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MOLECULAR PHYLOGENY OF PHYTOREMEDIATOR AQUATIC WEED *Pistia stratiotes* L. BY *rbcLa* BARCODING FROM BALLOKI HEADWORKS, PUNJAB, PAKISTAN

W. Abbas, M. Iqbal, Zaheer-ud-din Khan and M. U. F. Awan*

Department of Botany, GC University, Lahore, Pakistan
Corresponding author's Email: dr.umerfarooqawan@gcu.edu.pk

ABSTRACT

Pistia stratiotes L. is a pantropical aquatic weed, also known as water lettuce. It is native to tropical and subtropical lakes of Brazil and Chile. It is a floating stoloniferous plant that can influence aquatic ecosystems and limnological characteristics. *Pistia stratiotes* L. can be used to improve the quality of water and decrease the risk of eutrophication in aquatic ecosystems. Despite its ecological significance as a phytoremediator and its invasive distribution, it is necessary to investigate genotypic plasticity. The current study focuses on the molecular identification of *Pistia stratiotes* L. and explores its phylogenetic relationship with closely related species. Specific primers for *rbcLa* were applied for PCR amplification. PCR products after purification were subjected to *rbcLa* sequence barcode. Chromas was applied to analyse the chromatogram file. Sequence alignment was completed by multiple sequence alignment (MSA) and Clustal 2.1. Basic Logical Alignment Sequence Tool (BLAST) of the NCBI database was used to align the query sequence. The *rbcLa* marker barcode showed 99.46% accuracy for the identification of species. The DNA barcode based phylogenetic tree revealed that *Pistia stratiotes* L. is the only surviving specie of this genus and its closest relative is *Hydrilla verticillata* (L.f.) Royale belonging to the family Hydrocharitaceae.

Key words: Aquatic ecosystems, DNA barcode, *rbcLa*, Hydrophytes, Phylogeny.

INTRODUCTION

The traditional identification of plants requires complete morphological data including flowers and fruits (Hebert *et al*., 2003; Gonzalez *et al*., 2009; Düşen *et al*., 2018; Göktürk *et al*., 2019). Plant species identification by DNA barcoding is an innovative technique that not only identifies species but also reveal their phylogeny (Hebert *et al*., 2003). DNA barcoding is a molecular technique for proper species identification, differentiation and discovery of new species (Hollingsworth *et al*., 2011; Abdi *et al*., 2024). DNA barcodes are specific regions of DNA that can be amplified and sequenced regularly using universal primers. These standardized short DNA sequences serve as a unique identifier for species (Parmentier *et al*., 2013; Antil *et al*., 2023). Through extensive studies and comparing plant barcode sequences with international DNA barcode Data bases, it is easy to recognize unknown plant samples, as well as efficiently evaluate, understand, preserve and utilize biodiversity on a broad scale (Kress *et al*., 2015; Fatima *et al*., 2019; Üstüner *et al*., 2022; Letsiou *et al*., 2024)**.**

DNA barcoding plays a crucial role in plant genomic conservation and in creating genetic databases of rare species. It helps in preventing illegal trade and ensures the regulation of plant based products, including herbal remedies and other trade goods (Kress, 2017). The exploring plant DNA barcodes in a broad spectrum, is both delicate and challenging, especially when compared to the animal barcode region of the mitochondrial gene COI, which is proven ineffective in plants (Nehal *et al*., 2021). Additionally, Hybridization, limited sequence polymorphism, low rates of nucleotide substitution, and frequent gene flow between closely related species are significant obstacles in selecting a universal barcode for land plants (Hollingsworth *et al*., 2011), therefore the Consortium for Barcode of Life (CBOL) has determined barcoding regions from the genome of chloroplast (CBOL Plant Working Group, 2009). The chloroplast genome is smaller than that of the mitochondria and nucleus. cpDNA in higher plants evolves more slowly and undergoes fewer internal changes (Mo *et al*., 2020). This slow rate of evolution makes cpDNA suitable for studying plants beyond the family level (Shneyer, 2009). The Plant Working Group of CBOL was established to identify plant DNA barcodes (Kress *et al*., 2005). By 2009, Plant Working Group of CBOL suggested "Barcodes" for plants, which cover chloroplast genome regions including coding areas (*matK, accD, ccsA* and *rbcL*) and non-coding areas (*trnh – psbA, atpF – atpH*)

(CBOL Plant Working Group, 2009). The combination of *matK* and *rbcL* is proposed as universal because of the easy recovery of the *rbcL* region and the discriminatory power of the *matK* sequence (Hollingsworth *et al*., 2011).

The ribulose – 1,5 – bisphosphate carboxylase/oxygenase (RubisCO) gene is of 1400 base pairs, plays a pivotal role in CO_2 fixation in plants and algae. RubisCo consisting of 8 large subunits (LSU) and 8 small subunits (SSU). The large subunit is encoded by gene *rbcL* of chloroplast and is the most abundant protein on Earth (Tabita *et al*., 2007). This gene is crucial for the initial stages of the Calvin cycle in plants and remained highly conserved throughout evolution. Unlike certain chloroplast encoded genes the RubisCO lacks introns which highlights one of the significant advantages of the *rbcL* gene. The *rbcL* gene along with the *matK* gene mostly serve as a marker for DNA barcode. The *matK* gene's higher variability enables better intra-specific investigation, while the conserved nature of *rbcL* provides greater discriminatory power across species (Kress and Erickson, 2007). Generally, to determine evolutionary relationship among spermatophytes, the APG (Angiosperm Phylogeny Group) system is applied but APG analysis gives low-resolution phylogenetic trees which prevent the distinguishing relatedness at the species level (Hermsen, 2020; Tokgöz *et al*., 2024; Yuceol *et al*., 2024). DNA barcoding offers a unique method to achieve this goal by creating standard DNA sequences, DNA barcoding can identify evolutionary relationships among various plant species within the same family. Once sequences are obtained the phylogenetic trees using DNA barcodes can be constructed even for the individuals with confusing morphological features. Moreover, based on sequence similarity, the families and genera of unidentified individuals can be inferred from Barcode of Life Data (BOLD) system and/or GenBank (Kress and Erickson, 2008; Antil *et al*., 2023; Chac and Thinh, 2023).

Hydrophytes are plants that can thrive in water either partially or completely submerged. The hydrophytes have adopted the aquatic way of life in subsequent successions. They have modified themselves to survive in an aquatic ecosystem. In aquatic ecosystems, fluctuation in temperature is insignificant and nutrients are primarily present in a soluble state (Hamid *et al*., 2020). *Pistia stratiotes* L. is a stoloniferous, perennial, and surface floating aquatic macrophyte. When its old leaves degenerate its fruit sink to the bottom of the water body, where they decay and liberating the seeds for growth of new plants. *Pistia stratiotes* L. is highly invasive due to its speedy growth. Its average biomass yield is 200 tons/ hectare. Its biomass doubles every 10 to 15 days depending on the temperature of water and nutrients (*Pistia Stratiotes* L. | Species, 2024).

Pistia stratiotes L. is a promising option for phytoremediation of heavy metal - polluted water bodies. It is effective in removing chromium, lead and nickel from waste water (Zahari *et al*., 2021; Samal and Dash, 2024). It was able to remove up to 99.31% of lead and 76.25% chromium at 1mgL^{-1} concentration within two-week time period. The plant was particularly efficient at concentrating lead in its roots, reaching up to 15,000 mg Kg^{-1} in plant tissue (Zahari *et al*., 2021).

The Ravi river is a transboundary river between Pakistan and India and is a vital component of Indus river basin (Kanwar *et al*., 2021). Balloki Headworks is built on the Ravi river and is located approximately 65 km southwest of Lahore, Punjab, Pakistan. This Headworks feeds two canal systems viz. Lower Bari Doab Canal and Balloki Sulemanki link canals. It was constructed in 1913 by the British government in the form of level crossing where supply from the upper Chenab was dropped into the Ravi river on the right bank (Balloki Barrage International Commission on Irrigation & Drainage (ICID), 2024). Balloki Headworks provides numerous wetlands that plays a crucial role in maintaining the balance of the ecosystem. Many plant species in these wetlands have been catalogued, but the approach of identifying them through DNA barcoding has been largely overlooked. This study was designed for the molecular identification of *Pistia stratiotes* L. and to construct its phylogenetic tree using the Maximum Likelihood method, employing the discriminatory power of *rbcLa* marker gene as standard barcode.

MATERIALS AND METHODS

Survey Site: The survey site was Balloki Headworks located near Phool Nagar, Kasur District, nearly 65 Km distant from Lahore (Kasur, 2024), with coordinates of 31.2222 North and 73.8591 East.

Specimen Collection: The *Pistia stratiotes* L. specimens were collected from aforementioned survey site during summer of 2023. Plant collection and subsequent preservation were done according to standard "surface inventory" method (Parsons 2001).

Plants collected from the study site were well-preserved after drying and by mounting on herbarium sheets by following customary herbaria methods. Then these preserved samples were deposited in Dr. Sultan Ahmed herbarium, Department of Botany, GC University Lahore, Pakistan after posting voucher numbers.

DNA Extraction: Extraction and purification of DNA was executed according to amended CTAB method as described by Yameen *et al*. (2024).

DNA Quantification: Quantification of DNA was made by Nano Drop microvolume spectrophotometer (Thermo

Fisher Scientific) at A_{260}/A_{280} according to the method described by García-Alegría *et al*. (2023).

Agarose Gel Electrophoresis: For the confirmation of isolated DNA, 1% Agarose gel electrophoresis was executed according to Armstrong and Schulz (2015).

PCR Amplification: Confirmed cpDNA extracts were amplified by PCR for the *rbcLa* genetic marker using specific primers, as described by Lorenz (2012). The details are as follows:

Figure 1. GPS Map of Balloki Headworks

Table 1. Primers used for amplification

Primer	Length	%GC	Melting temperature (tm) °C	5´ to 3´ sequence	Reference
rbcLa-F	26	42	61	ATGTCACCACAAACAGAGACTAAAGC	Levin *et al*. (2003)
rbcLa-R	19	47	53	GTAAAATCAAGTCCACCRCG	Kress and Erickson (2007)

Amplified gene was confirmed by comparison with 1 kb DNA ladder (Invitrogen – Thermo Fisher Scientific) using 1% Agarose gel electrophoresis. The purified PCR amplification products were forwarded to Macrogen Inc., Seoul, Republic of Korea for Sanger sequencing.

Data Analysis and Sequence Alignment: Subsequent upon gene sequencing, the chromatogram file was obtained which was analyzed by Chromas (Subbotin,2021). Afterward, align the sequences using the multiple sequence alignment (MSA) technique. Subsequently, BLAST (Basic Logical Alignment Search Tool) the gene sequence from the NCBI database and aligned this query sequence (Kuznetsov and Bollin, 2020).

Phylogenetic Tree - based Analysis: By ensuing maximum likelihood method, the phylogenetic tree was created based on *rbcLa* gene with 1000 bootstrap replicates in MEGA6 (Tamura *et al*., 2013). The phylogeny was deduced via Maximum Likelihood methodology derived from Tamura-Nei model (Tamura and Nei, 1993).

RESULTS

cpDNA Extraction and Quantification: cpDNA Quantification from *Pistia stratiotes* L. specimens was done through Nano - Drop micro volume spectrophotometer (Thermo Fisher Scientific) at A_{260}/A_{280}. The average value obtained was ~1.83 which represented the pristine quality of DNA samples. García-Alegría *et al*. (2023) also explained that a ratio of ~1.8 is generally considered as pure DNA.

Gel Electrophoresis of cpDNA: Further validation of isolated cpDNA from *Pistia stratiotes* L. was through 1.0% Agarose Gel electrophoresis as shown in Fig. 2. First well denoted by L is 1kb DNA ladder (Invitrogen – Thermo Fischer Scientific), Well No. 1, 2, 3 and 5, 6, showed undamaged DNA.

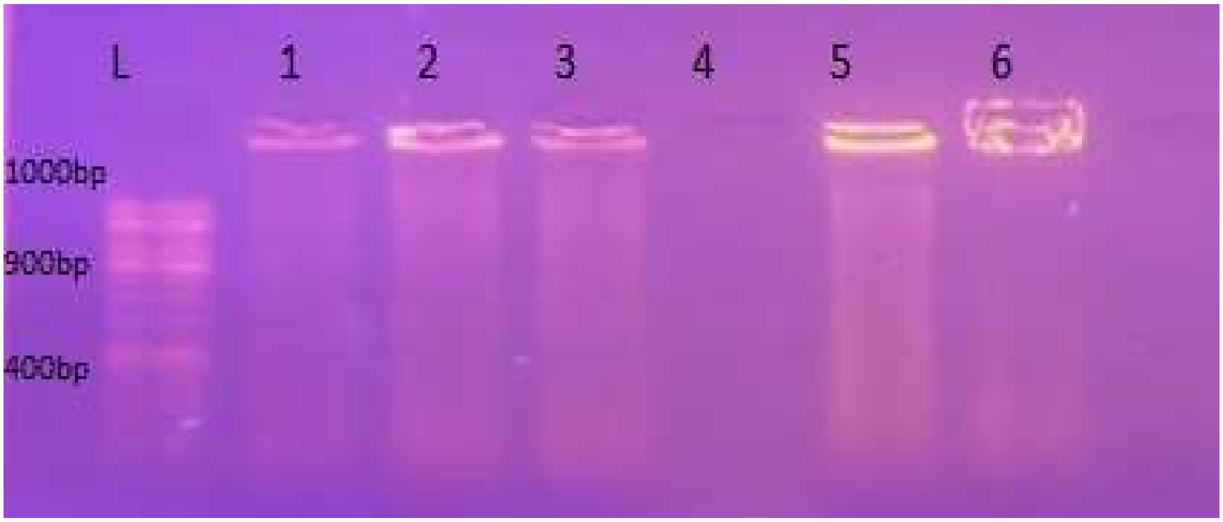

Figure 2. cpDNA extracted from *Pistia stratiotes* L.

PCR Amplification: The *rbcLa* marker was successfully amplified by utilizing following universal primers *rbcLa*-F: 5’- ATGTCACCACAAACAGAGACTAAAGC-3’ (Levin *et al*., 2003) and *rbcLa* – R: 5’ - GTAAAATCAAGTCCACCRCG - 3’ (Kress and Erickson, 2007**).**

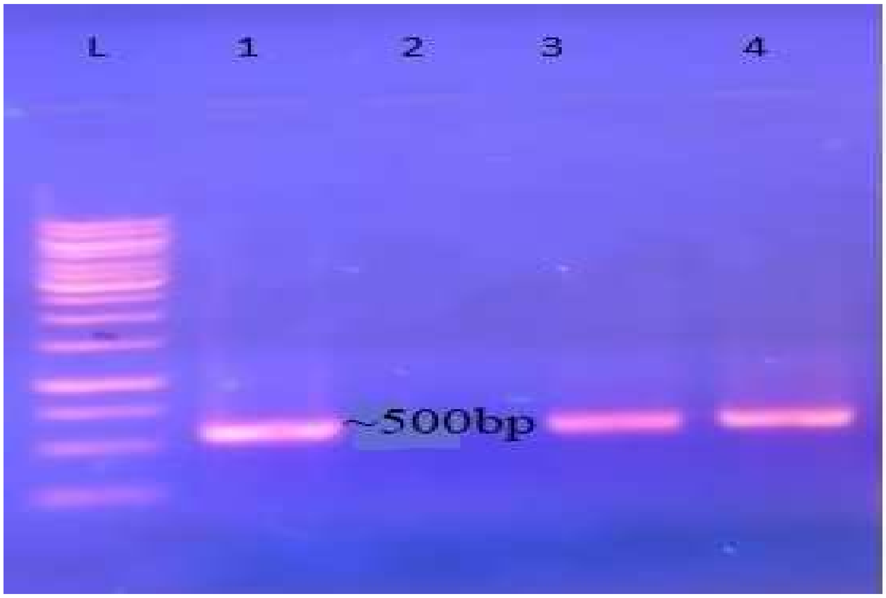

Figure 3: PCR amplification results for *rbcLa* observed on 1% agarose gel for *Pistia stratiotes* L.

The Figure. 3 illustrates the PCR amplification results for *rbcLa* observed on 1% agarose gel. Well L contain 1kb ladder (Invitrogen – Thermo Fisher Scientific). Well No. 1, 3, and 4 contain amplified marker and show clear bands without any smearing, corresponding to approximately 500bp.

Multiple Sequence Alignment (MSA): Subsequent DNA sequencing, the chromatogram file was evaluated by using Chromas software (Subbotin,2021)**.** Both chromatograms of forward and reverse sequences exhibited low - grade peaks in adjacent regions. To identify species by Basic Logical Alignment Sequence Tool (BLAST) of NCBI, forward and reverse sequences were segregated. By using the online reverse complement technique, single sequence was made from both forward and reverse sequences in which the forward sequence persists but reverse sequence changed into a reversed complement sequence (Schwartz *et al*., 2003; Henson *et al*., 2012). Afterward, the alignment was performed by Multiple Sequence Alignment (MSA) at Clustal 2.1 in Geneious. Discontinuous stars at both termini were shown in reverse complement due to primer site, unforeseen sequence, and variable nucleotides. The mid portion of reverse complement showed continuous stars (510 nucleotides) was selected as a DNA barcode for the data repository. Then NCBI's BLAST was used for species identification by this sequence (Arnold *et al*., 2020).

Species identification by BLAST: The obtained DNA sequence of *rbcLa* gene was 585bp; after cleaning it was reduced to 510bp with an average of 510±2. The base quality score of sequencing was 99.46%. Samples being examined were those that presented Max. Percentage Identity (PI%) (**Table 2**).

Query session No. **2953481,** 99.46% match with *Pistia stratiotes* L. which we already identified through morphological characteristics.

Taxonomic Identification of Species from *rbcLa* marker sequence data: The NCBI taxonomy browser revealed that plant is *Pistia stratiotes* L., commonly known as "water lettuce" and "shellflower". Its Taxonomic ID is 4477. In the NCBI BLAST database, it is classified under "monocots". Its taxonomic rank is "Species".

Table 2. BLAST results

Description	Values
Maximum Score	1018
Query cover	97%
E Value	0.0
Percentage Identity(PI)	99.46
Query Session No.	2953481

Table 3. Taxonomic hierarchy according to NCBI Taxonomy Browser

Nomenclatural Rank	Name
Domain	Eukaryota
Kingdom	Viridiplantae
Phylum	Streptophyta
Subphylum	Embryophyta
Division	Tracheophyta
Subdivision	Euphylophyta
Class	Magnoloipsida
Super order	Mesangiospermae
Order	Alismatales
Family	Araceae
Sub Family	Aroideae
Tribe	Pistiae
Genus	*Pistia*

Analysis by Phylogenetic Tree formation: The evolutionary lineage was deduced 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. **(Saitou and Nei, 1987; Tamura and Nei, 1993; Tamura *et al*., 2013**).

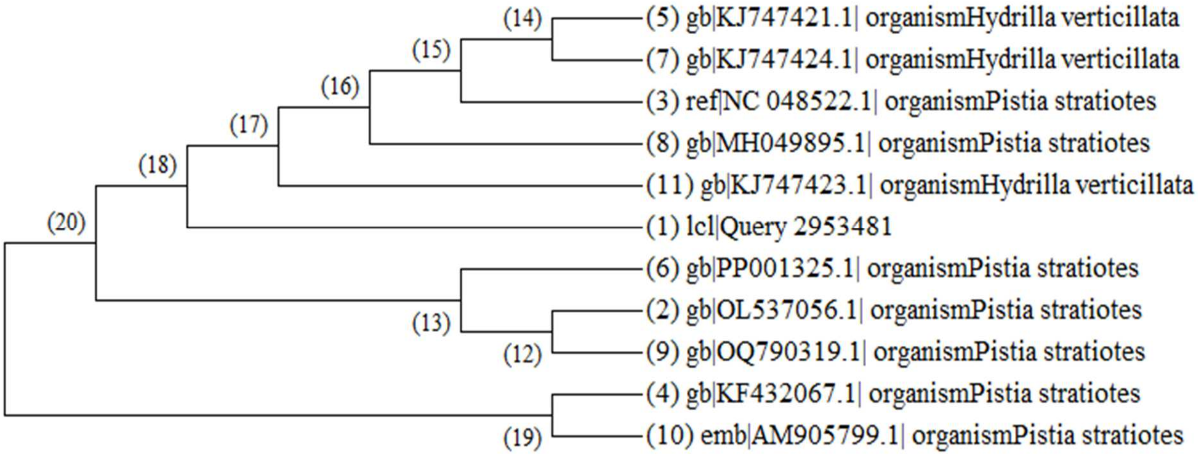

Figure 3. Evolutionary Relationships of Taxa

DISCUSSION

Pakistan is among the countries affected by global climate change, with erratic weather conditions and anthropogenic activities leading to the demolition of natural aquatic ecosystems (Hussain *et al*., 2020). Wetlands deliver crucial ecological functions for conservation of biodiversity and providing place for recreational activities (Janse *et al*., 2019). Punjab, Pakistan, is divided into tropical and subtropical regions, both rich in diverse flora and fauna at various organizational levels within the ecosystem. The genetic database of majority of its flora is unexplored specially the aquatic flora (Arshad *et al*., 2024). The aquatic plants have great ecological and medicinal importance. The restoration of natural water bodies is important for conservation of indigenous aquatic flora. Management of water bodies for conserving aquatic flora and fauna is not yet fully addressed in a way that will conserve genetic resources for farming and agriculture. There are very limited efforts to conserve aquatic genetic resources (FAO 2019).

Established waterbodies like dams and Headworks have extensive growth of hydrophytes which can cause serious economic and ecological damage, once they get in to it (Kaur *et al*., 2018). Their enormous growth in rivers, canals, barrages and dams affect hydropower generation and irrigation. Invasive hydrophytes also intimidate aquatic biodiversity by substituting indigenous flora and fauna there by producing irrevocable alterations to habitations (Dudgeon *et al*., 2006; Ghosh *et al*., 2021). Globally, the most notorious invasive aquatic plants include *Pistia stratiotes* L. (water lettuce), *Eichhornia crassipes* L. (water hyacinth), *Salvinia molesta* D.S. Mitch. and *Typha* (cattail) (Ghosh *et al*., 2021).

Pistia stratiotes L. (water lettuce) is an invasive hydrophyte and its ability to grow in waste water make this weed a potential resource for fodder, biomass for energy production, and bioremediation (Kaur *et al*., 2018). The conservation of local flora needs identification of species is the first step for establishment of genetic data bank. Species identification is a complicated process due to similarities in morphological characteristics (Páll-Gergely *et al*., 2024).

DNA barcoding has modernized systematics and can serve as a valuable tool for conservation, particularly in the study of invasive plants (Nath *et al*., 2024). This modern technique can help researchers to identify floral diversity more precisely for conserving local flora. This methodology is based on short DNA fragments to allot plant taxa to their clade with great precision (Cahyaningsih *et al*., 2024). The DNA barcoding technique has enormous prospective and could be helpful in production of novel materials for pharmaceutics and alternative energy resources (Constantinescu and Sicuia, 2020). The nuclear genome has *ITS* and mitochondrial genome has *COI* while chloroplast genome has *rbcL*, *matK*, *ycf3- trnS, and psbA-trnH* genes which are considered as highly conserved regions. These conserved genes are being utilized as standard DNA barcodes (CBOL 2009). Lonare *et al*. (2024) and Nath *et al*. (2024) recognized DNA barcoding as an effective tool for identification of native and invasive plant species. DNA barcoding has proved itself as a reliable way of plant identification process (Waterton and Wynne, 2013).

It was a successful attempt to establish the DNA barcode of commonly occurring aquatic plant, *Pistia stratiotes* L. (water lettuce) applying universal primers for *rbcLa* (Levin *et al*., 2003; Kress and Erickson, 2007). *rbcLa* was amplified successfully for *Pistia stratiotes* L. which implies it as a more appropriate DNA barcode for hydrophytes as well as terrestrial plants (Hollingsworth *et al*., 2009; Niemann *et al*., 2022; Espinosa *et al*., 2023) The BLAST results for conserved sequence query matched *Pistia stratiotes* L. Results of current investigation reveal that *rbcLa* is remarkably useful resource for recognizing species as reported by Espinosa *et al*. (2023) and Yameen *et al*. (2024). Therefore, *rbcLa* is enough for recognizing plant species of native as well as invasive nature, as stated by Dong *et al*. (2014) and Yameen *et al*. (2024).

Phylogeny in the form of a tree is interpreted as a better technique for species resolution than other nucleotide based techniques **(**Hollingsworth *et al*., 2016; Liu *et al*., 2021). *rbcLa* barcode - based phylogenetic tree had percentage of nodes having 99.46% bootstraps reinforces the concept that single barcode is idyllic in contrast to multi locus barcode for a minor data (Trujillo-Argueta *et al*., 2022).

Results of current study revealed that *Pistia stratiotes* L. and *Hydrilla verticillata* (L.f.) Royale are related to each other through a distant common ancestor as depicted by Figure. **3**. This phylogenetic relationship has ecological implications as this could be helpful to identify genetic traits that contribute to their invasiveness, such as rapid reproduction and adaptability to diverse aquatic environments (Colautti *et al*., 2017; Hohenlohe *et al*., 2021). As both of these plant species share similar ecological niche, they may out compete native species, leading to biodiversity loss (Ghosh *et al*., 2021). One more common characteristic of these plants is the formation of dense mats that block sunlight and deplete oxygen levels in aquatic ecosystems (Ismail *et al*., 2021).

DNA barcoding applying *rbcLa* genetic marker is an approved technology for the rapid identification of plant species, including invasive ones. It can facilitate early intervention before they establish dominance over native species, aiding in reintroduction efforts and restoring ecological balance (Kokou *et al*., 2021; Madeira *et al*., 2022).

Conclusion: Various plant species in aquatic ecosystems have been identified using traditional methods, but DNA barcoding has opened a new window in plant identification and establishing phylogenetic relationship. In this study, aquatic macrophyte *Pistia stratiotes* L. was identified using *rbcLa* DNA barcoding. The findings of this research will aid in expanding the application of DNA barcoding in aquatic plants. It was found that *rbcLa* is beneficial for the barcoding of plant species with the current universal primers, however, further research is required to enhance barcoding techniques and develop locally verified databases. This investigation demonstrated the *rbcLa* barcode as a valuable tool for biodiversity assessment and genetic conservation of aquatic plants.

Future Research Directions: The water bodies in Punjab, Pakistan, are receding due to rapid climate change, which is also wiping out aquatic floral biodiversity. Therefore, further investigations are needed to establish rapid and easy methods for establishing genetic database for plant conservation, which could aid in developing new plant varieties capable of tolerating changing environmental conditions.

Limitations of the Study: Recent study is mainly emphasized on the molecular phylogeny of *Pistia stratiotes* L., a well-known invasive hydrophyte so we specifically collect it from Balloki Headworks and canals originating from here. There were some challenges we faced during DNA extraction by CTAB method described by Doyle (1991) but then we use modified CTAB method as described by Yameen *et al.* (2024). During PCR amplification we face problems by *matK* and *ITS*2 marker which do not amplify with universal primers. For amplification of *rbcLa*, no specific problems were faced by universal primers and conditions described by Levin *et al.* (2003), Kress and Erickson (2007) and Lorenz (2012).

Ethical Approval: Not applicable

Author Contributions: MUF, ZK and MI contributed to the study conception and design. Material preparation, sample and data collection and analyses were performed by WA. The draft of manuscript was written by WA, and all authors commented on manuscript. All authors read and approved the final manuscript.

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Conflict of interest: Authors have no relevant financial or non-financial conflicts of interest to disclose.

Data Availability: All data from the current study are included in this manuscript. The data and materials will be available on request.

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REFERENCES

Abdi, G., S. Singh, S. Selvakumar, S.K. Dhar, G. Mudgal, P. Swaminathan and M.A. Tarighat (2024). DNA barcoding and its applications. In Advances in Genomics: Methods and Applications (pp. 91-117). Singapore: Springer Nature Singapore. https://doi.org/10.1007/978-981-97-3169-5_5

Antil, S., J.S. Abraham, S. Sripoorna, S. Maurya, J. Dagar, S. Makhija and R. Toteja (2023). DNA barcoding, an effective tool for species identification: a review. Mol. Bio. Rep. 50(1): 761-775. https://doi.org/10.1007/s11033-022-08015-7

Armstrong, J.A. and J.R. Schulz (2015). Agarose gel electrophoresis. Current Protocols Essential Laboratory Techniques. 10(1): 7-2. https://doi.org/10.1002/9780470089941.et0702s10

Arnold, N.D., D. Garbe and T.B. Brück (2023). Isolation, biochemical characterization, and genome sequencing of two high-quality genomes of a novel chitinolytic Jeongeupia species. Microbiol. Open. 12(4): e1372. https://doi.org/10.1002/mbo3.1372

Arshad, F., M. Shoaib, M.A. Jameel, M. Waheed, S.M. Haq, S. Iftikhar, and R.W. Bussmann (2024). Vegetation profile and ecological traits in irrigated plains of semi-arid regions: a case study of Okara, Punjab Pakistan. Eco. Front. 44(4): 700 – 711. https://doi.org/10.1016/j.ecofro.2024.01.003

Balloki Barrage | International Commission on Irrigation and Drainage (ICID). (2024, April 29). International Commission on Irrigation and Drainage (ICID). https://icid-ciid.org/award/his_details/37

Cahyaningsih, R., I. Martiansyah, S. Hidayat and M.R. Hariri (2024). DNA barcoding of medicinal plants for conservation purposes. In Ethnopharmacology and OMICS Advances in Medicinal Plants Volume 2: Revealing the Secrets of Medicinal Plants (pp. 163-212). Singapore: Springer Nature Singapore. https://doi.org/10.1007/978-981-97-4292-9_9

CBOL Plant Working Group (2009). A DNA barcode for land plants. Proceedings of the National Academy of Sciences of the United States of America, 106(31): 12794–12797. https://doi.org/10.1073/pnas.0905845106

Chac, L.D. and B.B. Thinh (2023). Species identification through DNA barcoding and its applications: a review. Bio. Bull. 50(6): 1143-1156. https://doi.org/10.1134/s106235902360229x

Colautti, R.I., J.M. Alexander, K.M. Dlugosch, S.R. Keller and S.E. Sultan (2017). Invasions and extinctions through the looking glass of evolutionary ecology. Phil. Trans. Roy. Soc. B: Bio. Sci. 372(1712): 20160031. https://doi.org/10.1098/rstb.2016.0031

Constantinescu, F. and O.A.B. Sicuia (2020). Phytonanotechnology and plant protection. In Phytonanotechnology (pp. 245-287). Elsevier. https://doi.org/10.1016/b978-0-12-822348-2.00013-9

Dong, W., T. Cheng, C. Li, C. Xu, P. Long, C. Chen and S. Zhou (2014). Discriminating plants using the DNA barcode rbc L b: an appraisal based on a large data set. Mol. Eco. Reso. 14(2): 336-343. https://doi.org/10.1111/1755-0998.12185

Doyle, J. (1991). DNA protocols for plants. In Molecular techniques in taxonomy (pp. 283-293). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-83962-7_18

Dudgeon, D., A.H. Arthington, M.O. Gessner, Z.I. Kawabata, D.J. Knowler, C. Lévêque, and C.A. Sullivan, (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. Bio Rev. 81(2): 163-182. https://doi.org/10.1017/9781139032759.009

Düşen, O., R.S. Göktürk, E. Kaya, U. Sarpkaya and B. Gürcan (2018). Morphological and molecular determination of a new Viola species (Violaceae) from Turkey. https://doi.org/10.11646/phytotaxa.369.1.4

Espinosa, P.A., J.N. Beisel, P. Verschuren and L. Hardion, (2023). Toward freshwater plant diversity surveys with eDNA barcoding and metabarcoding. Env. DNA 5(4): 648-670. https://doi.org/10.1002/edn3.407

FAO (Food and Agriculture Organization) (2019). The state of the world's aquatic genetic resources for food and agriculture. Rome, Italy: FAO Commission on Genetic Resources for Food and Agriculture assessments. https://doi.org/10.4060/ca5256en

Fatima, T., A. Srivastava, P.V. Somashekar, V.S. Hanur and M.S. Rao (2019). Development of DNA-based species identification and barcoding of three important timbers. Bul. National Res Cent. 43: 1-17. https://doi.org/10.1186/s42269-019-0116-8

García-Alegría, A.M., I. Anduro-Corona, C.J. Pérez-Martínez, M.A. Guadalupe Corella-Madueño, M.L. Rascón-Durán and H. Astiazaran-Garcia (2023). Quantification of DNA through the NanoDrop spectrophotometer: methodological validation using standard reference material and Sprague Dawley rat and human DNA. Int. J. Ana. chem. (1): 8896738. https://doi.org/10.1155/2020/8896738

Ghosh, R., C. Özaslan and P. Ray (2021). Invasive alien freshwater hydrophytes: Co-facilitating factors with emphasis on Indian scenario. 216 – 229. https://doi.org/10.5958/10.5958/0974-8164.2021.00042.3

Göktürk, R.S., O. Düşen, E. Kaya, B. Gürcan and U. Sarpkaya (2019). A new variety of Plocama calabrica (Rubiaceae) from Denizli (Turkey) confirmed by morphological and molecular ISSR markers. Acta Bot. Croa. 78(2): 142-146. https://hrcak.srce.hr/225648. https://doi.org/10.2478/botcro-2019-0016

Gonzalez, M.A., C. Baraloto, J. Engel, S.A. Mori, P. Pétronelli, B. Riéra, A. Roger, C. Thébaud and J. Chave (2009) Identification of Amazonian Trees with DNA barcodes. PLoS ONE, 4, e7483. https://doi.org/10.1371/journal.pone.0007483

Hamid, A., S.U. Bhat and A. Jehangir (2020). Local determinants influencing stream water quality. App. Wat. Sci. 10(1): 1-16. https://doi.org/10.1007/s13201-019-1043-4

Hebert, P.D.N., A. Cywinska, S.L. Ball and J.R. deWaard (2003) Biological Identifications through DNA Barcodes. Proceedings of the Royal Society B: Bio. Sci. 270: 313-321. https://doi.org/10.1098/rspb.2002.2218

Henson, J., G. Tischler and Z. Ning (2012). Next-generation sequencing and large genome assemblies. Pharmacogenomics, 13(8): 901-915. https://doi.org/10.2217/pgs.12.72

Hermsen, E.J. (2020). Overview of angiosperm phylogeny. https://www.Digitalatlasofancientlife.Org; Paleontological Research Institute. https://www.digitalatlasofancientlife.org/learn/embryophytes/angiosperms/angiosperm-phylogeny/ https://doi.org/10.1036/1097-8542.yb120239

Hohenlohe, P.A., W.C. Funk and O.P. Rajora (2021). Population genomics for wildlife conservation and management. Mol. Eco. 30(1): 62-82. https://doi.org/10.22541/au.158480040.06912807

Hollingsworth, P.M. (2009). Selecting barcoding loci for plants: evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. Mol. Eco. Res. 9(2): 439 - 457. https://doi.org/10.22541/au.158480040.06912807

Hollingsworth, P.M., D.Z. Li, M. van der Bank and A.D. Twyford (2016). Telling plant species apart with DNA: from barcodes to genomes. Philosophical

Transactions of the Royal Society B: Bio. Sci. 371(1702): 20150338. https://doi.org/10.1098/rstb.2015.0338

Hollingsworth, P.M., S.W. Graham and D.P. Little (2011). Choosing and using a plant DNA barcode. PloS one, 6(5): e19254. https://doi.org/10.1371/journal.pone.0019254

Hussain, M., A.R. Butt, F. Uzma, R. Ahmed, S. Irshad, A. Rehman and B. Yousaf (2020). A comprehensive review of climate change impacts, adaptation, and mitigation on environmental and natural calamities in Pakistan. Env. Monit. Asses. 192: 1-20. https://doi.org/10.1007/s10661-019-7956-4

Ismail, S.N., M.A. Hamid and M. Mansor (2021). An overview of macrophytes in the tropical wetland ecosystem. Indo. J. Lim. 2(1): 25-34. https://doi.org/10.51264/inajl.v2i1.12

Janse, J.H., A.A. Van Dam, E.M. Hes, J.J. de Klein, C.M. Finlayson, A. B. Janssen and J.T. Verhoeven (2019). Towards a global model for wetlands ecosystem services. Cur. Opin. Env. Sust. 36: 11-19. https://doi.org/10.1016/j.cosust.2018.09.002

Kanwar, V.S., S.K. Vashisht, A. Kanoungo, R. Pathak, R. and M. Kanwar (2021). Himachal Pradesh. In Geotechnical Characteristics of Soils and Rocks of India (pp. 279-294). CRC Press. https://doi.org/10.1201/9781003177159-14

Kasur. (2024, November 15). https://Lahoredivision.Punjab.gov.Pk/. https://lahoredivision.punjab.gov.pk/ divisionatglance_majorattractions_kasur

Kaur, M., M. Kumar, S. Sachdeva and S.K. Puri (2018). Aquatic weeds as the next generation feedstock for sustainable bioenergy production. Biores. Tech. 251: 390-402. https://doi.org/10.1016/j.biortech.2017.11.082

Kokou, K., K.M. Afiademanyo and L.K. Agboyi, (2021). Invasive alien species in Togo (West Africa). Invasive Alien Species: Observations and Issues from Around the World. 1: 291-312. https://doi.org/10.1002/9781119607045.ch10

Kress, W.J. (2017). Plant DNA barcodes: applications today and in the future. J. Sys. Evol. 55(4): 291-307. https://doi.org/10.1111/jse.12254

Kress, W.J. and D.L. Erickson (2007). A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. PLoS One, 2(6): e508. https://doi.org/10.1371/journal.pone.0000508

Kress, W.J. and D.L. Erickson (2008). DNA barcodes: genes, genomics, and bioinformatics. Proceedings of the National Academy of Sciences. 105(8): 2761-2762. https://doi.org/10.1073/pnas.0800476105

Kress, W.J., K.J. Wurdack, E.A. Zimmer, L.A. Weigt and D.H. Janzen (2005). Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences. 102(23): 8369-8374. https://doi.org/10.1073/pnas.0503123102

Kress, W.J., C. García-Robledo, M. Uriarte and D.L. Erickson, (2015) DNA Barcodes for Ecology, Evolution, and Conservation. Tr. Eco. Evo. 30: 25-35. https://doi.org/10.1016/j.tree.2014.10.008

Letsiou, S., P. Madesis, E. Vasdekis, C. Montemurro, M.E. Grigoriou, G. Skavdis and A.G. Tzakos (2024). DNA barcoding as a plant identification method. App. Sci. 14(4): 1415. https://doi.org/10.20944/preprints202312.0536.v1

Levin, R.A., W.L. Wagner, P.C. Hoch, M. Nepokroeff, J.C. Pires, E.A. Zimmer and K.J. Sytsma (2003). Family-level relationships of Onagraceae based on chloroplast *rbcL* and *ndhF* data. Am. J. Bot. 90(1): 107-115. https://doi.org/10.3732/ajb.90.1.107

Liu, Y., M. Zhang, X. Chen, Y. Hu, J. Gao and X. Zhang (2021). Developing an efficient DNA barcoding system to differentiate between Lilium species. BMC Pl. Bio. 21: 1-13. https://doi.org/10.1186/s12870-021-03229-6

Lonare, N., G. Patil, S. Waghmare, R. Bhor, H. Hardikar and S. Tembe (2024). DNA Barcoding of Invasive Terrestrial Plant Species in India. Mol. Biotech. 1-8. https://doi.org/10.1007/s12033-024-01102-z

Lorenz, T.C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. J. Vis. Exp. (63): 3998. https://doi.org/10.3791/3998

Madeira, P.T., F.A. Dray Jr and P.W. Tipping (2022). The phytogeography and genetic diversity of the weedy hydrophyte, *Pistia stratiotes* L. Bio. Inv. 24(8): 2613-2634. https://doi.org/10.1007/s10530-022-02798-3

Mo, Z., W. Lou, Y. Chen, X. Jia, M. Zhai, Z. Guo and J. Xuan (2020). The chloroplast genome of *Carya illinoinensis*: genome structure, adaptive evolution, and phylogenetic analysis. Forests. 11(2): 207. https://doi.org/10.3390/f11020207

Nath, S., J.T. VanSlambrouck, J.W. Yao, A. Gullapalli, F. Razi and Y. Lu (2024). DNA barcoding of terrestrial invasive plant species in Southwest Michigan. Plant Direct. 8(6): e615. https://doi.org/10.1002/pld3.615

Nehal, N., B. Choudhary, A. Nagpure and R.K. Gupta (2021). DNA barcoding: a modern age tool for detection of adulteration in food. Crit. Rev. Biotech. 41(5): 767-791. https://doi.org/10.1080/07388551.2021.1874279

Niemann, H.J., B.S. Bezeng, R.D. Orton, R.M. Kabongo, M. Pilusa and M. Van der Bank (2022). Using a DNA barcoding approach to facilitate biosecurity: Identifying invasive alien macrophytes traded within the South African aquarium and pond plant industry. S. Afr. J. Bot. 144: 364-376. https://doi.org/10.1016/j.sajb.2021.08.041

Páll-Gergely, B., F.T. Krell, L. Ábrahám, B. Bajomi, L.E. Balog, P. Boda and A. Móra (2024). Identification crisis: a fauna-wide estimate of biodiversity expertise shows massive decline in a Central European country. Biodiv. Cons. 33(13): 3871-3903. https://doi.org/10.1007/s10531-024-02934-6

Parmentier, I., J. Duminil, M. Kuzmina, M. Philippe, D.W. Thomas, D. Kenfack, G.B. Chuyong, C. Cruaud and O.J. Hardy (2013). How effective are DNA barcodes in the identification of African Rainforest Trees? PLoS ONE. 8: e54921. https://doi.org/10.1371/journal.pone.0054921

Parsons, J. (2001). Aquatic plant sampling protocols. http://www.Ecy.Wa.Gov; State of Washington Department of Ecology. Retrieved January 6, 2025, from http://www.ecy.wa.gov/biblio/0103017.html

Pistia stratiotes L. Species (2024). India biodiversity portal. https://indiabiodiversity.org/species/show/230798

Saitou N. and M. Nei (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Bio. Evol. 4:406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Samal, K. and R.R. Dash (2024). Experiments and modelling to develop a *Pistia stratiotes* L. based Floating Vegetated System (FVS) for the removal of heavy metals (Pb, Zn, Cr, Cu, Ni). Sci. Tot. Env. 926: 171981. https://doi.org/10.1016/j.scitotenv.2024.171981

Kuznetsov, A. and C.J. Bollin (2020). NCBI genome workbench: desktop software for comparative genomics, visualization, and GenBank data submission. In Multiple Sequence Alignment: Methods and Protocols (pp. 261-295). New York, NY: Springer US. https://doi.org/10.1007/978-1-0716-1036-7_16

Schwartz, S., L. Elnitski, M. Li, M. Weirauch, C. Riemer, A. Smit and W. Miller (2003). MultiPipMaker and supporting tools: alignments and analysis of multiple genomic DNA sequences. Nucl. Ac. Res. 31(13): 3518-3524. https://doi.org/10.1093/nar/gkg579

Shneyer, V.S. (2009). DNA barcoding is a new approach in comparative genomics of plants. Russ. J. Gen. 45: 1267-1278. https://doi.org/10.1134/s1022795409110027

Subbotin, S.A. (2021). Phylogenetic analysis of DNA sequence data. In Techniques for work with plant and soil nematodes (pp. 265-282). Wallingford UK: CABI. https://doi.org/10.1079/9781786391759.0015

Tabita, F.R., T.E. Hanson, H. Li, S. Satagopan, J. Singh and S. Chan (2007). Function, structure, and evolution of the RubisCO-like proteins and their RubisCO homologs. Microbiol. Mol. Bio. Rev. 71(4): 576-599. https://doi.org/10.1128/mmbr.00015-07

Tamura K., G. Stecher, D. Peterson A. Filipski and S. Kumar (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Bio. Evol. 30: 2725-2729. https://doi.org/10.1093/molbev/mst197

Tamura, K. and M. Nei (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Bio. Evol. 10(3): 512-526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

Tokgöz, H.B., Ö. Çetin, H.B. Kaya, C. Akkale, H. Yildirim, A.F. Pirhan and F. Altan (2024). Genetic diversity of Lilium candidum natural populations in Türkiye evaluated with ISSR and M13-tailed SSR markers. Plant Sys. Evol. 310(1): 5. https://doi.org/10.1007/s00606-023-01886-8

Trujillo-Argueta, S., R.F. Del Castillo and A. Velasco-Murguía, (2022). Testing the effectiveness of *rbcLa* DNA-barcoding for species discrimination in tropical montane cloud forest vascular plants (Oaxaca, Mexico) using BLAST, genetic distance, and tree-based methods. PeerJ. 10: e13771. https://doi.org/10.7717/peerj.13771

Üstüner, H., M. Yavuz, R.S. Göktürk, E. Kaya and S. Galatali (2022). Morphological and molecular notes of Gypsophila pilulifera. https://doi.org/10.11646/phytotaxa.556.2.1

Waterton, C. and B. Wynne (2013). Barcoding nature: shifting cultures of taxonomy in an age of biodiversity loss. Routledge. https://doi.org/10.4324/9780203870440

Yameen, B., W. Abbas, M.U.F. Awan, A. Tahir, S. Mohammad and M. Shoib (2024). *RbcLa* marker based identification and phylogenetic analysis of Kasuri methi (*Trigonella foenum-graecum* L.): a native plant of Kasur district of Punjab (Pakistan). Gen. Reso. Crop Evol. 1-11. https://doi.org/10.1007/s10722-024-02044-w

Yuceol, F., O. Çecen, R.S. Gokturk, E. Kaya and H. Agar (2024). Valeriana alanyense (Caprifoliaceae): a new species from Southwest Anatolia (Turkey) based on morphological and molecular data. Phytotaxa. 653(1): 79-90. https://doi.org/10.11646/phytotaxa.653.1.6.

Zahari, N.Z., N.S. Fong, F.N. Cleophas and S.A. Rahim (2021). The potential of *Pistia stratiotes* L.in the phytoremediation of selected heavy metals from simulated wastewater. Int. J. Tech. 12: 613-624. https://doi.org/10.14716/ijtech.v12i3.4236.