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RESEARCH PAPER

Morphological and Molecular Phylogenetic Analyses Reveal a New Record to the Flora of Iraq: *Azolla filiculoides*

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A B S T R A C T:

Azolla filiculoides is an aquatic fern native to Americas. The species has dispersed through Europe and Asia. Recently, it has been identified in northern part of Iran. However, there is no evidence confirms the presence of *Azolla sp.* in Kurdistan region of Iraq. The aim of the present study was to identify the species of *Azolla* found in Tanjaro river/northern part of Iraq. We studied morphological characteristics including trichomes, leaf branches, and three phylogenetic positions such as *rbcl*, *rps4* and *trnG-trnR* of the species. The morphological and phylogeny investigations were compared with other identified species (reference species). Our results showed that the morphological characteristics are more similar to *Azolla filicluloides* than other species. Based on the phylogenetic trees, the position of the observed species was closer to *Azolla filiculoides* than others. These results indicated that the *Azolla* species found in Tanjaro river is more likely *c.*

KEY WORDS: Ferns, *Azolla*, Molecular Markers, Phylogeny, Morphology, Kurdistan, Iraq
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1.INTRODUCTION :

Azolla species are small aquatic ferns belong to family Azollaceae, they grow on the surface of fresh water of ponds, lakes or streams, these plants were named for the first time by Lamarck in 1783 (Sood et al., 2008, Papaefthimiou et al., 2008). *Azolla* is associated symbiotically with prokaryotic cyanobacterium conferring high rates of nitrogen fixation, which is used as a biofertilizer with rice crops in several countries specifically in Asia (Bhuvaneshwari and Singh, 2015, Papaefthimiou et al., 2008). Based on its morphological and reproductive patterns, *Azolla* has seven distinct species that are grouped into two sections including Rhizosperma and Euazolla. The first section includes *A. nilotica and A. pinnata* (Plazinski et al., 1988), whereas the latter section consists of five species of *Azolla*, namely *A. filiculoides*, *A. caroliniana*, *A. mexicana*, *A. rubra*, *A. microphylla* (Lumpkin and Plucknett, 1980). Following the introduction of *Azolla*, it is readily transported locally by activities of human, animal and waterfowl that they are subsequently considered facilitators (Brochet et al., 2009).

Previous molecular studies have suggested a taxonomic treatment according to the recognition of the two above-stated sections only (Pereira et al., 2011). The knowledge of fern biology and the processes that govern the evolution of land plants and specifically ferns are limited because of a dearth of genomic information. The genome of *A. . filicluoides* is relatively 0.75 Gb (Obermayer et al., 2002), a characteristic distingush them from other ferns, a group which is notorious for large genomes that are averagely 12 Gb (Sessa and Der, 2016). Many studies use plastid intergenic (*trnG-trnL*), and genes (*rps4* and *rbcL*) sequences to do phylogentic analysis and allow us to better

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identify a certain species of *A*zolla in relation with other species (Metzgar et al., 2007, Lu et al., 2015, Madeira et al., 2019). The most notorious member of this group of ferns, *A. filiculoides* is the most damaging invasive alien in several parts of the world. This species has been introduced into parts of Africa, South East Asia and northern Iran for use as a natural biofertilizer for rice (Lumpkin and Plucknett, 1980). However, there is no previous evidence confirming the presence of *Azolla* into northern parts of Iraq.

Proper identification of the host and invasive *Azolla sp.* is critical in biological control studies and conservation strategies (Madeira et al., 2016). However, the identification of *Azolla sp.* is notoriously difficult (Evrard and Van Hove, 2004). Morphological similarity and diminutive stature of *Azolla sp.* have caused a long history of mistaken identifications and have occasionally led to taxonomic confusion (Reid et al., 2006). Fortunately, numerous molecular taxonomies for *Azolla sp.* have been investigated in recent years. Such publications have helped scientists to clarify the taxonomy, and have also provided molecular barcodes for the identification of field samples of *Azolla* (Lu et al., 2015, Madeira et al., 2016, Li et al., 2018, Madeira et al., 2019)

The aim of the present study was to confirm a thorough morphological and molecular analysis of *Azolla* in the north part of Iraq in order to understand which species is present. This identification is of importance to reveal a new plant of the flora of Iraq and to world map of ferns, specifically *Azolla sp.*

2. MATERIALS AND METHODS

2.1. Study Area

Samples of the aquatic fern *Azolla* sp. were collected from three different sites of freshwater of an irrigation drainage of Sharazoor area at different times (figure 1, A and B). The collection sites are specifically located in the Northern part of Iraq, Kurdistan Region, Al-Sulaymaniyah Governorate. Its latitude is 35.566864 with a longitude of 45.416107. The Global Positioning System (GPS) data of Al-Sulaymaniyah governorate is 35° 34' 0.7104" N and 45° 24' 57.9852" E. Geographic positions of the collection sites were recorded using GPS (Garmin 72, USA). The respective sample was collected in the field where located specifically on Tanjaro river (Figure 2).

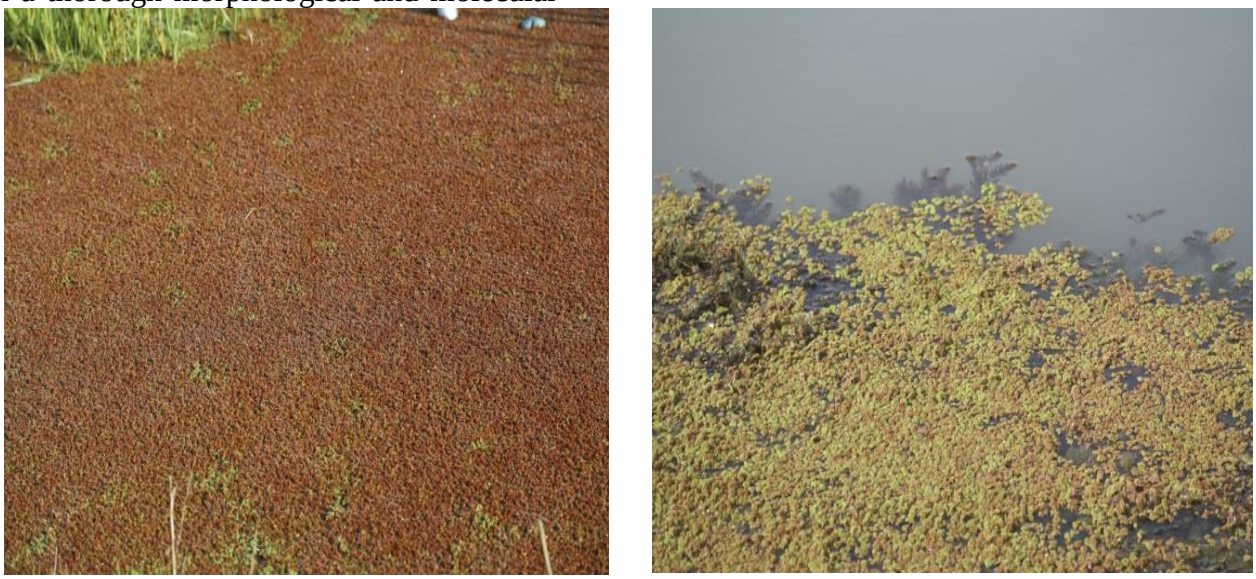

Figure 1. Density of the *Azolla* sp. in the studied area, (A) Dense mat (B) thin mat of *Azolla*

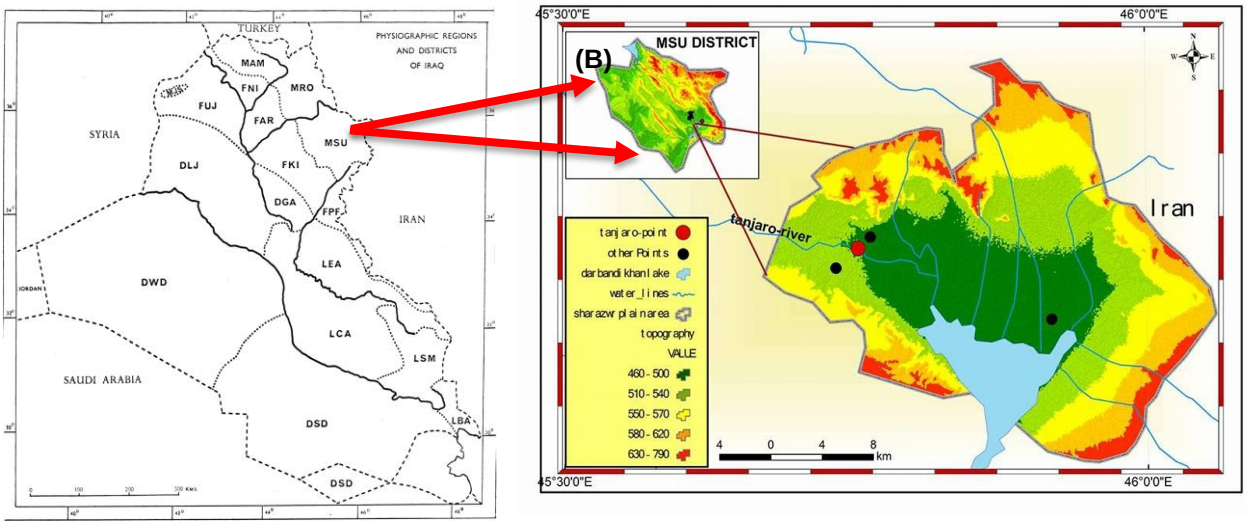

Figure 2: (A) Map of physiographic regions and districts of Iraq (Guest et al., 1966), (B) the geographic distribution map of the studied *Azolla sp.* in MSU (Sulaimania district)

2.2. Morphological Study

Trichomes, leaf branches, leaf surfaces and leaf margin shapes were morphologically examined using the stereo microscope (Meiji Model RZ, USA). In addition, in terms of getting a new herbarium number, a part of the sample was preserved in 70% Ethyl alcohol and subsequently sent to the Iraqi National Herbarium, Ministry of Agriculture-Baghdad.

2.3. Molecular Phylogeny Analysis

DNeasy® Plant Mini kit (Qiagen, UK) was used to extract DNA from the studied sample following the manufacturer's instructions. According to protocols that were previously established (Pryer et al., 2004, Metzgar et al., 2007), three plastidal amplifications (Table 1), *rbcl*, *rps4* and *trnG-trnR* were attempted for the respective sample using the polymerase chain reaction (PCR) (Eppendorf™ Mastercycler™ Nexus Thermal Cycler). The PCR reaction was a mixture of 20 µl containing 10 ng (2 µl) of the template, 10 µl 2X BioMix PCR master mix (Bioline, UK,), 6 µl (10 pmol) of forward and reverse primer (German) and 2 µl of free RNAse water with the following steps: one cycle at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min; and ended with one cycle at 72 °C for 10 min. PCR reactions were run at 90 volts for 45 minutes in a horizontal gel electrophoresis unit (Fisher scientific, USA). The gel was visualized under a UV lamp and followed by capturing a clear picture using a Nikon digital camera.

PCR products were purified and sequenced, the sequencing reactions were investigated for both strands of all three purified PCR products using HiSeq4000 (Illumina, San Diego, USA) of Macrogen Inc., Korea. Information of primers used for amplification and sequencing reactions are shown in table 1.

All sequences were examined and aligned for identities and contaminations using the National Center for Biotechnology Information nucleotide-nucleotide BLAST (blastn) search (Altschul et al., 1997) table 2. To construct phylogenetic trees, DNA sequences were aligned using Clustal W in Bioedit version 7.0.5.3 (Hall, 1999). The alignments include 1595 bp (rbcL), 308 bp (rps4), and 1080 bp (trnG-trnR). The aligned genetic sequences were concatenated into one mega database using Mesquite (Maddison and Maddison, 2018). The phylogenetic trees were built using Mega X software version 10.1 (Kumar et al., 2018). The maximum likelihood tree was produced using Hasegawa-Kishino-Yano with Gamma distribution (HKY+G) model as best fit substitution model (it had low Corrected Akaike information criterion value in compare with other models).

Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise

distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3444)) Maximum parsimony tree was generated using 500 replicates with Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000). The initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 7 nucleotide sequences. The Neighbor-Joining (NJ) tree was built using Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004).

Table 1: Primers used to amplifying and sequencing DNA amplicons in *Azolla sp*. retrieved from (Metzgar et al., 2007).

Locus	Primer	Sequence (5' to 3')	Amplicon size (bp)
rbcL	ESRBCL1F ESRBCL1361R	ATGTCACCACAAACGGAGACTAAAGC TCAGGACTCCACTTACTAGCTTCACG	**1300**
rps4	RPS5F TRNSR	ATGTCCCGTTATCGAGGACCT TACCGAGGGTTCGAATC	**1200**
trnG-trnR	TRNG1F TRNG63R	GCGGGTATAGTTTAGTGGTAA GCGGGAATCGAACCCGCATCA	**1300**
	TRNG353R TRNR22R	TTGCTTMTAYGACTCGGTG CTATCCATTAGACGATGGACG	**1200**

Table 2. GeneBank accession number of the species used in this study. All species had *trnG-trnR, rbcL, and rps4* genetic sequences in genebank. Except *Azolla nilotica* had only *rps4*. The symbol (×) means no genetic information exist in genebank.

No.	Species	*t*rnG-trnR	rbcL	rps4
1.	*A.filiculoides*	JX280884.1	KM360662.1	EF520913.1
2.	*A. caroliniana*	EF520894.1	EF520919.1	EF520906.1
3.	*A. pinnata*	EF520902.1	AM177355.1	EF520907.1
4.	*A. nilotica*	×	×	EF520912.1
5.	*A. mexicana*	EF520897.1	EF520922.1	EF520911.1
6.	*A. microphylla*	EF520896.1	EF520921.1	EF520908.1

3. RESULTS

Morphological results of the present study were observed that the trichome types were unicellular, although bicellular trichomes are rarely recognized on the upper leaf lobe of the studied species (figure 3, A). In addition, green, yellowish or dark-red in colour, adventitious roots, branched protostelic stem and two rows tile-like bilobate leaves are morphological characteristics of the studied *Azolla* (figure 1, A and B, and figure 3, B, C and D).

In addition, clear sequences of the respective genes were used to produce phylogenetic trees in order to genetically identify the studied species of *Azolla*. Results showed that the most likely tree

(Figure 4) produced from maximum likelihood (ML) analysis of the combined genetic sequences (*rbcl, rps4* and *trnG-trnR*) was well resolved (bootstrap test, 500 replicates). Based on ML tree, *Azolla sp.* is sister to *A. filiculoides* (bootstrap value=64%).

In addition, the most parsimonious tree (388 steps. consistency index= 0.828125, retention index = 0.810345), and composite index = 0.787371) (Figure 5) built from maximum parsimony analysis where all the branches were well resolved (no polytomies, Bootstrap values ≥ 50 %). Results also showed that position of *A.* species is in the same clade where *A. pinnata, A. nilotica, and A. filiculoides* are exist and it is much closer to the latter species than others.

In a similar pattern with parsimony tree, the Neighbor-Joining tree (Figure 6) also revealed that the position of *Azolla sp.* is in the same clade of *A. filiculoides, A. nilotica, and A. pinnata*. The branch of *Azolla sp.* and *A. filiculoides* is considerably resolved (polytomy, Bootstrap value <50 %).

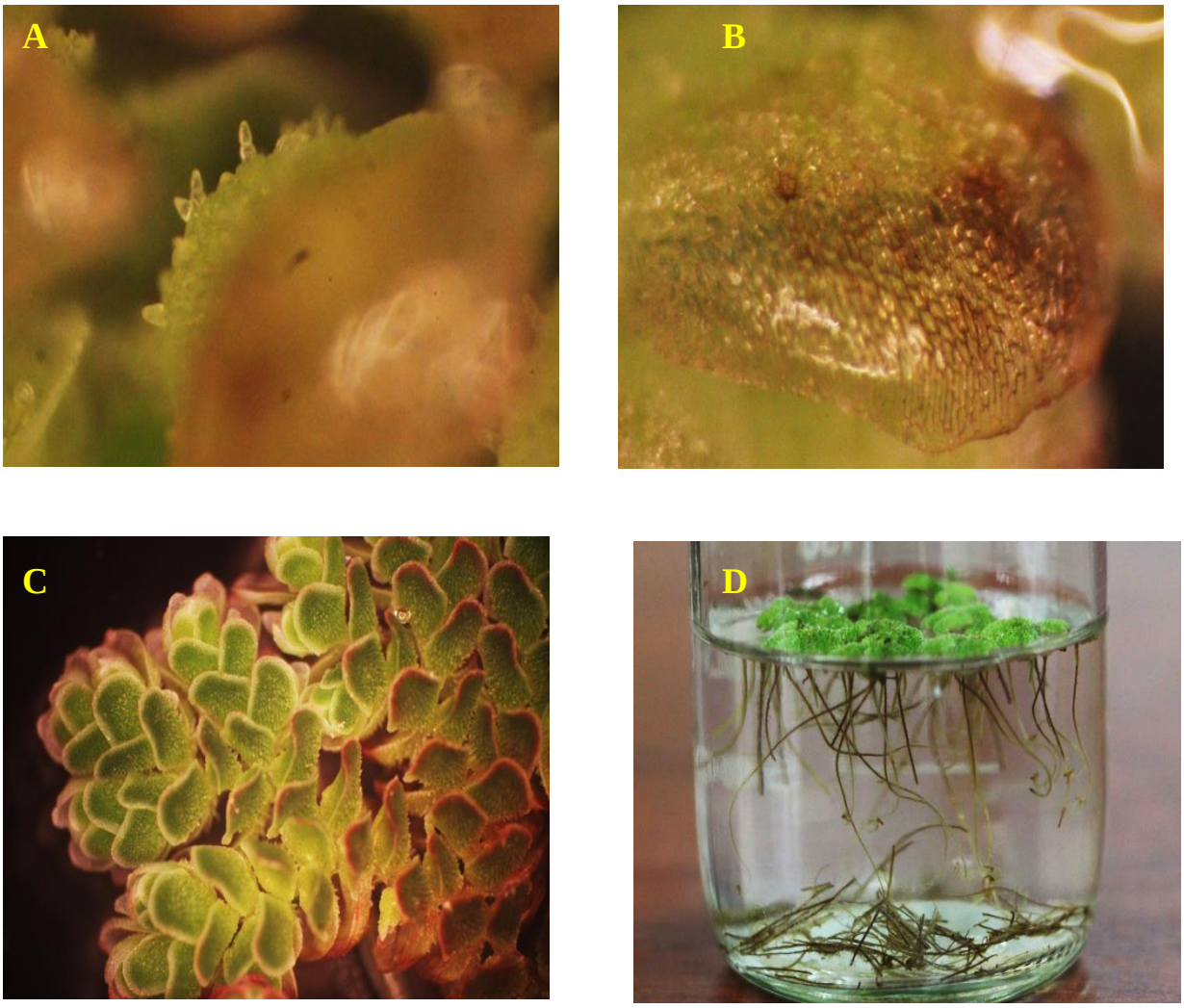

Figure 3: (A) leaf surface, (B) unicellular and bicellular trichomes, (C) branching pattern and leaf margins, (D) root length of *Azolla sp.*

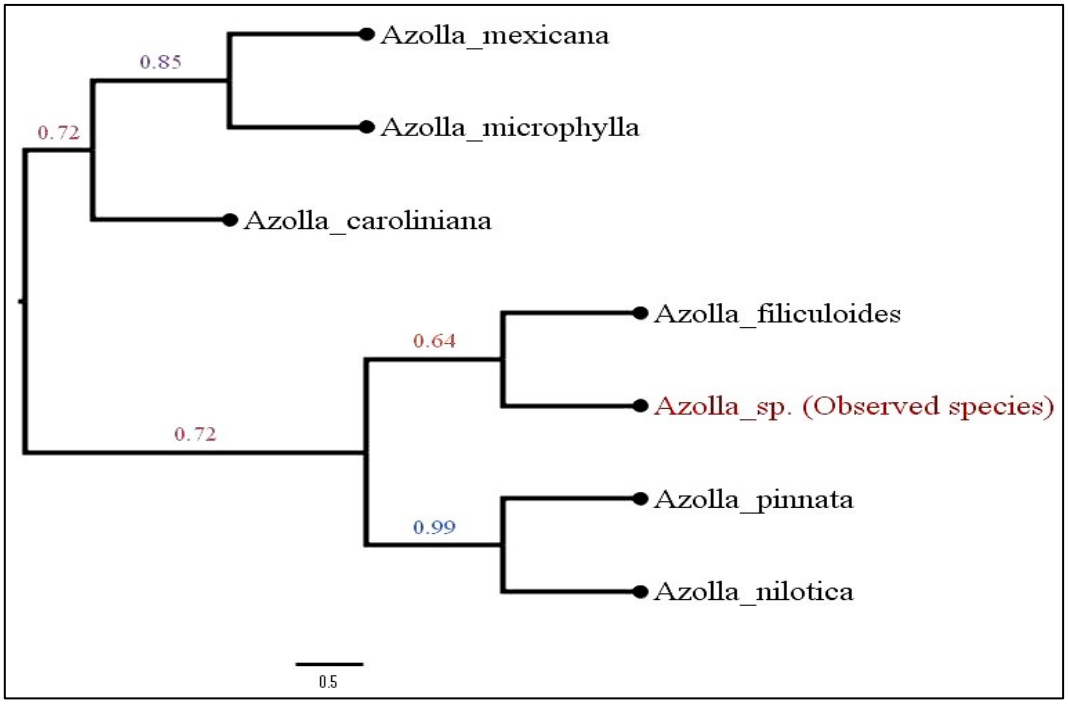

Figure 4. Maximum Likelihood method based on the Tamura-Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2985 positions in the final dataset.

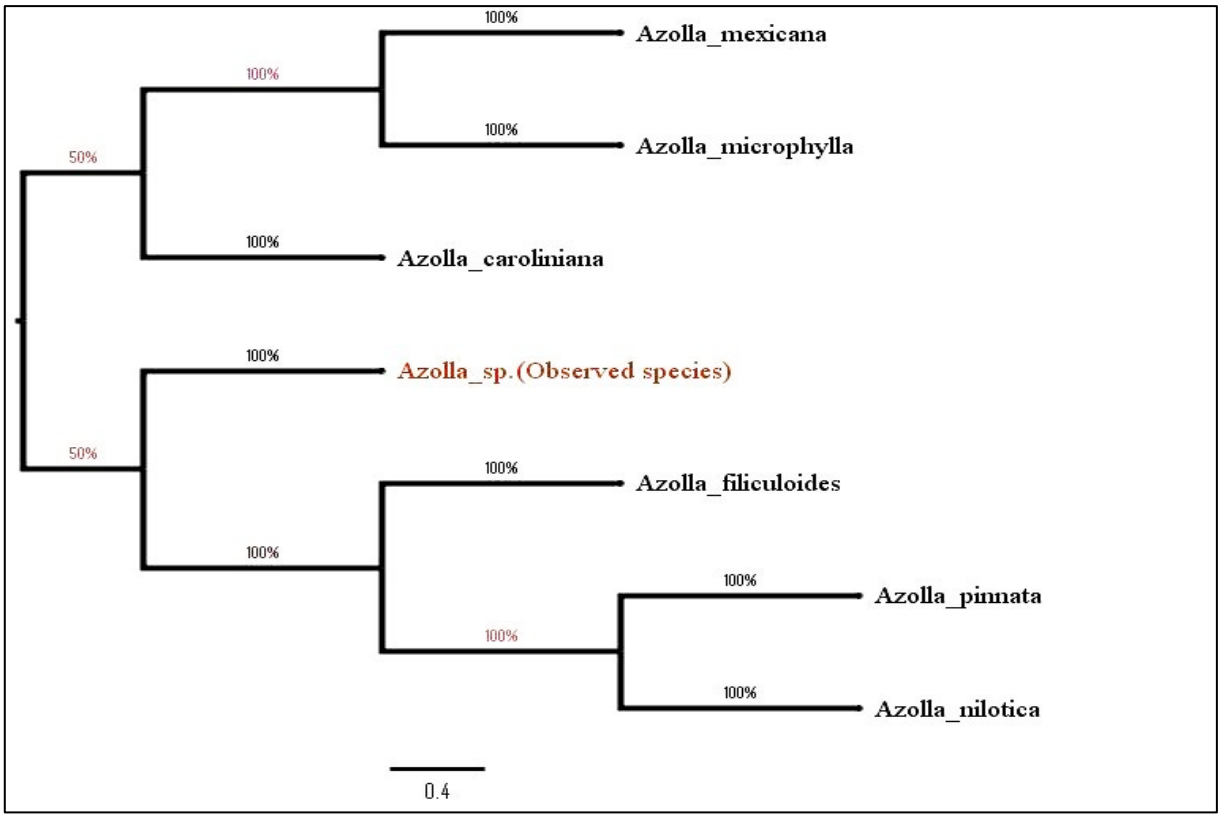

Figure 5. Maximum parsimony tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2985 positions in the final dataset.

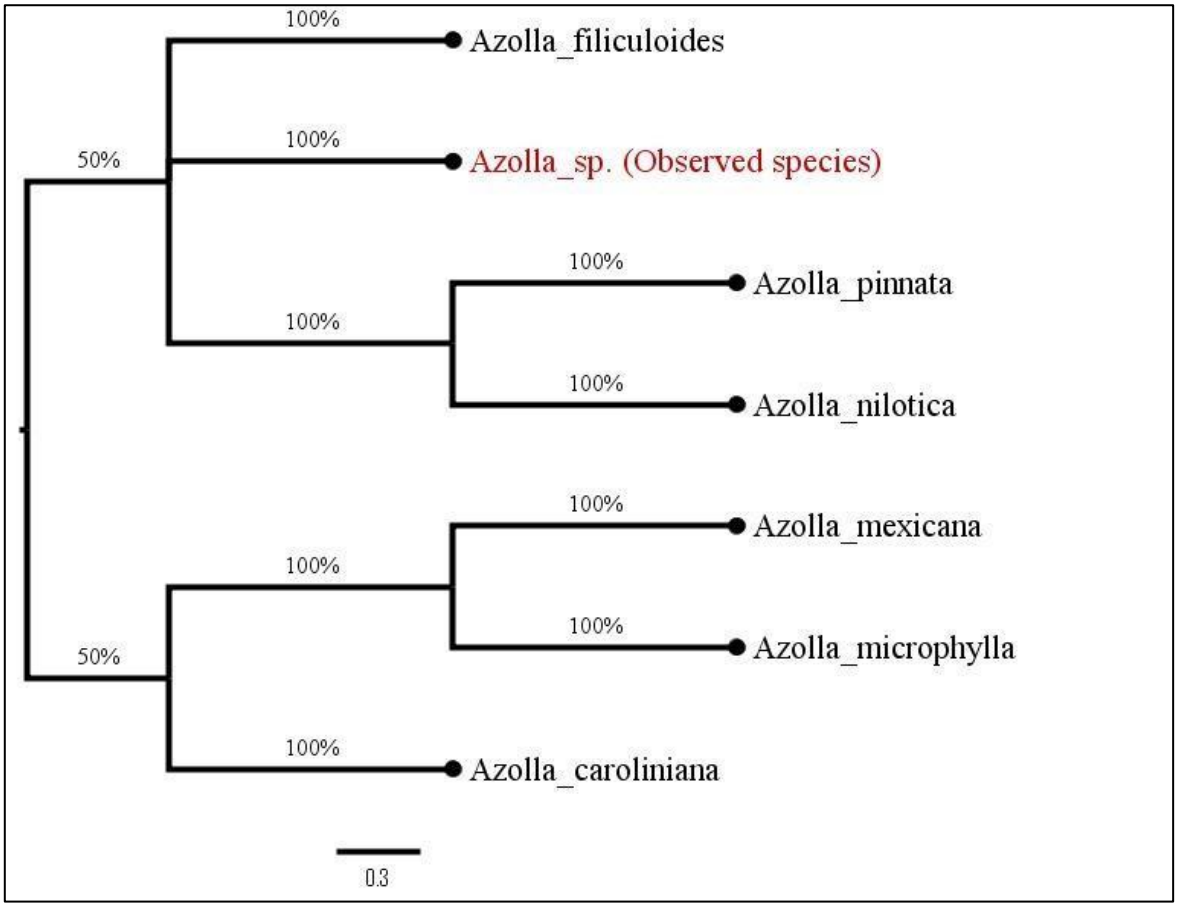

Figure 6. Neighbor-Joining tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2985 positions in the final dataset.

4. DISCUSSION

The existence of this plant has never been recorded in Iraq and it has probably aliened or invaded the region at least 20 years ago. According to our investigations with local farmers, it is confirmed that this plant exists in the study area since 1996. The respective *Azolla sp.* had been successfully registered at the National Herbarium of Iraq and had been given the specimen number 58730. Despite the registration of the studied fern, there were no clear evidence confirms its species type. Previous studies have shown a large consensus in regards to the unique status of *A. filiculoides*, which is the presence of unicellular trichomes on upper leaf lobes (Evrard and Van Hove, 2004). However, trichome structures of each *A. caroliniana, A. mexicana, A. microphylla* have nearly been characterized to be bicellular (Lumpkin and Plucknett, 1982, Jonsell, 2000). Although the cellular number(s) of the respective *Azolla* is dubitable, leaf branches and leaf surfaces, root pattern, leave colours indicate that this species is more likely A. *filiculoides* (Myśliwy and Szlauer-Łukaszewska, 2017).

Morphological results were mostly similar to previous studies of *A. filiculoides*, but making decision in regards to the species type of the respective *Azolla* is relatively hard. To overcome this issue, phylogenetic analysis was investigated considering three position of the chloroplast DNA.

Based on phylogenetic and morphological results, it is indicated that the *Azolla* species found in Tanjaro River is more likely *Azolla filiculoides*. According to maximum likelihood (ML) tree (figure 4), the respective species of *Azolla* is more closely related to *Azolla filiculoides* (collected form GeneBank) than other species. In addition, the result of Maximum Parsimony (MP) and

Neighbor-Joining (NJ) trees (Figure 5 and 6) confirm that what ML tree revealed, in which the *Azolla* in the Tanjero River is *Azolla filiculoides*. The existence of such wetland exotic species (non-native species) for the first time in the study area could be due to many reasons. For example, the climatic conditions of the river could be optimum for their growth and propagations. The existence of same species has been observed in north of Iran (Hashemloian and Azimi, 2009). The study area is adjacent to the northern part of Iran. This means that the species might been dispersed through water from Iran to north of Iraq. The other factor behind the species dispersal is human activities such as trading, tourism and animal. The role of migratory ducks in the long-distance dispersal of native plants and the spread of exotic plants in Europe (Brochet et al., 2009). The introduction of this new species into Kurdistan/Iraq flora has useful and harmful patterns at same time. It could be useful by providing ecological benefits to the river. For example, this species is a good nitrogen fixer and it can purify the water from heavy metals (Brouwer et al., 2018). In addition, it could be a good replacer of protein for fishes (Mosha, 2018). On the other hand, the high dispersal rate of the species within a short period may compete other aquatic species and vanish them from their natural habitat (Myśliwy and Szlauer-Łukaszewska, 2017).

5. CONCLUSION

In conclusion, this study has shown the importance of incorporating phylogenetic analyses beside morphological studies in taxonomic studies. The existence of *Azolla filiculoides* in north Iraq add a new record to flora of Iraq. This new record has two benefits: increasing the plant biodiversity in Iraq and for ecological and agricultural managements. This finding might pave the way for ecologists to do further works on such species. For example, species richness, estimation the invasive status of the species and its effects on availability of water and soil nitrogen. Furthermore, the existence of such species in the rivers will improve the growth of fishes. Fisheries can use this species as a natural protein source instead of commercial one to propagate their wealth.

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