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PHYLOGENETIC INSIGHTS IN *TRAPA NATANS* L.: EVALUATING ITS2 AS A MOLECULAR MARKER

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Abstract

Water chestnut is an aquatic species belonging to Lythraceae family, widely valued for its medicinal and nutritional properties. DNA barcoding is an efficient technique which requires minimal tissue sample for species recognition. The aim of this work was the precise identification and phylogenetic assessment of Water chestnut based on ITS2 DNA barcode. DNA was isolated using the GeneJet plant genomic DNA kit and quantified via a Nano - drop microvolume spectrophotometer, and assessed for quality by 1% agarose gel electrophoresis. The nuclear molecular marker, ITS2 was amplified through for PCR. The ITS2 amplicons were purified and subjected to Sanger sequencing. The sequence was then aligned using Multiple Sequence Alignment (MSA) to identify conserved regions. This conserved ITS2 region was used for species identification through NCBI's BLAST, confirming the species as *Trapa natans* L. by achieving a 99.41% correct identification rate. Phylogenetic tree based analysis was performed using MEGA6 with the ITS2 barcode, revealing a close relationship between *Trapa natans* L. *Trapa acornis* Osbeck., *Trapa jeholensis* Nakai., *Trapa natans* L. var. *Bispinosa* (Roxb.) Makino and *Trapa natans* L. var. *Quadrispinosa* (Roxb.) Makino, indicated by a branch length of 0.0000 at their nodes. This confirms ITS2 as a powerful nuclear barcoding region for identifying *Trapa* species and its utility for analysing and managing *Trapa* germplasm collections due to its biparental inheritance. But ITS2 does not elucidate cytoplasmic inheritance patterns due to lack of complementary cpDNA marker data. Future studies should employ "Integrative Taxonomy", combining ITS2 data with chloroplast markers to detect natural hybridization and maternal lineages.

Key words: Hydrophyte; Integrative taxonomy; Molecular phylogeny; DNA Barcode; ITS2

Introduction

Biodiversity is essential for sustaining ecological balance on Earth. It encompasses the existence of life within ecological complexes, including the variations among and between species (Diaz & Malhi, 2020). Although approximately 9 million species contribute to Earth's biodiversity but only a small fraction have been formally described. Quantifying daily extinction rates is extremely challenging, compounded by the fact that scientists have documented only 10-15% of the planet's total biodiversity (Halder & Jha, 2023). Consequently, an estimated 86-91% of Earth's species remain unidentified, contributing factors include the lack of reliable identification techniques. Traditional taxonomic methods, relying solely on morphological traits, often lead to misidentification, particularly for cryptic species or those with phenotypic plasticity (Hending, 2025).

DNA barcoding addresses this challenge by utilizing molecular techniques to differentiate species based on conserved DNA sequences. It employs unique molecular markers and standardized DNA fragments from specific gene loci to analyse diverse samples (Zhu *et al.*, 2022). These DNA barcodes are genetic sequences stored in digital libraries, providing accessible references for identifying unknown species. Ideal barcodes are short DNA sequences that are easily amplified (Gostel & Kress, 2022). DNA barcoding is applicable to all organisms, including animals, plants, and microbes (Braun *et al.*, 2022; Solovyeva *et al.*, 2023; Yameen *et al.*, 2024; Abbas *et al.*, 2025). At first, it was developed to recognize microbes, but now this technique is applicable to all life forms (Antil *et al.*, 2023; Chac & Thinh, 2023). DNA barcoding is significantly important for verifying the authenticity of biological products (Kress *et al.*, 2015), and is particularly valuable for identification of medicinal plant species, supporting their conservation and ensuring product originality (Mishra *et al.*,2016; Vassou *et al.*, 2016; Yu *et al.*, 2021). The foremost advantage of DNA barcoding is that it requires only minimal tissue samples (Paranaiba *et al.*, 2019).

Trapa natans L. commonly named as "Singhara is an annual herbaceous hydrophyte, belongs to Lythraceae family, endemic to tropical, subtropical, and temperate regions of Eurasia, but it has also become invasive in parts of North America and Australia (Lambrechts *et al.*, 2020). The plant has a flexible submerged stem covered with numerous pectinate root-like structures. Its leaves are rhomboidal in shape and clustered on the upper stem, measure 4 to 5 cm in length and have reddish - purple

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undersides. Its flowers are solitary, white, and sparse, while the fruits are somewhat rectangular bear sharp, spiny horns (Palm *et al*., 2024). It is cultivated globally across approximately 603,076 hectares (Jana, 2020).

Trapa natans L. holds remarkable medicinal value. Traditionally, it has been used in Chinese ethnomedicine for its anti-inflammatory, analgesic, antidiabetic, antimicrobial, antiulcer, neuroprotective, and immunomodulatory activities (Rajput & Singh, 2023), and it is also consumed as an herbal tea in some regions of Japan (Iwaoka *et al.*, 2021). In Pakistan, its nutty fruit is eaten after boiling and removing its pericarp. Ethnomedicinally, its fresh fruit is used to treat male and female sexual disorders and also anti – inflammatory agent (Ali *et al*., 2020). Recent studies indicate that alcohol extracts of *Trapa natans* L. fruit shells demonstrate efficacy in treating diabetes mellitus, cataracts, immune dysregulation, bacterial infections, and stomach ulcers (Garg *et al.*, 2020). Fruit and leaf extracts exhibit anticancer and antioxidant activities (Xia *et al.*, 2017).

Plant identification traditionally relied on morphology, DNA analysis now enables molecular characterization and the development of bio-inventories (Miller *et al.*, 2016). Plant DNA barcoding utilizes chloroplast DNA markers (e.g., *rbcL*, *matK*, *trnL*, *trnH-psbA*) and nuclear DNA markers (e.g., ITS, ITS2). The Consortium for the Barcode of Life (CBOL) Plant Working Group recommends the highly conserved *rbcL* and *matK* regions as universal core barcodes for plants (Kress, 2017; Bidyananda *et al.,* 2024). DNA barcoding has proven accuracy for cryptic species discovery, taxa detection, and phylogenetic studies (Mahima *et al*., 2020). For animals, the mitochondrial gene *CO1* is effective, whereas multi-locus barcoding is standard for plants (Antil *et al.*, 2023).

The Internal Transcribed Spacer 2 (ITS2) is a widely used DNA barcode, typically 200–300 base pairs long, located between the 5.8S and 28S rRNA genes, is a highly conserved nuclear DNA segment (Espinosa *et al.*, 2024). It contains conserved regions that facilitate universal primer design and the discrimination of closely related species (Nafisi *et al.,* 2023). Its ease of PCR amplification, even from degraded DNA, enhances its utility across taxa (Sokołowska *et al.,* 2022). ITS2 demonstrates high interspecific divergence and low intraspecific variation, making it highly effective for distinguishing plant species (Zheng *et al.*, 2021).

DNA barcoding enables the construction of phylogenetic trees for specimens that lack morphological characterization, allowing for molecular identification of unknown taxa. Furthermore, sequence similarity searches against databases like the GenBank and Barcode of Life Data (BOLD) system enable inference of family and genus affiliations for unidentified specimens. In spite of the ecological and taxonomical importance of *Trapa* species, no molecular phylogenetic studies employing DNA barcoding have previously been undertaken, highlighting a critical gap addressed by the present investigation. The current study aims to identify and assess the phylogeny for *Trapa natans* L., using the Maximum Likelihood method while employing the discriminatory power of ITS2 gene as standard barcode for angiosperms.

Materials and Methods

Survey site: The survey site was Khanki headworks on the River Chenab, along with its adjoining wetlands, spanning 4 km^2, located near Wazirabad District, Punjab, Pakistan, with coordinates of 32^o.2426 North and 73^o.580 East.

Specimen collection: Plant samples (Fig. 1) were collected from the eight designated survey sites during autumn 2024. A total of 15 specimens were collected and preserved for each site according to the standard "surface inventory" method (Parsons 2001). Four plant specimens were preserved by drying and mounting on herbarium sheets following standard herbarium techniques. Specimens in duplicate were deposited in the Dr. Sultan Ahmad Herbarium, Department of Botany, GC University, Lahore, Pakistan, under the assigned voucher No. GC–Herb–Bot–4912. A total of 100 healthy plant samples were preserved by freezing at -20^{o}C, for DNA extraction, 10 young and healthy plants were selected.

DNA isolation and quantification: DNA was isolated and purified from leaves of “Water chestnut” samples using the GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Quantification of DNA was made by Nano-Drop Lite Plus micro volume spectrophotometer (Thermo Fisher Scientific) at A_{260}/A_{280} (Carvalho *et al*., 2018). For the confirmation of the quality of DNA, electrophoresis was performed on 1% agarose gel using isolated DNA from plant specimens (Armstrong & Schulz, 2015).

PCR amplification: The isolated DNA samples were amplified by method as described by Lorenz (2012) for the ITS2 genetic marker with some modifications. A total of 25 µL PCR reaction mixture was prepared by adding 12 µL master mix, 10 µL nuclease free water, 0.5 µL reverse primer, 0.5 µL forward primer, DNA template 2 µL for reaction. Primers used for PCR amplification were S2F (ATGCGATACTTGGTGTGAAT) and S3R (GACGCTT CTCCAGACTACAAT) (Chen *et al*., 2010). Denaturation temperatures were 95ºC for 2 minutes and 30 seconds respectively. Annealing temperature was 55ºC for 30 seconds. Extension temperatures were 72ºC for 1 minute and 2 minutes respectively. All the reactions were performed for 30 cycles. Amplicons were confirmed by comparing with 1 kb DNA ladder *(Invitrogen – Thermo Fisher Scientific)* through 1% agarose gel electrophoresis. Purified PCR amplicons were forwarded to 1st Base (Apical Scientific) Selangor, Malaysia for Sanger sequencing. Consequent upon DNA sequencing, the chromatogram was evaluated by using Chromas software (Al-Shuhaib & Hashim, 2023). Sequence cleaned and trimmed by using Justbio web tool (Hosted Tools - Justbio) and BioEdit version 7.7.1.

Identification through basic local alignment sequence tool (BLAST): Prior to identify species by Basic Local Alignment Sequence Tool (BLAST) of NCBI, forward sequence was used to get its complementary reverse sequence by online reverse complement technique (Bioinformatics. com). Single sequence was made from both forward and reverse sequences where the forward sequence remains intact but reverse sequence changed into a reversed complement sequence. Afterward, assessed the alignment of the sequence using Multiple Sequence Alignment (MSA) at ClustalW in Geneious Prime to get conserved portion as DNA barcode. Subsequently, BLAST (Basic Local Alignment Search Tool) the conserved gene sequence from the NCBI database and align this query sequence (Kuznetsov & Bollin, 2020).

Line barcode and QR code generation: The line barcode of conserved gene sequence was developed by BIO-RAD DNA Barcode Generator [https://biorad-ads.com/dnabarcodeweb/] and TEC – IT QR code generator [https://barcode.tec-it.com/en/QRCode](Guo *et al.*, 2022).

The Bio–Rad barcode generator (Bio-Rad DNA Barcode Generator) is an elegant online tool that transform DNA sequences into visual barcodes. After Providing a sequence name, insert the DNA sequence using FASTA syntax and select the “add” button on the interface. The tool converts each nucleotide into a colour coded bar. These colours are arranged linearly to reflect the sequence order, creating a barcode like image. The resulting barcode is a graphical representation of DNA sequence.

Phylogenetic tree-based analysis: The phylogenetic tree was generated based on ITS2 gene with 1000 bootstrap replicates in Molecular Evolutionary Genetic Analysis version 6 (MEGA6) by following maximum likelihood method derived from Tamura–Nei model (Tamura & Nei, 1993; Tamura *et al*., 2013).

Results

DNA isolation and quantification: Average ratio of DNA quantification at A_{260}/A_{280} was ~1.8 which represented the quality DNA from all plant specimens. Additional authentication of isolated DNA from *Trapa natans* L. specimens was carried out through 1% Agarose gel electrophoresis as shown in Fig. 2. Each well was labelled by 1 to 11 number. Well No. 1 had 1kb DNA ladder (Invitrogen – Thermo Fischer Scientific), while all other have DNA extracts from plant specimens. Well No. 4, 5, 6, 7, 8, 9 and well No. 11 showed fine bands of genomic DNA at top (Fig. 2).

Polymerase chain reaction (PCR): ITS2 nuclear marker was successfully amplified by PCR as shown in Fig. 3. Well No. 1, 3, 4 and 5 have bright bands. The observed bands were located in between 400 and 500 bp.

Size of obtained DNA sequence was of ~469 bp. Chromas showed that nucleotide peaks from 1 to 42 and 450 to 469 overlap with each other with repeating peaks showing abrupt sequence, while 43 to 449 shows good peaks. Sequence trimmed by using justbio web tool (Hosted Tools - Justbio) and BioEdit version 7.7.1 and became of ~340 bp.

Multiple sequence alignment (MSA): After gene cleaning, assessed the alignment by multiple sequence alignment at ClustalW in Geneious Prime. During this procedure conserved region was shown as continuous stars, which was selected as DNA barcode for data repository. Then NCBI’s BLAST was used for species identification by this sequence.

Conserved gene sequence of ITS2 for *Trapa natans* L.:

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>ATCGAGTCTTGACGCAAGTTGCGCCTGAAGCCT
TATGGCTGAGGGCACGCCTGCCTGGGTGTCACTC
AACATTCCGCCCACACCGTCGGTCTCCCTCTCAC
GGGCCTGCGACATGGTGGGGAGTGGACGATGGC
CTCCCGTGGCCTCCTGGCTGTGGCTGGCCGAAAA
AATGAGCGATGGAGCGATGCTCTCCGCGACGCA
CGGTGGTGGTATCAACTTCCCTCGGCAGGCAGTC
GGGAGTCATCTCTCCTAGGTTCTCAAAGGGGCCT
CTATCATCGAGACACATGCGACCCCAGGTCAGGC
GGGGCCACCCGCTGAGTTTAAGCATATCAA
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BLAST analysis: Above mentioned conserved gene sequence was used for species identification by NCBI’s BLAST. The sequence showed a 100% query coverage and 99.41% similarity index with *Trapa natans* L. All observed values of BLAST analyses were mentioned in the Fig. 4.

Fig. 1. Plant samples collected from adjoining wetlands of Khanki headworks on the River Chenab.

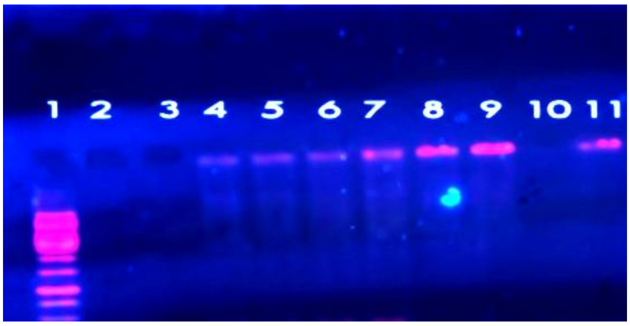

Fig. 2. Isolated DNA of *Trapa natans* L. visualized by 1% agarose gel.

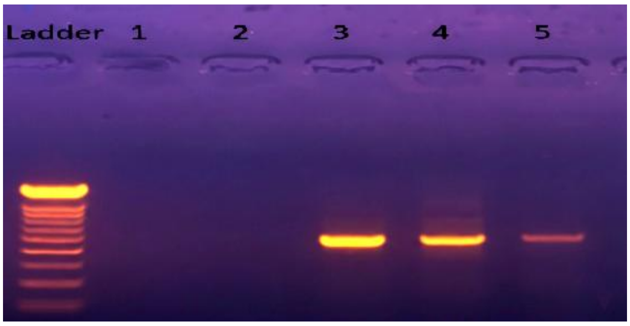

Fig. 3. Amplified PCR product of ITS2 marker as visualized on 1% agarose gel.

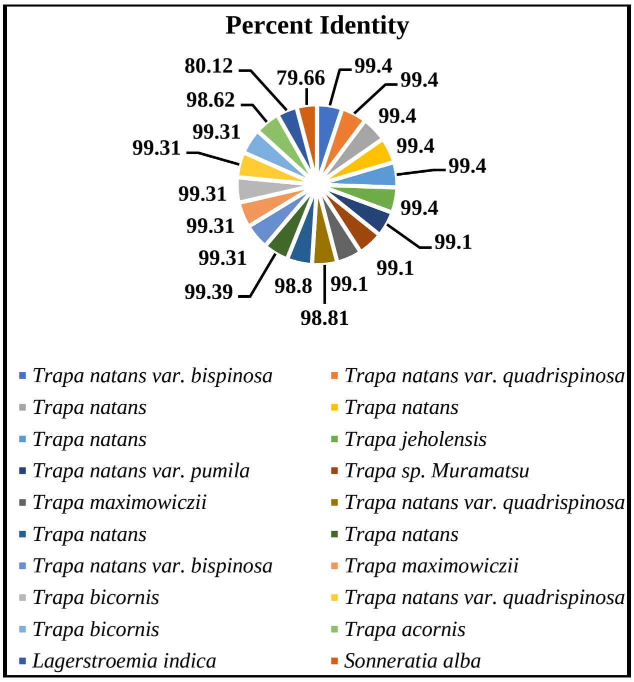

Fig. 4. Percentage identification of query coverage by BLAST.

ITS2 marker of Trapa natans

0 340

Fig. 5. Line barcode and QR code of ITS2 marker for identification of *Trapa natans* L.

Taxonomic identification of species from ITS2 marker sequence data: By utilizing ITS2 marker sequence, the NCBI taxonomy browser revealed that plant is *Trapa natans* L., commonly known as "Water Chestnut". In the NCBI BLAST database, its taxonomic ID is 22666. It is classified under "Eudicotes" and its taxonomic rank is "Species".

Line DNA barcode and QR code generation: Line barcode and QR code of conserved gene sequence was generated by BIO-RAD DNA Barcode Generator [https://biorad-ads.com/dnabarcodeweb/] and TEC – IT QR code generator [https://barcode.tec-it.com/en/QRCode] as shown in Fig. 5.

Features of Conserved gene sequence used as barcode: Line barcode of conserved ITS2 gene sequence generated by BIO-RAD DNA Barcode Generator (https://biorad-ads.com/dnabarcodeweb/) revealed the following features as shown in Table 1.

Table 1. Features of conserved ITS2 gene sequence.

Region	Number of conserved sites	Number of variable sites	G + C %
ITS2	340	3	60

Phylogenetic tree based analysis: The evolutionary descent was constructed using maximum likelihood approach based on the Tamura – Nei model, and was utilized to construct a phylogenetic tree with MEGA6 software, employing 1000 bootstrap repeats.

The tree shown (Fig. 6) is rooted, indicating a common ancestor at the base from which all other species evolved, representing evolutionary relationship among various species of genus *Trapa* with genera *Lagerstroemia* and *Sonneratia* serving as outgroups. The tree is divided into two primary lineages stemming from a common ancestor, consisting of an in – group and an outgroup. The in – group consists majority of the samples belong to the genus *Trapa*, including various subspecies and varieties. The outgroup consists of *Sonneratia alba* Sm., *Lagerstroemia indica* L. and *Lagerstroemia tomentosa* C. Presel. These also belong to the Lythraceae family, serving as a reference point to root the *Trapa* phylogeny.

The tree exhibits a high degree of polytomy i.e. very short internal branch lengths (i.e. 0.000) within the *Trapa* genus, proposing highly conserved genetic sequences among these taxa. The top major clade contains a large cluster of *Trapa natans* L. variants and its closely related species like *T. acornis* Osbeck. and *T. jeholensis* Nakai. A smaller distinct clade towards the bottom contains *T. natans var. pumila* Nankano ex Verdc., *T. maximowiczii* Korsh., *Trapa sp. Muramatsu*, and *T. incisa* Sieb. & Zucc. Unlike the top major clade, the lower clade containing *Trapa incisa* Sieb. & Zucc. and *Trapa* sp. *Muramatsu* shows small but measurable distances of 0.0036. This suggests that *Trapa incisa* Sieb. & Zucc. which is often characterized by smaller, more triangular leaves and smaller fruits may be more evolutionarily distinct or represent an older lineage within the genus compared to the "standard" *Trapa natans* L. complex. The branch length of 0.1619 at the base of the *Trapa* cluster indicates a significant genetic divergence between the genus *Trapa* and its relatives *Lagerstroemia* and *Sonneratia.* The outgroup *Sonneratia alba* Sm. shows the longest terminal branch of 0.0892, indicating it as the most genetically distinct taxon in this set.

The *Trapa* genus likely underwent a rapid expansion and diversification into different morphotypes like the different horn shapes seen in *T. natans* L. vs. *T. bicornis* Osbeck. so quickly that their genomes haven't had enough time to accumulate distinct mutations. This suggests a recent adaptive radiation.

BLAST analysis of the ITS2 sequence against the GenBank database (NCBI) identified the top 20 matches, primarily *T. maximowiczii* Korsh., *T. jeholensis* Nikai., and *T. natans* L. (including varieties *bispinosa* (Roxb.) Makino, *quadrispinosa* (Roxb.) Makino and *pumila* Nankano ex Verdc.). The maximum alignment score was 606 (minimum 601), with 100% query cover indicating complete alignment. The extremely low E-value (5e-169 to 2e-167) confirmed high sequence similarity. The percentage identity was 99.41% for *T. natans* L. and 99.10% for *T. jeholensis* Nikai., indicating minor genetic variation. The ITS2 locus was further validated as an effective barcode through multiple sequence alignment.

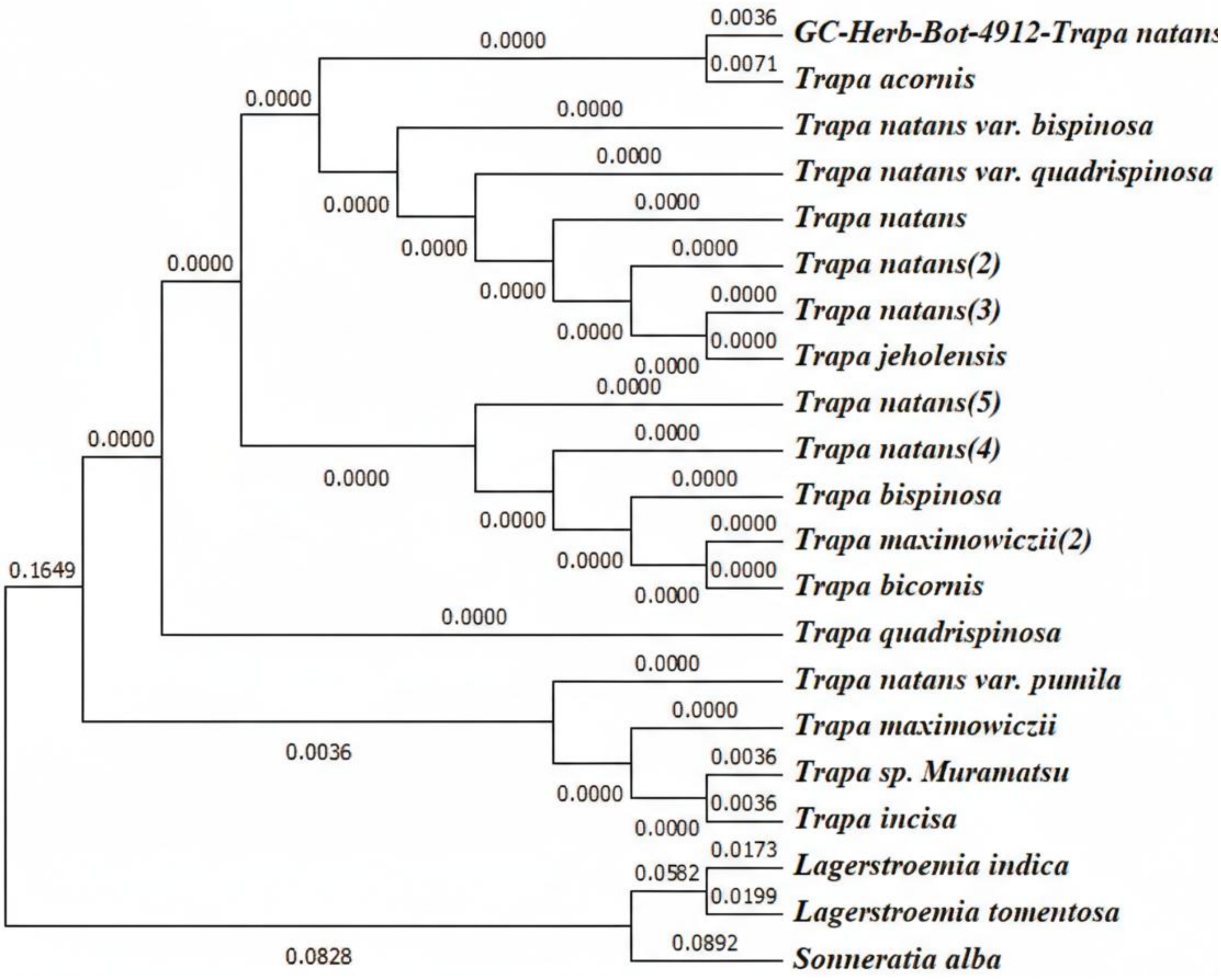

Fig. 6. Evolutionary relationships of taxa based on ITS2 DNA barcode.

Discussion

Taxonomic knowledge is essential for effectively sharing research insights across scientific domains, including medicine, agriculture and forestry (Musvuugwa *et al.*, 2021). Classical taxonomy, primarily based on morphological characteristics, faces limitations, such as reduced accuracy in identifying cryptic species or those exhibiting phenotypic plasticity (Zaman *et al.,* 2025). Moreover, morphological resemblance does not always indicate phylogenetic affiliation, as convergent evolution can lead genetically distinct species to appear similar (Wiens, 2004). Accurate morphological identification also requires broad expertise to avoid misidentification among look alike species (Bianchi & Gonçalves, 2021). Advancement in DNA based techniques have revealed numerous taxonomic errors based solely on morphology (Sheth & Thaker, 2017). Therefore, integrative taxonomy has emerged, coalescing morphology with molecular, ecological, and behavioural characteristics. DNA barcoding based phylogenetic analysis enhance precision and address the shortcomings of classical taxonomy (Miralles *et al.*, 2024).

Trapa natans L. is notable for its globally confusing morphology (Takano & Kadono, 2005). It was described by Linnaeus (1753) (family Trapaceae), it was later placed in Onagraceae (Palm *et al*., 2024). But BLAST results of ITS2 barcode firmly position *Trapa* species within the family Lythraceae, order Myrtales (Xue *et al*., 2017).

In this study, nuclear marker - ITS2 was applied to identify *Trapa natans* L., as ITS2 has proven more efficient than chloroplast genes *rbcL* and *matK* for the development and utility of universal primers, sequencing success, species resolution and establishing phylogenetic relationship (Al–Juhani, 2019). Our results confirm that ITS2 is readily amplified and sequenced due to conserved regions suitable for universal primer design. It serves as a universal plant DNA barcode and a complementary locus to *CO1* for animals (Mao *et al*., 2021; Pere *et al*., 2024), boasting a high species identification success rate (Chen *et al*., 2010; Devi *et al*., 2022; Basavaraju *et al*., 2023). ITS2 exhibits significant interspecific divergence with minimal intraspecific variation, making it highly effective for distinguishing plant species (Zheng *et al.,* 2021) and reliable for biodiversity assessments, identifying rare/endangered species, and authenticating medicinal plants and adulterants (Ibrahim *et al*., 2024). Results of the current study are in concurrence with Al–Juhani, (2019) and Acharya *et al*., (2022), revealing that ITS2 DNA barcoding is a superior approach in species identification and determination of phylogenetic relationship of medicinally important plants like *Trapa natans* L. over chloroplast genomic marker like *rbcLa* and *matK*.

Phylogenetic tree interpretation provides superior species resolution compared to other nucleotide-based methods (Kapli *et al.,* 2020; Liu *et al.,* 2021). The ITS2-based phylogenetic tree, with nodes supported by 100% bootstrap values, reinforces that a single barcode can be effective for resolving species in smaller datasets where molecular and morphological distinctions are challenging (Birch *et al.,* 2017; Acharya *et al.,* 2022; Abbas *et al.,* 2025). The ITS2 revealed a monophyletic phylogeny of *Trapa* species and serves as a reliable marker for evaluating genetic divergence. The current findings proposed a scientific foundation for species recognition, genetic conservation, and the safe utilization of this medicinally and ecologically significant plant.

Although ITS2 is powerful single nuclear marker for *Trapa* identification and phylogenetics, as it provides biparental data, absence of complementary chloroplast DNA marker data leaves cytoplasmic inheritance patterns unexplored. Future studies should employ "Integrative Taxonomy", combining ITS2 data with chloroplast marker *trnH-psbA* to detect natural hybridization and maternal lineages. To move beyond species identification, further research should utilize microsatellites or High Throughput Sequencing (HTS) to assess the gene flow between fragmented populations in different wetlands of Pakistan. Such data will be crucial for developing site specific conservation strategies for these ecologically sensitive wetlands.

Conclusion

Precise plant identification is vital for a wide range of biological fields and studies including agriculture, medicine, biodiversity assessment and conservation. This study successfully validated the ITS2 nuclear region as a highly efficient and reliable DNA barcode for molecular identification and phylogenetic reconstruction of medicinally significant aquatic plant *Trapa natans* L. (water chestnut). The results confirmed that the ITS2 barcode alone serves as a universal marker for species – level identification of aquatic plants and provides superior species resolution and primer universality compared to cpDNA markers such as *rbcL* and *matK*. Ultimately, this molecular framework provides a critical scientific basis for the genetic conservation and sustainable ecological management of *Trapa* species and other aquatic plants.

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Data Availability: All data from the current study are included in this manuscript.

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