla filiculoides* Lam. *American Fern Journal*, *104*(2), 58-66. https://doi.org/10.1640/0002-8444-104.2.58

Kumar, A., Mishra, P., Singh, S. C., & Sundaresan, V. (2014). Efficiency of ISSR and RAPD markers in genetic divergence analysis and conservation management of *Justicia adhatoda* L., a medicinal plant. *Plant Systematics and Evolution*, *300*(6), 1409-1420. https://doi.org/10.1007/s00606-013-0970-z

Kumar, U., Rout, S., Kaviraj, M., Swain, P., & Nayak, A. K. (2021). Uncovering morphological and physiological markers to distinguish *Azolla* strains. *Brazilian Journal of Botany*, *44*(3), 697-713. https://doi.org/10.1007/s40415-021-00725-9

Kumar, V., Kumar, P., Singh, J., & Kumar, P. (2020). Potential of water fern (*Azolla pinnata* R.Br.) in phytoremediation of integrated industrial effluent of SIIDCUL, Haridwar, India: Removal of physicochemical and heavy metal pollutants. *International Journal of Phytoremediation*, *22*(4), 392-403. https://doi.org/10.1080/15226514.2019.1667950

Lestari, S., Roeswitawati, D., Setyobudi, R., Soni, P., Syafrani, S., Maftuchah, M., Suhesti, E., Putri, A., Savitri, E., Basyaruddin, M., & Pakarti, T. (2024). *Azolla* for water and land phytoremediation against heavy metals: a mini review. *BIO Web of Conferences, 104,* Article 00042. https://doi.org/10.1051/bioconf/202410400042

Lumpkin, T. A., & Plucknett, D. L. (1980). *Azolla*: Botany, physiology, and use as a green manure. *Economic Botany*, *34*(2), 111-153. https://doi.org/10.1007/BF02858627

Lydia, N., Basamba, T. A., Nyakoojo, C., Mustafa, A. S., Saidi, N., Mutumba, G. M., & Ssenku, J. E. (2023). Ecological distribution and genetic diversity of *Azolla* in Uganda. *BMC Plant Biology*, *23*(1), Article 131. https://doi.org/10.1186/s12870-023-04146-6

Madeira, P. T., Centre, T. D., Coetzee, J. A., Pemberton, R. W., Purcell, M. F., & Hill, M. P. (2013). Identity and origins of introduced and native *Azolla* species in Florida. *Aquatic Botany*, *111*, 9-15. https://doi.org/10.1016/j.aquabot.2013.07.009

Madeira, P. T., Hill, M. P., Dray Jr, F. A., Coetzee, J. A., Paterson, I. D., & Tipping, P. W. (2016). Molecular identification of *Azolla* invasions in Africa: The *Azolla* specialist, *Stenopelmus rufinasus* proves to be an excellent taxonomist. *South African Journal of Botany, 105,* 299-305. https://doi.org/10.1016/j.sajb.2016.03.007

Madeira, P. T., Dray Jr, F. A., & Tipping, P. W. (2019). Molecular identification of *Azolla* in the Yangtze River watershed, China. *Aquatic Botany, 159,* Article 103149. https://doi.org/10.1016/j.aquabot.2019.103149

Mantang, W., Mantiri, F. R., & Kolondam, B. J. (2018). Identifikasi tumbuhan paku air (*Azolla* sp.) secara morfologi dan molekuler dengan menggunakan gen *rbcL* [Identification of water ferns (*Azolla* spp.) based on morphological traits and the *rbcL* gene marker]. *Jurnal Bios Logos,* 8(2), 38-44. https://doi.org/10.35799/jbl.8.2.2018.21445

Meiriani, & Tarigan, D. (2024). Utilisation *Azolla* liquid organic fertiliser with goat manure to enhance organic okra (*Abelmoschus esculentus* L. Moench) production. *IOP Conference Series: Earth and Environmental Science*, *1302,* Article 012030. https://doi.org/10.1088/1755-1315/1302/1/012030

Metzgar, J. S., Schneider, H., & Pryer, K. M. (2007). Phylogeny and divergence time estimates for the fern genus *Azolla* (Salviniaceae). *International Journal of Plant Sciences*, *168*(7), 1045-1053. https://doi.org/10.1086/519007

Najibulloh, A. S. I., Yusuf, A. F., Chasani, A. R., & Daryono, B. S. (2025). Genetic diversity and population structure of *Azolla* inferred by trnL-trnF intergenic region and morphological data in Yogyakarta, Indonesia. *Asia-Pacific Journal of Molecular Biology and Biotechnology, 33*(3), 66-77. https://doi.org/10.35118/apjmbb.2025.033.3.08

Nasir, N. A. N. M., Zakarya I.A., Kamaruddin S.A., Islam A.K.M.A. (2022a) Advances and future prospects on biotechnological approaches towards *Azolla* for environmental sustainability. Pertanika *Journal Tropical Agricultural Science 45*(3), 595-609. https://doi.org/10.47836/pjtas.45.3.04

Nasir, N. A. N. M., Kamaruddin S.A., Zakarya I.A., Islam A.K.M.A. (2022b) Sustainable alternative animal feeds: recent advances and future perspective of using *Azolla* as animal feed in livestock, poultry, and fish nutrition. *Sustain Chem Pharm, 25*, Article 100581. https://doi.org/10.1016/j.scp.2021.100581

Nazareno, A. G., Knowles, L. L., Dick, C. W., & Lohmann, L. G. (2021). By animal, water, or wind: can dispersal mode predict genetic connectivity in riverine plant species? *Frontiers in Plant Science, 12*, Article 626405. https://doi.org/10.3389/fpls.2021.626405

Peakall, R., & Smouse, P. E. (2012). GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics*, *28*(19), 2537-2539. https://doi.org/10.1093/bioinformatics/bts460

Pereira, A. L., Martins, M., Oliveira, M. M., & Carrapiço, F. (2011). Morphological and genetic diversity of the family *Azolla*ceae inferred from vegetative characters and RAPD markers. *Plant Systematics and Evolution*, *297*(3-4), 213-226. https://doi.org/10.1007/s00606-011-0509-0

Pereira, A. L., Teixeira, G., Sevinate-Pinto, I., Antunes, T., & Carrapiço, F. (2001). Taxonomic re-evaluation of the *Azolla* genus in Portugal. *Plant Biosystems 135*(3), 285-294. https://doi.org/10.1080/11263500112331350920

Perkins, S. K., Peters, G. A., Lumpkin, T. A., & Calvert, H. E. (1985). Scanning electron microscopy of perine architecture as a taxonomic tool in the genus *Azolla* Lamarck. *Scanning Electron Microscopy, 4*, 1719-1734.

Prevost, A., & Wilkinson, M. J. (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars: *Theoretical and Applied Genetics*, *98*(1), 107-112. https://doi.org/10.1007/s001220051046

Rebai, A. (2023). Sampling in populations for whole genome sequencing: How to capture diversity while ensuring representativeness? *International Journal of Modern Anthropology*, *2*(19), 1045-1049. https://doi.org/10.4314/ijma.v2i19.1

Reid, J. D., Plunkett, G. M., & Peters, G. A. (2006). Phylogenetic relationships in the heterosporous fern genus *Azolla* (*Azolla*ceae) based on DNA sequence data from three noncoding regions. *International Journal of Plant Sciences*, *167*(3), 529-538. https://doi.org/10.1086/501071

Reitsema, R. E., Wolters, J.-W., Preiner, S., Meire, P., Hein, T., De Boeck, G., Blust, R., & Schoelynck. J. (2020). Response of submerged macrophyte growth, morphology, chlorophyll content and nutrient stoichiometry to increased flow velocity and elevated $CO_2$ and dissolved organic carbon concentrations. *Frontiers in Environmental Science, 11,* Article 527801. https://doi.org/10.3389/fenvs.2020.527801

Reynolds, C, Miranda, N.A.F., Cumming, G.S. (2015). The role of waterbirds in the dispersal of aquatic alien and invasive species. *Diversity and Distributions*, *21*(7), 744-754. https://doi.org/10.1111/ddi.12334

Rivaes, R. P., Feio, M. J., Almeida, S. F. P., Calapez, A. R., Sales, M., Gebler, D., Lozanovska, I., & Aguiar, F. C. (2022). River ecosystem endangerment from climate change-driven regulated flow regimes. *Science of The Total Environment, 818,* Article 151857. https://doi.org/10.1016/j.scitotenv.2021.151857

Roldán-Ruiz, I., Dendauw, J., Van Bockstaele, E., Depicker, A., & De Loose, M. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). *Molecular Breeding*, *6*(2), 125-134. https://doi.org/10.1023/A:1009680614564

Ronce, O. (2007) How does it feel to be like a rolling stone? Ten questions about dispersal evolution. *Annual Review of Ecology, Evolution, and Systematics, 38,* 231-253. https://doi.org/10.1146/annurev.ecolsys.38.091206.095611

Sadeghi, R., Zarkami, R., Sabetraftar, K., & Damme, P. V. (2013). A review of some ecological factors affecting the growth of *Azolla* spp. *Caspian Journal of Environmental Sciences (CJES)*, *11*(1), 65-76. http://hdl.handle.net/1834/10480

Sarah, D. P., & Ratnam, K. (2020). *Azolla* as lowcost supplement and live stock feed for Ompok Pabda. *International Journal of Research and Review, 1*(8), 63-66.

Saunders, R. M. K., & Fowler, K. (1992). A morphological taxonomic revision of *Azolla* Lam. section *Rhizosperma* (Mey.) Mett. (*Azolla*ceae). *Botanical Journal of the Linnean Society*, *109*(3), 329-357. https://doi.org/10.1111/j.1095-8339.1992.tb00277.x

Susilo, A., Utami, I., & Suryanto, A. (2024). Cultivation of *Azolla microphylla* as an Alternative feed for goats in Kalikesur Village, Kedungbanteng District, Banyumas Regency. *JAKADIMAS (Jurnal Karya Pengabdian Masyarakat), 2*(1), Article 10101. https://doi.org/10.33061/jakadimas.v2i1.10101

Svenson, H. K. (1944). The new world species of *Azolla*. *American Fern Journal*, *34*(3), 69-84. https://doi.org/10.2307/1545228

Thepsilvisut, O., Srikan, N., Chutimanukul, P., Marubodee, R., & Ehara, H. (2024). developing guidelines for *Azolla microphylla* production as compost for sustainable agriculture. *Resources, 13*(11), Article 158. https://doi.org/10.3390/resources13110158

Trakhtenbrot, A., Nathan R., Perry G., Richardson D.M. (2005). The importance of long-distance dispersal in biodiversity conservation. *Diversity and Distributions, 11*(2)*,* 173-181. https://doi.org/10.1111/j.1366-9516.2005.00156.x

Umali, L. J., Duncan, J. R., & Burgess, J. E. (2006). Performance of dead *Azolla filiculoides* biomass in biosorption of Au from Wastewater. *Biotechnology Letters*, *28*(1), 45-50. https://doi.org/10.1007/s10529-005-9686-7

Utomo, R., Noviandi, C. T., Umami, N., & Permadi, A. (2019). Effect of composted animal manure as fertiliser on productivity of *Azolla Pinnata* Grown in Earthen Ponds. *OnLine Journal of Biological Sciences*, *19*(4), 232-236. https://doi.org/10.3844/ojbsci.2019.232.236

Wagner, G. M. (1997). *Azolla*: A review of its biology and utilisation. *The Botanical Review*, *63*(1), 1-26. https://doi.org/10.1007/BF02857915

Weising, K., Nybom, H., Pfenninger, M., Wolff, K., & Kahl, G. (2005). *DNA fingerprinting in plants: principles, methods, and applications* (2nd ed.). CRC Press. https://doi.org/10.1201/9781420040043

Wiryati, G. (2021). Study of the utilisation of *Azolla microphylla* as alternative feed by *Tilapia farmers in* Panjalu Ciamis, West Java Province, Indonesia. *E3S Web of Conferences*, *322*, Article 02007. https://doi.org/10.1051/e3sconf/202132202007

Yusuf, A. F., Alfiyani, A., Salsabila, T. A. S., Kusnanda, P. S., Hidayati, I. N., & Daryono, B. S. (2023). Assessment of maturity stage and stability of new Indonesian melon cultivar 'Melona' based on ISSR markers and morphological characteristics. *Biodiversitas*, *24*(1). https://doi.org/10.13057/biodiv/d240137

Yusuf, A. F., Wibowo, W. A., & Daryono, B. S. (2022). Genetic stability of melon (*Cucumis melo* L. cv. Meloni) based on inter-simple sequence repeat and phenotypic characteristics. *Biodiversitas*, *23*(6). https://doi.org/10.13057/biodiv/d230631

Zhong, Z., Huang, Q., Huang, Y., Huang, X., Chen, Z., Feng, D., & Tang, L. (2011). Biological characteristics seven crossbred *Azolla* and ISSR markers. *Chinese Journal of Tropical Crops,* 32(10), 1873-1877. https://doi.org/10.3969/j.issn.1000-2561.2011.10.017