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Research Article

Isolation and Identification of *Azolla* Species from Selected Regions of Iraq

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ABSTRACT

Azolla fern, rich in proteins (up to 30%), carbohydrates, and vitamins, holds nutritional, environmental, and economic importance as a biofertilizer, animal feed, and water purifier. Its occurrence in Iraq has been reported in Kalar, the Hawr al-Hartha marshes near Basra, and the Tigris River, with studies from 2015 to 2023 confirming its local distribution. In the present study, three *Azolla* samples were collected from different locations: Abu Ghraib district in Baghdad province, Karbala province, and Salah al-Din province. DNA was extracted and amplified to assess the genetic diversity of the available *Azolla* species. PCR analysis revealed that the ferns *A. microphylla* and *A. nilotica* each produced a single DNA band. Sequence analysis showed 99.2% similarity of one sample with *A. microphylla* (accession number PQ587101.1), thereby identifying it as *A. microphylla*. Another sample showed 99% similarity with *A. nilotica* (accession number PQ587100.1), confirming its identification as *A. nilotica*. A phylogenetic tree was constructed using the NCBI-BLASTn program. Comparative analysis of the nucleotide sequences with reference data from the National Center for Biotechnology Information (NCBI), using BioEdit software, indicated that the *A. nilotica* isolate shared 99% similarity with an American isolate, while *A. microphylla* showed 99% similarity with Indian isolates. This study provides new insights into the biodiversity of *Azolla* in Iraq and identifies its species through molecular characterization.

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Introduction

Azolla is a free-floating aquatic fern that grows in the fresh-water of lakes, marshes and ponds and is described as being cosmopolitan (Amro, 2022). The fronds of *Azolla* are generally small, with a diameter ranging from 1-2.5 cm, although some species can attain sizes of up to 15 cm or more (Qiu and Yu, 2003). The presence of specific leaf cavities that hold the nitrogen-fixing cyanobacterium *Anabaena azollae makes up one of Azolla's unique traits* (Jama et al., 2023). Because of this symbiotic relationship, *Azolla* is able to fix atmospheric nitrogen straight within leaves, enhancing aquatic ecosystem and providing a natural source of biofertilization (Yang et al., 2022).

According to taxonomy, *Azolla* is an affiliate of the salviniaceae family, having two main subgenera (Roy et al., 2016). The first, *Azolla* (or *Euazolla*), includes four species: *A. caroliniana*, *A. mexicana*, *A. filiculoides*, and *A. microphylla*, all of which are native to the Americas. The second subgenus, *Rhizosperma*, consists of two species: *A. nilotica* and *A. pinnata*, both of which are native to Africa (Al-Saadi et al., 2024; Mosha, 2018). Recent molecular studies have further expanded the classification of the

genus. A recent study (Chang et al., 2020) showed the presence of a newly identified *Azolla* species known as *A. caroliniana* or *A. japonica* in Taiwan, in addition to the more widespread *A. pinnata*. Based on phylogenetic analysis using DNA sequencing, this taxon was more similar to *A. caroliniana* than to *A. japonica*, demonstrating important phylogenetic relationships within the genus.

Although global research on *Azolla* has advanced considerably (Maulood et al. (2022), it has been noted that there is still a lack of research on a number of issues related to non-flowering plants in Iraq and the Middle East. Although Iraq is home to more than 180 species of algae and no less than 20 species of ferns, the discovery of the aquatic fern *Azolla* and the demonstration of its environmental and economic importance was recently made in Basra Governorate, first in 2016 and again in Maysan in 2017. (Al-Tameemi and Al-Kanani, 2020). Recently, it was found in the Tigris River near the body and in the wetlands of the Dalmaj Reserve (Salim and Sleem, 2021). Its rapid proliferation can block sunlight penetration, reduce dissolved oxygen, and alter aquatic biodiversity by suppressing algae and macrophytes (Al-Mandeel, 2023).

The growth and distribution of *Azolla* are influenced by several abiotic factors, particularly water quality and temperature. It typically flourishes during early spring and late summer but exhibits reduced growth during colder months such as late autumn (Al-Tameemi & Al-Kanani, 2020). Morphological variation among populations has been attributed to both geographical location and subtle phenotypic differences among species (Kodit et al., 2023).

Beyond its ecological role, *Azolla* has attracted increasing attention for its economic and agricultural applications. Due to its high protein content and rich nutrient profile, *Azolla* is widely used as a low-cost, sustainable feed for livestock, poultry, and fish, reducing the reliance on expensive commercial feed sources (Ahmad and Tariq, 2021). Its ability to fix atmospheric nitrogen makes it an attractive organic fertilizer, minimizes the demand for synthetic nitrogen inputs in rice cultivation and other cropping systems. Furthermore, *Azolla* has been explored for its potential in phytoremediation, where it can absorb heavy metals and other pollutants from contaminated water bodies (Alebachew Chekol et al., 2024).

The integration of *Azolla* cultivation into farming systems has been shown to provide multiple benefits. For example, co-cultivation of *Azolla* with ducks and fish in integrated rice-fish-duck systems improves nutrient cycling, enhances soil fertility, reduces labor costs, and increases overall farmers' income (Mustabi et al., 2021). Moreover, its availability throughout the year, especially in the summer when traditional fodder is difficult to obtain, makes it a viable option to overcome the challenges related to fodder scarcity. (Alebachew Chekol et al., 2024).

Given its ecological adaptability and socio-economic importance, the current study was designed to collect *Azolla* populations from different regions of Iraq, classify them using molecular classification and identify the existing species. This will provide a foundation for further ecological, genetic, and applied research on *Azolla* in Iraq and contribute to developing sustainable management and utilization strategies.

Materials and Methods

Study area

Fresh and small leaf samples were collected from plants across three geographical locations in the provinces of Baghdad, Karbala, and Salah al-Din. The samples were obtained from the river within the central habitat, comprising a total of 30 plants. Each sample was placed in a plastic container, labeled with its number and site name, and transported to the Molecular Genetics Laboratory for classification and identification of species.

Morphological study

A morphological analysis of leaf and branch traits was carried out, including examinations of leaf surfaces and margins, with the objective of assigning a new accession number for depositing the plant in the herbarium. The samples were preserved in 70% ethanol prior to deposition in the Iraqi National Herbarium, under the Ministry of Agriculture in Baghdad.

Molecular and phylogenetic study

DNA extraction and gel electrophoresis

Approximately 200 mg of leaf tissue was soaked in distilled water after storage at 4°C for one day, then finely ground in a porcelain mortar with liquid nitrogen. DNA extraction was performed using the DNeasy® Plant Mini Kit (Qiagen, UK), based on the manufacturer's instructions. A NanoDrop spectrophotometer was utilized for assessing the concentration and purity of the analyzed DNA (Thermo Fisher Scientific, Madison, USA), measuring absorbance at 260 and 280 nm. Genomic DNA integrity was verified by 1% agarose gel electrophoresis (Al-Ahmer

et al., 2017). The extracted DNA was stored at -20°C until further use.

DNA amplification and gel electrophoresis

DNA amplification was conducted using a plastidial amplification primer set (Maloukh et al., 2017). The rbcL region was targeted using polymerase chain reaction (PCR) with an Eppendorf™ Mastercycler™ Nexus Thermal Cycler. PCR reactions were prepared in a 25 µl total volume, consisting of 2X BioMix PCR Master Mix (Bioline, UK), 2 µl (10 pmol) of forward and reverse primers (German), and 10 ng (2 µl) of template DNA, adjusted with nuclease-free water. Amplification was performed in an XP Thermal Cycler (Hangzhou Bioer Technology Co., Ltd., China) under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 50 sec; followed by a final extension at 72°C for 5 sec. PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. Horizontal electrophoresis was run for 45 min at 90 V (Auto, Japan). DNA bands were visualized under a UV lamp, and images were captured with a Nikon digital camera.

Sanger sequencing and phylogenetic tree

Purification and sequencing of PCR products were carried out, with both strands of the purified products sequenced according to the standard operating procedures of Macrogen Inc. (Geumcheon, Korea). Sequence quality, identity, and possible contamination were assessed using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

Table 1. Primers used for DNA amplification and sequencing in *Azolla* spp.

Locus	Sequence (5' to 3')	Time (C°)	Amplicon size (bp)
rbcl	F:ATGTCACCACAAACAGAGACTAAAGC	60	599 bp
	R: GTAAAAATCAAGTCCACCRCG	72	

Results

Morphological characterization of *Azolla* species

Samples of *Azolla*, identified as distinct species, were subjected to detailed visual and morphological examination. The morphological features of the examined *Azolla* (Figure 1) were characterized by a green, yellowish, or dark-red frond coloration, two rows of bilobate, tile-like leaves, adventitious roots, and a branched protostelic stem. These diagnostic traits, particularly the variation in pigmentation and the distinctive bilobed leaf arrangement, serve as key criteria for accurate identification and taxonomic classification of *Azolla* species. Such morphological characteristics are not only important for systematic studies but also provide insights into their ecological adaptability and potential applications in agriculture, environmental management, and biotechnology.

Molecular characterization of *Azolla* species

DNA isolation and quality assessment

Genomic DNA was successfully extracted from the tissues of three *Azolla* plant samples. The DNA appeared as a distinct single band under UV light after staining with ethidium bromide, confirming its intact nature (Figure 2). The concentration of the extracted DNA ranged from 55 to 200 ng/µl, while the purity, as measured by the A260/A280 ratio, was within the acceptable range of 1.8-2.0. These results indicated that the DNA quality and yield were suitable for downstream molecular applications, including PCR amplification and sequencing.

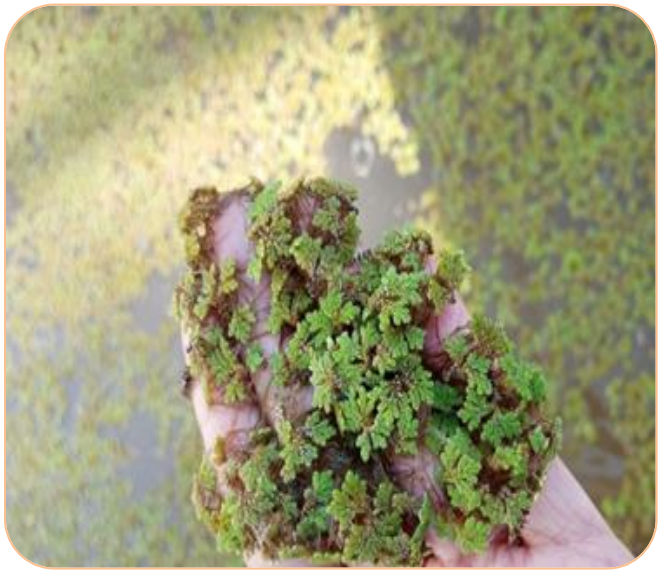


Figure 1. Leaf surface, leaf margins, branching pattern, and root length of *Azolla* sp.

PCR amplification and phylogenetic analysis

PCR amplification of the extracted DNA generated amplicons of approximately 599 bp in all three *Azolla* samples, as confirmed by agarose gel electrophoresis

(Figure 3). The presence of clear and specific bands demonstrates the successful amplification of the target DNA region. Subsequent sequencing and phylogenetic tree analysis revealed the genetic relationships among the studied *Azolla* species, thereby complementing the morphological identification. This molecular evidence provides a robust framework for species-level characterization and evolutionary analysis of *Azolla*.

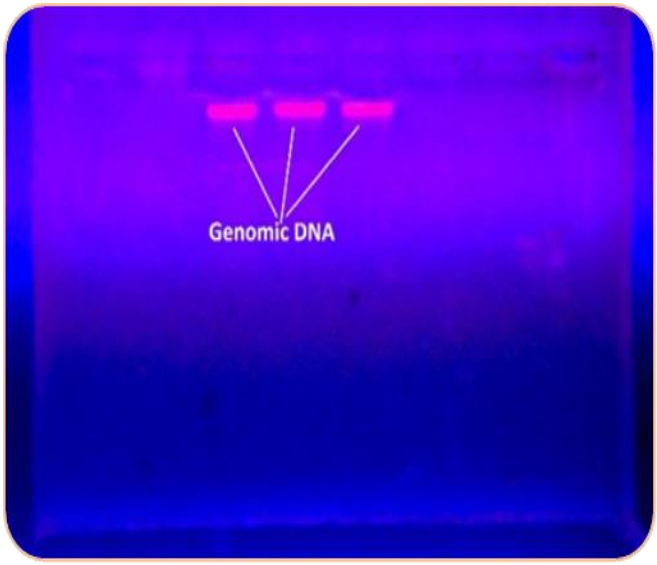


Figure 2. DNA separation on a 1.5% agarose gel at 70 V for 1 h.

Determination of nitrogenous base sequences, bioinformatics, and phylogenetic analysis

For the genomic characterization of *Azolla* species, the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (rbcL locus) was selected as the target marker due to its high reliability in molecular systematics of ferns and related taxa. PCR amplification was successfully performed, and amplicons from three positive samples were purified and sequenced using the Sanger sequencing method. The obtained nucleotide sequences were analyzed using BioEdit software, followed by comparison with reference sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database.

Sequence alignment revealed a 99% identity between the amplified DNA fragments of *A. nilotica* and the American strain available in GenBank. Similarly, the *A. microphylla* sequences showed a 99.2% similarity with Indian strains (Figure 4). These results confirm the high degree of conservation in the rbcL region among geographically distinct populations of *Azolla* species.

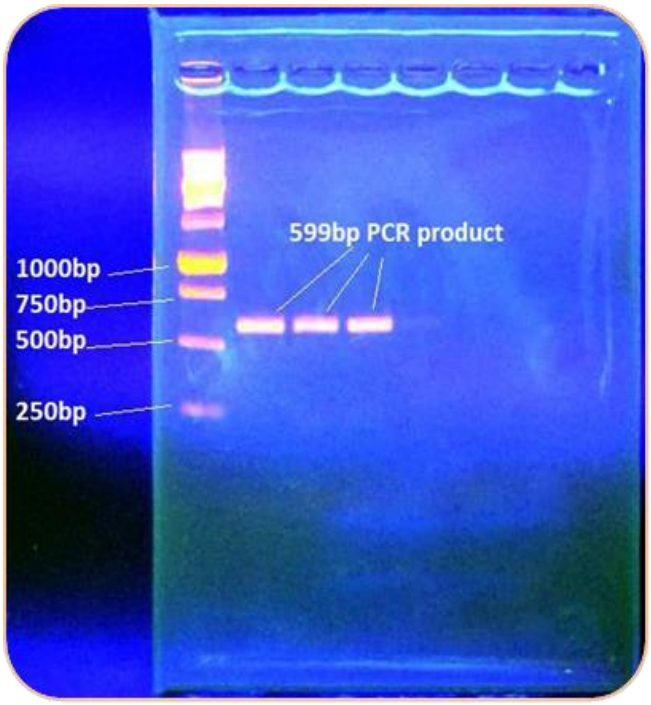


Figure 3. Gel electrophoresis of PCR-amplified rbcL gene on 2% agarose gel stained with ethidium bromide, run at 90 V for 45 min, and visualized under a UV transilluminator. M: 100-1500 bp DNA ladder. The PCR yielded a product of 599 bp.

Phylogenetic relationships were further examined through evolutionary tree analysis (Figure 5). The constructed tree included seven outgroup taxa, with *Salvinia* identified as the closest relative to *Azolla*. Within this framework, *A. oblongifolia* appeared most closely related to the studied species, reflecting their shared ancestry. In contrast, *S. crenata* was positioned as genetically distant from *Azolla*. Other ferns, including *Cibotium regale*, *Gymnosphaera ramispina*, *Sphaeropteris brunoniana*, and *Cyathea lepifera*, also occupied distant clades, emphasizing the evolutionary divergence of *Azolla* from these lineages.

Importantly, the phylogenetic tree demonstrated that *A. nilotica* and *A. microphylla* cluster as sister taxa, branching from a common node but forming distinct lineages. This suggests that although they share a recent common ancestor, they have undergone independent evolutionary differentiation.

The generated sequences have been deposited in the NCBI GenBank under the accession numbers MF177090.1 (*A. nilotica*) and *MF177089.1 (*A. microphylla*), ensuring their availability for future comparative and evolutionary studies.

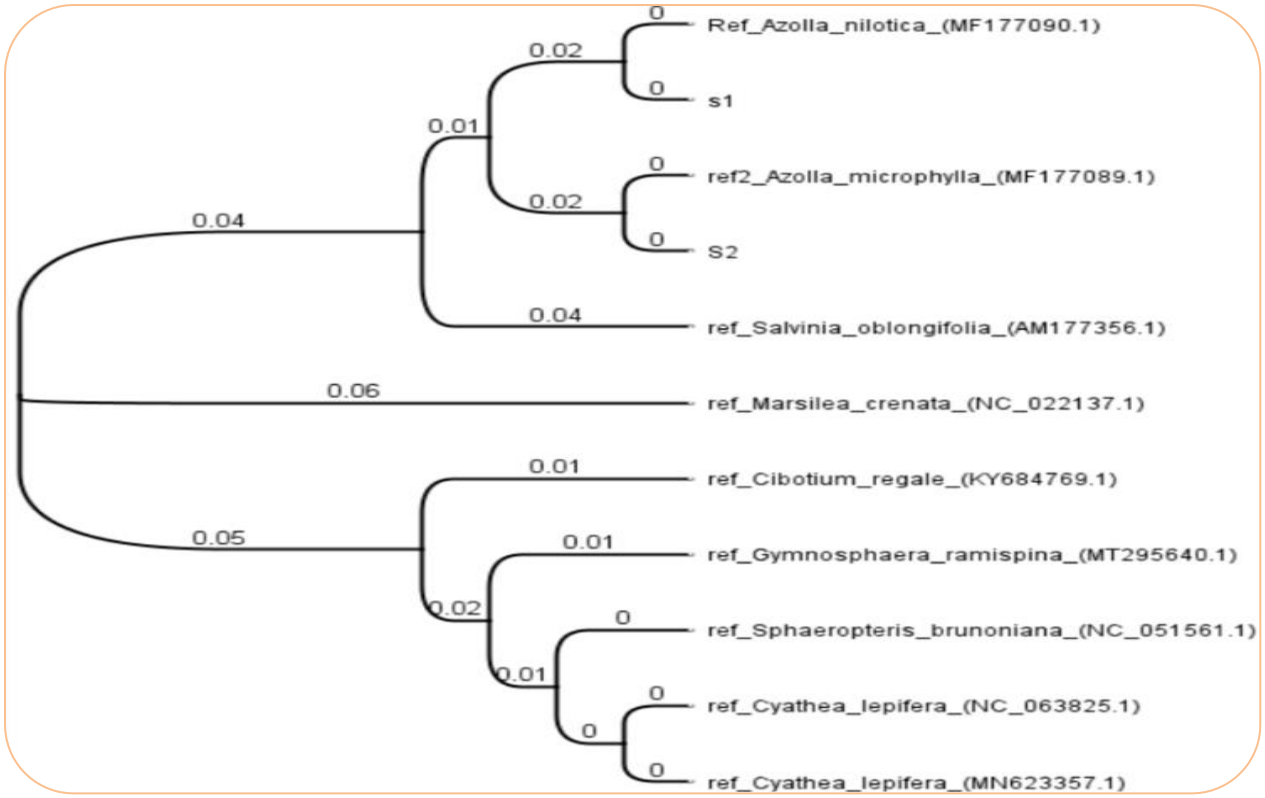


Figure 4. Comprehensive phylogenetic tree showing the separation of sequences among *Azolla* genes. The numbers next to Sample S1 and Sample S2 correspond to the reference numbers recorded in GenBank.

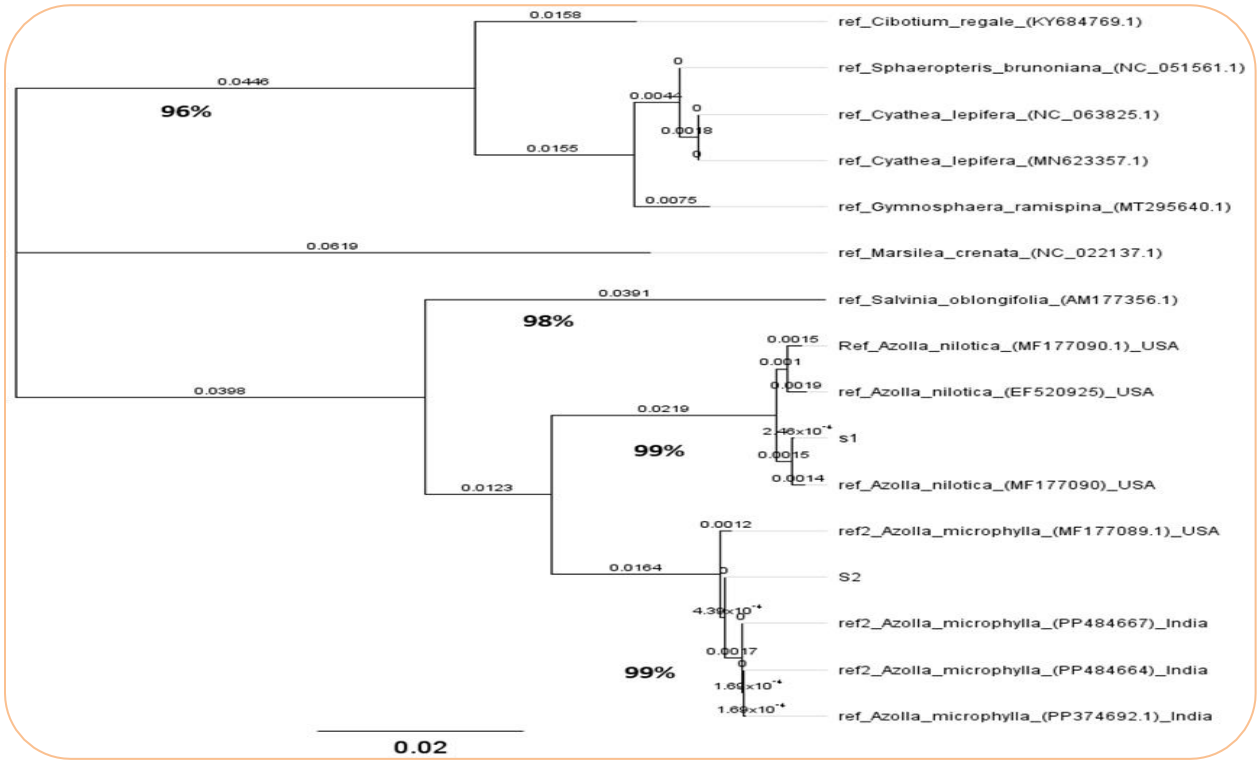


Figure 5. Comprehensive phylogenetic tree showing the gap sequences among *Azolla* genes. The numbers on the right indicate the reference accession numbers registered in the GenBank database.

Discussion

Research on *Azolla* species in Iraq remains scarce and is virtually nonexistent in the regions where the current samples were collected. Historical records indicate that the first *Azolla* specimen was deposited in the National Herbarium of Iraq under specimen number 58730 in 1996, as reported by Mahmood et al., (2020). Although this specimen was registered, its precise species identity was not conclusively verified.

Previous studies have emphasized the distinct taxonomic status of *A. nilotica*, which is characterized by a superior leaf lobe larger than the inferior lobe, a broadly ovate to elliptic shape, and an apex ranging from rounded to widely pointed (Birks, 2002). Nevertheless, *A. microphylla* has been classified as quite little (1-5 mm). Leaf dimensions are almost symmetrical. The species acclimatizes to dry and semi-arid (xerophytic) environments (Krueger, 2023).

Despite uncertainties regarding cellular counts, morphological traits such as branching patterns, leaf surface features, root development, and coloration strongly suggest that the specimens collected in this study are more closely aligned with *A. nilotica* and *A. microphylla*. These findings are broadly consistent with earlier morphological investigations; however, species-level identification of *Azolla* remains challenging. Difficulties arise due to overlapping morphological traits, reproductive structures, and physiological variations, as also reported by Kumar et al. (2021).

To overcome these challenges, molecular characterization was prioritized, as it provides higher resolution in differentiating closely related species. Phylogenetic analysis targeting three chloroplast DNA regions revealed strong sequence identity with reference accessions in international databases. Two samples showed high similarity with *A. nilotica* (99%) and *A. microphylla* (99.2%), whereas one sample remained unclassified due to degraded DNA. Collectively, the findings confirm the presence of four *Azolla* species in Iraq: A. microphylla, *A. nilotica*, *A. pinnata*, and *A. filiculoides*.

From an evolutionary perspective, *Azolla* is believed to have coexisted with the cyanobacterial symbiont *Anabaena* for over 100 million years, predating the emergence of flowering plants in terrestrial ecosystems (Li et al., 2018). Iraq's climatic conditions, temperature, rainfall, and wind regimes, are largely suitable for Azolla cultivation, although relative humidity remains a limiting factor (Hamid et al., 2024).

The ecological and agricultural roles of *Azolla* are dual in nature. On the positive side, it contributes to atmospheric nitrogen fixation (de Boer and van Ittersum, 2018), serves as a protein-rich supplement to fishmeal, as reported by Korsa et al. (2024), and contributes to water purification through heavy metal removal. For example, in the Al-Tuwaitha area of Baghdad, *Azolla* reduced heavy metal concentrations by 49-79% within 14 days (Rezooqi et al., 2021). Furthermore, field studies in Ninawa Governorate reported that 70% of farmers used *Azolla* as animal feed, with moderate to widespread distribution of the plant (Al-Hafidh and Lafta, 2024). Moreover, *Azolla* application has been associated with improved crop performance; wheat yield increased by 80% following NPK supplementation (Alati et al., 2019).

However, dense surface mats formed by *Azolla* can negatively impact aquatic ecosystems by reducing light penetration, limiting gas exchange, and inducing hypoxia, which may stress or even kill aquatic organisms as indicated by Akomolafe and Rahmad,(2018). These contrasting effects highlight the need for balanced management strategies to maximize *Azolla*'s benefits while minimizing its ecological risks.

Conclusion

This study demonstrates that combining morphological and molecular approaches provides a comprehensive and reliable framework for classifying *Azolla* species. Distinct traits such as frond coloration, adventitious root systems, and leaf lobe characteristics were documented. Molecular analysis of the rbcL plastid gene confirmed 99% similarity with *A. nilotica* and 99.2% with *A. microphylla*. These results represent the first molecularly validated records of these species in Iraq, with corresponding data deposited in the NCBI GenBank database.

The findings emphasize the genetic diversity of *Azolla* in Iraq and highlight the necessity of further research, particularly to characterize the unidentified sample. This work provides a foundation for future ecological, agricultural, and biotechnological studies, as well as for the sustainable utilization and conservation of *Azolla* species in Iraq.

Authors' Contributions

WMA was responsible for sample collection, performing the experimental procedures, and preparing the initial draft of the manuscript; SKA contributed to the experimental design, provided guidance during the research, and critically revised the manuscript for

important intellectual content; Both authors reviewed and approved the final version of the manuscript prior to submission.

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Conflict of Interest
The authors declare no conflict of interest.

Sustainable Development Goals Targeted
SDG 2: Zero Hunger
SDG 13: Climate Action
SDG 15: Life on Land

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