JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP
Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises
(Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/43/3/MRN-2030

Molecular phylogeny of *Ruppia maritima* L. from Chilika Lake, Odhisa using nuclear and plastid genes

E. Dilipan[1,2]*, D. Arulbalachandran[2], J. Rajkumar[3] and M. Ramachandran[2]

[1]Department of Biotechnology, Selvam College of Technology (Affiliated to Anna University) Namakkal-637 003, India
[2]Department of Botany, Periyar University, Salem-636 011, India
[3]National Centre for Sustainable Agriculture and Wetland Research Foundation Trust, Perambalur-621 212, India

***Corresponding Author Email :** dilipanev@gmail.com

Received: 17.06.2021 *Revised*: 19.09.2021 *Accepted*: 05.01.2022

Abstract

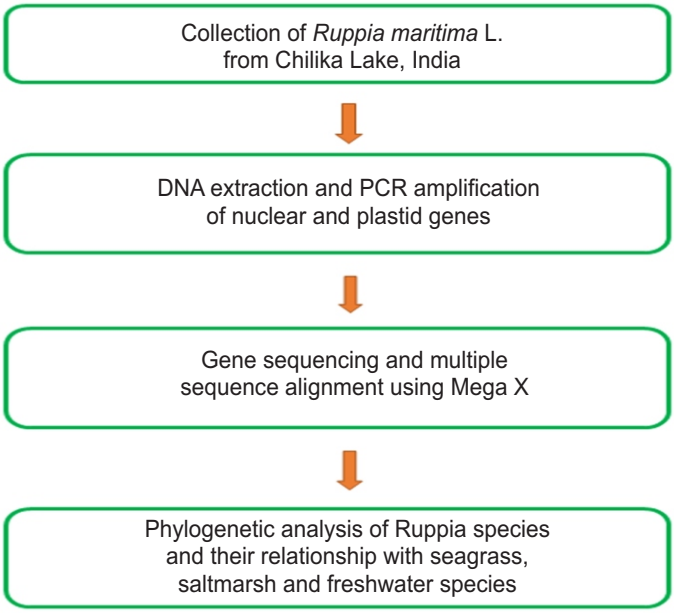
Aim: The present study aims to confirm the molecular identification of *Ruppia maritima* L. from Chilika Lake, Odhisa using different molecular markers and find out the evolutionary lineage of *Ruppia* species.

Methodology: For molecular identification, *R. maritima* was collected from Chilika Lake and the rbcL, matK, and ITS gene sequences were investigated and analyzed using BLAST to verify the gene fragment. Phylogenetic tree was constructed using MEGA X software following Neighbor joining (NJ) and Maximum parsimony (MP) methods.

Results: The nucleotide (*ITS*) and plastid gene (*rbcL* and *matK*) sequences of *Ruppia maritima* were examined. The tree topology of *R. maritima rbcL* and *matK* gene sequences revealed a paraphyletic cluster at 99 and 100% bootstrap. The conserved region was high in *matK* (74.8%), followed by *rbcL* (45.2%) and *ITS* (19.1%) with seagrass and freshwater species. *ITS* had the most significant proportion of variable sites (79.7%), followed by *rbcL* (52.4%) and *matK* (25.2%). The ITS region showed less parsimonious-informative character (66.1) than *rbcL* and *matK* region.

Interpretation: The study concludes that the *R. maritima* was identified through molecular markers, which was a closed lineage with seagrasses than freshwater species.

Key words: Chilika Lake, Phylogenetic identification, *Ruppia maritima,* Salt marsh, Seagrass



How to cite : Dilipan, E., D. Arulbalachandran, J. Rajkumar and M. Ramachandran: Molecular phylogeny of *Ruppia maritima* L. from Chilika Lake, Odhisa using nuclear and plastid genes. *J. Environ. Biol.*, **43**, 412-419 (2022).

Introduction

Ruppia maritima L. is a saline tolerant species distributed near freshwater to hypersaline condition (Lazar and Dawes, 1991; Koch *et al.,* 2007), which is generally restricted to shallow waters such as coastal lagoons and brackish water defined by fine texture sediments and high saline fluctuations (Mannino *et al.,* 2015). *Ruppia* meadows in shallow habitats play a vital ecological role and provide shelter and food for benthic communities, fishes, and marine mammals (Hemminga and Durarte, 2000; Lopez-Calderon *et al.,* 2010). The distribution of *R. maritima* population is confined to salinity zones and withstands abrupt saline variation (Koch *et al.,* 2007). *Ruppia maritima* thrives in nutrient-rich environments, which may be stressful for other seagrass species (Burkholder *et al.,* 1994).

Other seagrass species had previously inhabited the locations, but storms (Cho and May, 2008), salinity variations (Fourqurean *et al.,* 2003), and other severe weather events caused *R. maritima* to recolonize (Johnson *et al.,* 2003). Based on a bioregional model, *R. maritima* L. is expected to distribute in all the regions (Short *et al.,* 2007). However, identifying *Ruppia* species has been confusing because of the simplified morphology and high phenotypic plasticity, which makes it challenging to name *Ruppia* species. Moreover, *Ruppia maritima* L. was commonly reported in the early nineteenth century from the tidal marshes around salt works from India (Hooker, 1894), and patchy distribution of the same was reported to very few localities (Jagtap, 1991).

Recently, Pattnaik *et al.* (2020) reported the distribution of *R. maritima* L. meadows in Chilika Lake. However, *R. rostellata* is considered a synonym for *R. maritima* (McCann, 1945), and *R. rostellata* was morphologically identified in the Gulf of Kutch (Rodrigues *et al.,* 2009). These morphological characteristics often exhibit significant phenotypic variability across taxa, between taxon populations, and even within populations, leading to taxonomic uncertainty (Van Vierssen *et al.,* 1981; Hara, 1983; Aedo and Fernandez Casado, 1988). These kinds of confusion over taxonomy and synonym of *Ruppia* species due to morphological variations in different geographical regions pave the way to the taxonomic study of the genus using molecular techniques (Mishra *et al.,* 2017). In view of the above, the present study was conducted to confirm the molecular identification of *Ruppia maritima* L. from Chilika Lake, Odhisa using different molecular markers and find out the evolutionary lineage of *Ruppia* species.

Materials and Methods

Sample collection and preservation: The marine angiosperm *Ruppia maritima* L. was collected from the outer channel (19o41'35.71"N; 85o25'4.20"E) of Chilika Lake (Odhisa), India, from a depth of 1-2 m during March 2019. The plant materials were washed thoroughly in seawater to remove debris and sediments and rinsed with double distilled water. The whole leaf material was then cut and immersed in NaCl/CTAB solution (Storchova *et al.,* 2000) for long-term storage. For further analysis, the preserved samples were transported to the Botany Department's laboratory at Periyar University, Salem, India.

DNA extraction and gene amplification: The genomic DNA was extracted from fresh leaves (200 mg) of *Ruppia maritima* L. based on the methodology supplied by the manufacturer of HipurA™ Super Plant DNA purification kit (Himedia, Code: MB571; Mumbai, India). For the molecular identification of *R. maritima*, *rbcL, matK,* and *ITS* gene sequences were investigated according to Lucas *et al.* (2012). The following primer pairs were utilized: 5`-GTAAAATCAAGTCCACCRCG-3` and 5`-ATGTCACCACAAACAGAGACTAAAGC-3` (Kress and Erickson, 2007) for *rbcL* fragment of 599 bp; 5`-TAATTTACGATCAATTCATTC-3` and 5`-GTTCTAGCACAAGAAAGTCG-3` (Ford *et al.,* 2009) for a *matK* gene of 945 bp; 5`-CCTTATCATTTAGAGGAAGGAG-3` (ITS5a) (Stanford *et al.,* 2000; Kress *et al.,* 2005) and 5`-TCCTCCGCTTATTGATATGC-3` (ITS4) (White *et al.,* 1990) for *ITS* of 900 bp. PCR reaction was performed in a PCR Thermal Cycler (Cyberlab, Smart PCR) with GoTaq G2 Green PCR master mix (Promega, Cat: M7822; Madison, USA) in a total volume of 1× concentration of 25 µl reaction mixture by adopting the following PCR program: 5 min 95 °C, 40 cycles of 60 s at 95 °C, 60 sec at 50 °C, 120 sec at 72 °C, followed by 10 min of final extension at 72 °C. PCR fragments of respective genes were sequenced by capillary electrophoresis using an ABI 3730xl DNA analyzer, and the gene sequence (Xcelris Labs, ISO certified: 9001 2015, Ahmedabad, India) was obtained.

Bioinformatic analysis: The obtained raw sequence data was edited with DNA Baser ver. 5.15. The sequence files obtained were assembled and analyzed using ClustalW algorithm. The assembled sequences were manually edited to obtain a consensus sequence. The consensus sequence of *rbcL, matK* and *ITS* were analyzed using BLAST to verify the gene fragment. Dendrogram was constructed using MEGA X (Kumar *et al.,* 2018) software following neighbor joining (NJ) and maximum parsimony (MP) methods. The bootstrap consensus trees inferred from 500 replicates were taken to represent the evolutionary history (Saitou and Nei, 1987) of the taxa analyzed. Branches with <50 % bootstrap replicates were collapsed and significant bootstrap values were shown next to the branches (Felsenstein, 1985). The evolutionary distances were estimated by Kimura 2-parameters (Kimura 1980) based on the number of base substitutions per site. The maximum parsimony trees were obtained using a close neighbor interchange algorithm (Nei and Kumar, 2000) with search level one, and the random addition of sequences (ten replicates) in which the initial trees were obtained.

Results and Discussion

Alismatales are found in various habitats, including fresh, brackish, marine environments, depending on the habitat of most species in a genus, and have evolved convergent traits as adaptations to the marine environment (Li and Zhou, 2009).

Table 1: Nucleotide sequence characteristic analyzed using Mega X.

Barcodes	Aligned Length (bp)	Conserve	Variable Site	Parsimony Informative character	Conserve	Variable Site	Parsimony Informative	Total Individual
ITS	826	158	658	546	19.1	79.7	66.1	20
matK	937	701	236	163	74.8	25.2	17.4	25
rbcL	699	316	366	52	45.2	52.4	7.44	19

Table 2: Vegetative and reproductive characters of freshwater and marine angiosperms. (Den Hartog, 1970; Den Hartog *et al.,* 1987; Ramamurthy *et al.,* 1992; Kannan and Thangaradjou, 2008; Dilipan *et al.,* 2020)

Species	Leaf sheath	Gamete type	Inflorescence	Pollen type	Pollination	Habitat
Vallisneria asiatica	absent	Dioecious	Cymose, axillary	Spherical	Epihydrophily	Fresh water
Vallisneria americana	Weakly correlated with leaf	Dioecious	Solitary, axillary	Spherical	Epihydrophily	Fresh water
Potamogeton natans	Truncate, open leaf sheath	Monoecious	Spike, terminal	Spherical	Anemophily	Fresh water
Potamogeton pusillis	Tubular sheath	Monoecious	Spike, terminal	Spherical	Anemophily	Fresh water
Potamogeton crispus	Stipular sheath, obtuse apex	Monoecious	Spike, terminal,	Spherical	Anemophily	Fresh water
Cymodocea serrulata	Auriculae acute, broadly triangular	Dioecious	Cymose, terminal	Filiform	Hypohydrophily	Marine
Cymodocea rotundata	Obconical, auriculae acute	Dioecious	Cymose, terminal	Filiform	Hypohydrophily	Marine
Halodule pinifolia	Linear, convolute	Dioecious	Solitary, terminal	Filiform	Hypohydrophily	Marine
Halodule uninervis	Linear	Dioecious	Solitary, terminal	Filiform	Hypohydrophily	Marine
Halodule wrightii	Linear, convolute	Dioecious	Solitary, terminal	Filiform	Hypohydrophily	Marine
Syringodium isoetifolium	Biauriculate	Dioecious	Cymose, terminal	Filiform	Hypohydrophily	Marine
Syringodium filiforme	Biauriculate	Dioecious	Cymose, terminal	Filiform	Hypohydrophily	Marine
Posidonia oceanica	Bicuspidate	Monoecious	Cymose, terminal	Filiform	Hypohydrophily	Marine
Posidonia australis	Biauriculae, obtuse	Monoecious	Cymose, terminal	Filiform	Hypohydrophily	Marine
Phyllospadix iwatensis	Auriculae obtuse	Dioecious	Spadix, terminal	Filiform	Hypohydrophily	Marine
Ruppia maritiama	Acute-acuminate	Monoecious	Racemose	Boomerang	Anemophily	Brackish water
Ruppia cirrhosa	Basal	Monoecious	Racemose	Boomerang	Anemophily	Brackish water
R. megacarpa	Rounded auricles	Monoecious	Racemose	Boomerang	Anemophily	Brackish water

According to Short *et al.* (2007), the bioregional model of seagrass distribution, *R. maritima* L. is predicted to exist in all biogeographical areas. However, due to the difficulties of correctly recognizing species and the traditional usage of *R. maritima* as a catch-all taxon, *R. maritima* distributional range may have been overestimated, and *Ruppia* species diversity may have been underestimated (den Hartog and Kuo, 2006; Triest and Sierens, 2014). In this study, the genomic DNA was extracted from *R. maritima* and optimized for PCR reactions. The recommended primer pairs of *rbcL, matK* and *ITS* (Lucas *et al.,* 2012) worked well after optimization. Thereafter, PCR amplification successfully produced 691 bp *ITS*, 606 bp *rbcL*, and 860 bp *matK*, respectively. When *Ruppia* sequence was examined in blastn, it exhibited more similarity with seagrass

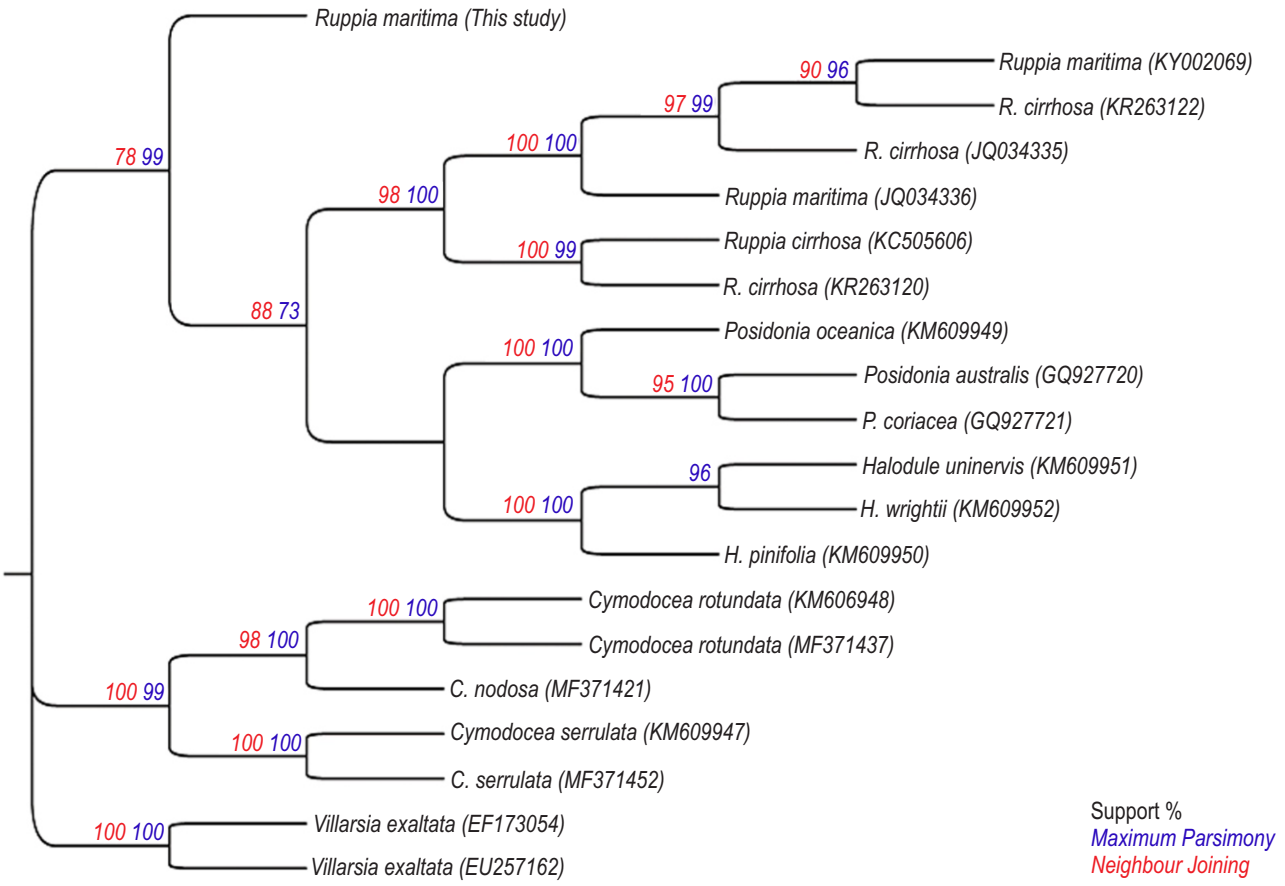


Fig. 1: Neighbor-joining and maximum parsimony dendrogram showing phylogenetic relationship of Internal Transcribed Spacer (*ITS*) sequences of *Ruppia maritima* L. with representative species of freshwater, and seagrasses from GenBank database. The numbers at nodes indicate levels of bootstrap support (%) of 500 resampled datasets; values (45 %) are shown.

sequences (~99 percent) than freshwater species (~85 percent), since BLAST can be used as a rapid identification tool (von Crautlen *et al.,* 2011). There are two possibilities of seagrass origin, either they have descended from a freshwater hydrophyte or from a primitive stock or a saltmarsh type (Dilipan *et al.,* 2018). However, morphological and physiological evidence showed that salt and wave tolerance are shared between seagrasses and saltmarshes, supporting the saltmarsh type (Larkum and den Hartog, 1989). In this study, the nucleotide (ITS) and plastid gene (*rbcL* and *matK*) sequences of *Ruppia maritima* were analyzed, and the multiple sequence alignment with clustalW program results showed that 826 bp of *ITS*, 699 bp of *rbcL*, and 937 bp of *matK*.

The matK had the highest percentage of the conserved region (74.8%), followed by *rbcL* (45.2%) and ITS (19.1%) with seagrass and freshwater species (Table 1). However, the highest percentage of variable site was obtained by *ITS* (79.7%), followed by *rbcL* (52.4%) and *matK* (25.2%). Besides, *ITS* had the highest parsimony-informative character (66.1) than *rbcL* and *matK* region (Table 1). The data incongruence between DNA types obtained from the nuclear and plastid gene sequences concluded that the varied pattern might be due to hybridization (Triest and Sierens, 2013; Mishra *et al.,* 2017). Morphological and phylogenetic reports revealed the complex evolutionary history of the genus *Ruppia* with polyploidization and hybridization (Ito *et al.,* 2013; Triest and Sierens, 2014; Martinez-Garrido *et al.,* 2017). Earlier research found that *rbcL* bootstrap value could not resolve Cymodoceaceae, Posidoniaceae, and Ruppiaceae and suggested that additional gene sequences be used in future studies to resolve these polytomies (Li and Zhou, 2009). We conducted molecular analysis on the nuclear (ITS) and plastid (*rbcL* and *matK*) genes, as well as six morphological characters (habitat, leaf sheath, gamete, inflorescence, pollen, and pollination) of each species (Table 2), in order to gain a better understanding of *Ruppia maritima* evolutionary lineages. Tomlinson (1982) reported that no unique morphological characteristics do not distinguish seagrasses from other aquatic plants, except pollen types such as filiform pollen (Zosteraceae, Posidoniaceae, and Cymodoceaceae), while others have strings of spherical pollen (*Thalassia, Halophila*). Besides,

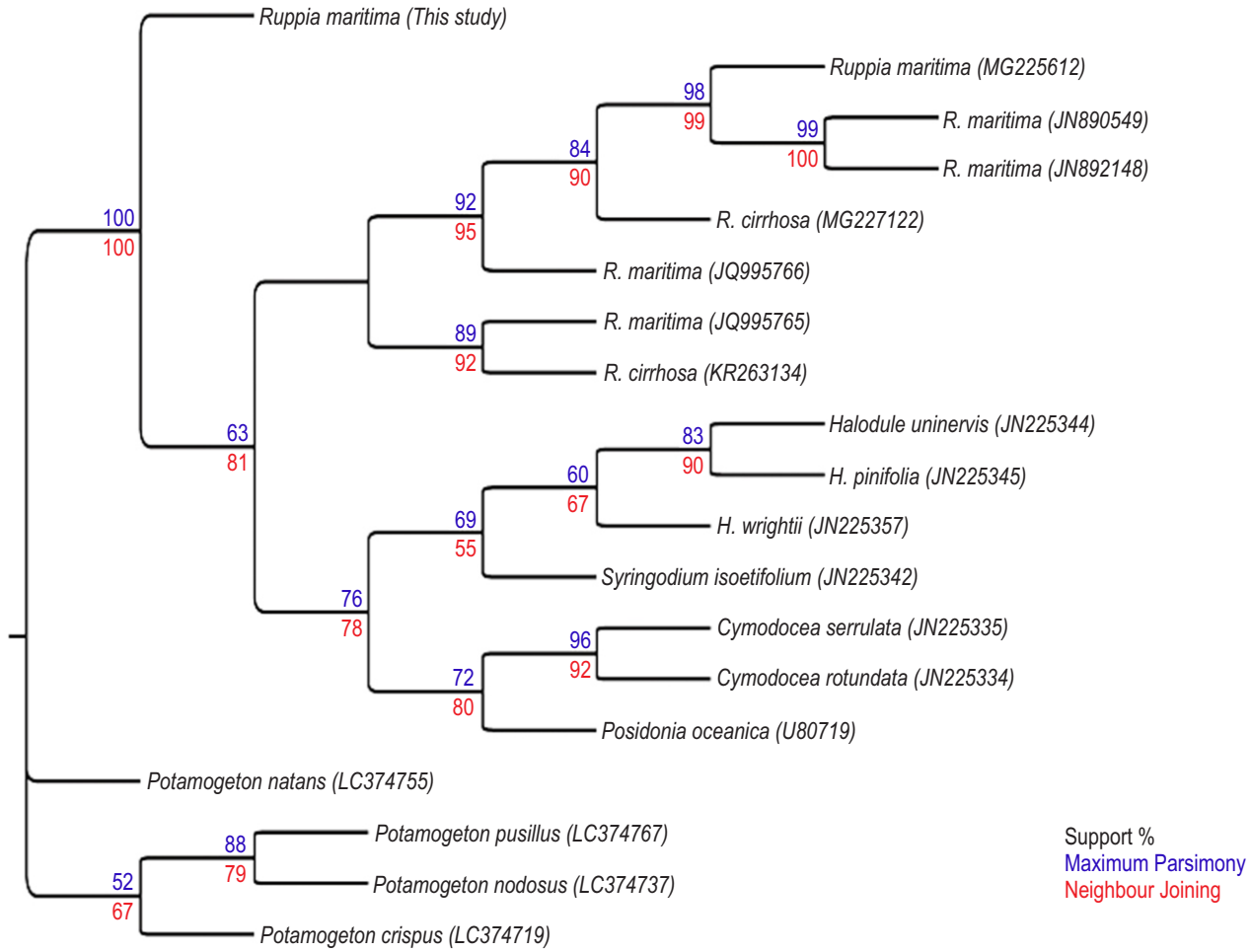


Fig. 2: Neighbor-joining and maximum parsimony dendrogram showing the phylogenetic relationship of *rbcL* sequences of *Ruppia maritima* L. with representative species of freshwater, and seagrasses from GenBank database. The numbers at nodes indicate levels of bootstrap support (%) of 500 resampled datasets; values (45 %) are shown.

Cymodoceaceae, Zannichelliaceae, Posidoniaceae, and Zosteraceae were identified as distinct clades of seagrass, while Hydrocharitaceae was grouped with the other two (Ruppiaceae were merged with Potamogetonaceae). However, *Ruppia* species have a boomerang pollen type, which is distinct from marine and freshwater species. Moreover, the phylogenetic tree obtained from the molecular markers separated the *Ruppia* clade from other aquatic angiosperms. The present study revealed that the *Ruppia* species might have evolved as intermediatory species between marine and freshwater, which adapt to seasonal changes. The cladogram based on Internal Transcribed Spacer gene sequence analysis involved 20 nucleotide sequences with a total length of 826 nucleotide positions in the alignment to construct neighbor-joining (NJ) and maximum parsimony method (Fig. 1), which supported the clusters at 78% and 99% bootstrap value with other *Ruppia* and seagrass species, respectively, based on the optimal tree with the sum of branch length 1.4. The *matK* and *rbcL* sequences of *Ruppia maritima* are already contained in the GenBank database provide a promising identification at least to a genus level with a database providing comparable data for nearly 90% of the considered seagrass taxa. The presently proposed DNA barcodes (*rbcL* and *matK*) can be used to initiate barcoding of all land and water plants, with some families as exceptions (Roy *et al.,* 2010; Lucas *et al.,* 2012). The recommended two-locus DNA barcode consisting of *rbcL* and matK (Hollingsworth *et al.,* 2009) was suggested to be the best compromise compared to all other loci tested.

In this study, the tree topology of *rbcL* and *matK* gene sequences of *R. maritima* showed that the cluster was grouped in a paraphyletic manner (Fig. 2, 3) at 99% and 100% bootstrap, respectively. Besides, the phylogenetic tree analysis inferred from *ITS, rbcL,* and *matK* using NJ and MP methods revealed that all species generated a morphological group. In comparison with nuclear and plastid genes of *R. maritima*, the *ITS* region showed

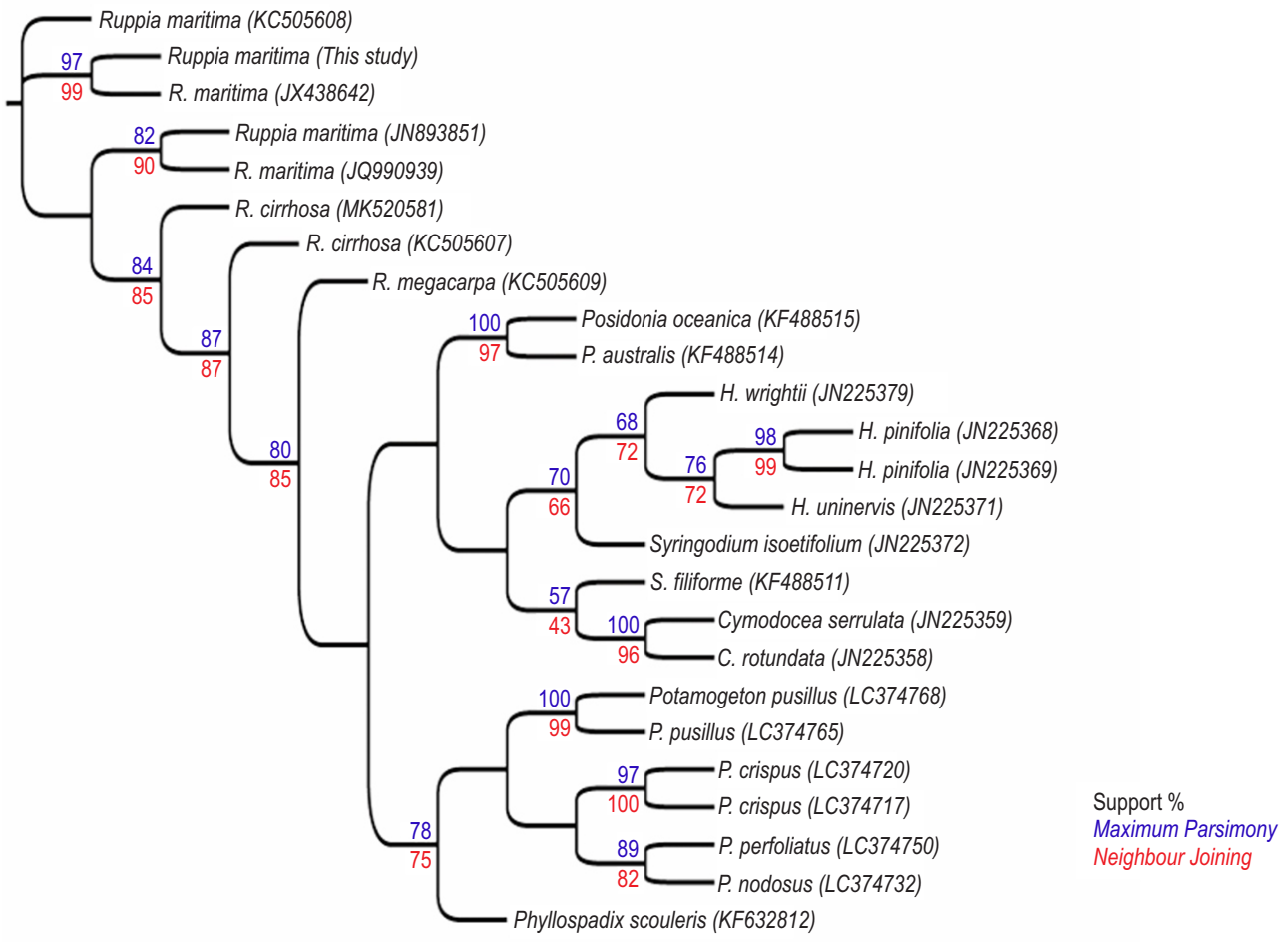


Fig. 3: Neighbor-joining and maximum parsimony dendrogram showing phylogenetic relationship of *matK* sequences of *Ruppia maritima* L. with representative species of freshwater, and seagrasses from GenBank database. The numbers at nodes indicate levels of bootstrap support (%) of 500 resampled datasets; values (45 %) are shown.

high parsimony-informative than plastid genes; however, the plastid genes obtained high conserved region, which depicts that the chloroplast genes of *Ruppia* species are in close relationship with seagrass species than freshwater species. The *rbcL* and *matK* tree topology indicate it as the closest extant sister group as Potmogetonaceae and Posidonaceae (Les *et al.,* 1997). The *rbcL* and *matK* data indicate a relatively high degree of molecular divergence separating the two clades, representing an argument against a taxonomic merger of these groups and further reflects the distinctness of seagrass families indicated previously by morphological phylogenetic studies (Dahlgren *et al.,* 1985).

The present study reveals the results of different trees; a single locus cannot resolve the well-described species correctly, or the molecular marker is not robust using only a few specimens. However, a rapid system for identification close to species level is provided using *rbcL* and *matK*. The distribution of *R. maritima* species in Chilika Lake must be adapted to overcome local conditions such as intertidal areas, shallow turbid waters, eutrophic waters, etc., where the sampled location of *R. maritima* was visited by a large number of waterbirds, the leading migratory bird group in Chilka Lake (Sundaravadivelu *et al.,* 2019). *Ruppia* species are known to be dispersed by seabirds and ocean currents (Figuerola *et al.,* 2002; Triest and Sierens, 2013).

The study concludes that *R. maritima* was identified through molecular markers. It has a closed lineage with seagrasses than freshwater species; still, different markers needs to be tested from a different population of *Ruppia* species to confirm the evolutionary lineage between marine and freshwater.

Acknowledgments

Authors thank the Department of Botany, Periyar University, Salem, India, for continuing support to utilize the infrastructure and instruments for this investigation. Dr. E. Dilipan (File no. PDF/2017/000338) is grateful to SERB-DST for providing fund through NPDF program. We would like to thank Dr.

R.N. Samal, Scientist, Chilika Development Authority, Bhuvaneswar, Odhisa, India for his hospitality and arrangement for collecting plant samples.

Add-on Information

Authors' contribution: E. Dilipan: Carried out research work and draft the manuscript; **D. Arulbalachandran:** Supervised the research work and checked manuscript; **J. Rajkumar:** Carried out field work and checked manuscript; **M. Ramachandran:** Carried out field and laboratory work.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

References

Burkholder, J.M., H.B.Jr. Glasgow and J.E. Cooke: Comparative effects of water-column nitrate enrichment on eelgrass *Zostera marina*, shoalgrass *Halodule wrightii*, and widgeon grass *Ruppia maritima*. *Mar. Ecol. Prog. Ser.,* **105**, 121-138 (1994).

Cho, H.J. and C. May: Short-term spatial variations in the beds of *Ruppia maritima* (Ruppiaceae) and *Halodule wrightii* (Cymodoceaceae) at Grand Bay National Estuarine Research Reserve, Mississippi, USA. *J. Missis. Acade. Sci.*, **53**, 2-3 (2008).

Dahlgren, R.M.T., H.T. Clifford and P.F. Yeo: The Families of the Monocotyledons: Structure, Evolution and Taxonomy. 1st Edn., Springer-Verlag Berlin Heidelberg, **XII**, 520 (1985).

den Hartog, C.: The Seagrasses of the World. North-Holland Publishing, Amsterdam (1970).

Dilipan, E., E.P. Nobi and J. Rajkumar: Manual for identification of seagrasses of India. 1st Edn., White Falcon Publishing, Chandigarh, India (2020).

Dilipan, E., C. Lucas, J. Papenbrock and T. Thangaradjou: Tracking the phylogeny of seagrasses: Inferred from 18S rRNA gene and ancestral state reconstruction of morphological data. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.,* **88**, 497-504 (2018).

Felsenstein, J.: Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783–791 (1985).

Figuerola, J., A.J. Green and L. Santamaria: Comparative dispersal effectiveness of wigeon grass seeds by waterfowl wintering in south-west Spain: Quantitative and qualitative aspects. *J. Ecol.,* **90**, 989-1001 (2002).

Ford, C.S., K.L. Ayres, N. Toomey, N. Haider, J. Van Alphen Stahl, L.J. Kelly, N. Wikström, P.M. Hollingsworth, R.J. Duff, S.B. Hoot and R.S. Cowan: Selection of candidate coding DNA barcoding regions for use on land plants. *Bot. J. Linn. Soc.,* **159**, 1-11 (2009).

Fourqurean, J.W., J.N. Boyer, M.J. Durako, L.N. Hefty and B.J. Peterson: Forecasting responses of seagrass distribution to changing water quality using monitoring data. *Ecol. Appl.,* **13**, 474-489 (2003).

Green, E.P. and F.T. Short: World Atlas of Seagrasses. Univ of California Press, Berkeley, USA, 324 pages (2003).

Hemminga, M.A. and C.M. Duarte: Seagrass Ecology. Cambridge University Press. The Edinburgh Building, Cambridge, UK (2000).

Hollingsworth, P.M., L.L. Forrest, J.L. Spouge, M. Hajibabaei and S. Ratnasingham: A DNA barcode for land plants. *Proc. Natl. Acad. Sci. U.S.A.,* **106**, 12794-12797 (2009).

Hooker, J.D.: Impatiens L. In: Flora of British India (Ed.: J.D. Hooker). **Vol. VI**, L. Reeve & Co., Great Britan, 791 pages (1894).

Ito, Y., T. Ohi-Toma, J. Murata and N. Tanaka: Comprehensive phylogenetic analyses of the *Ruppia maritima* complex focusing on taxa from the Mediterranean. *J. Plant Res.,* **126**, 753–762 (2013).

Jagtap, T.G.: Distribution of seagrasses along the Indian Coast. *Aquat. Bot.,* **40**, 379-386 (1991).

Johnson, M.R., S.L. Williams, C.H. Lieberman and A. Kolbak: Changes in the abundance of the seagrasses *Zostera marina* L. (eelgrass) and *Ruppia maritima* L. populations in San Diego, California, following an El Niño event. *Estuaries*, **26**,106-115 (2003).

Kannan, L. and T. Thangaradjou: Identification and assessment of biomass and productivity of seagrasses. In: Proceedings of the National Training Workshop on Marine and Coastal Biodiversity Assessment for Conservation and Sustainable Utilization (Eds.: J.K. Patterson Edward, A. Murugan and J. Patterson). SDMRI Publication, Tuticorin, India 10, pp. 9-15 (2006).

Kimura, M.: A simple for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* **16**, 111–120 (1980).

Koch, M.S., S.A. Schopmeyer, C. Kyhn-Hansen, C.J. Madden and S. Peters: Tropical seagrass species tolerance to hypersalinity stress. *Aquat. Bot.,* **86**, 14-24 (2007).

Kress, W.J. and D.L. Erickson: A two locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH psbA* spacer region. *PLoS ONE,* **2**, e508 (2007).

Kress, W.J., K.J. Wurdack, E.A. Zimmer, L.A. Weigt and D.H. Janzen: Use of DNA barcodes to identify flowering plants. *PNAS*, **102**, 8369–8374 (2005).

Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura: MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.,* **35**, 1547 (2018).

Larkum, A.W.D. and C. den Hartog: Evolution and biogeography of seagrasses. In: Biology of Seagrasses (Eds.: A.W.D. Larkum, A.J. McComb and S.A. Shepherd). Elsevier, Amsterdam, pp. 112–156 (1989).

Lazar, A.C. and C. Dawes: A seasonal study of the seagrass *Ruppia maritima* L. in Tampa Bay, Florida. Organic constituents and tolerances to salinity and temperature. *Bot. Mar.,* **34**, 265-269 (1991).

Les, D.H., M.A. Cleland and M. Waycott: Phylogenetic studies in Alismatidae, II-evolution of marine angiosperms (seagrasses) and hydrophily. *Syst. Bot.,* **22**, 443–463 (1997).

Li, X., and Z. Zhou: Phylogenetic studies of the core Alismatales inferred from morphology and *rbcL* sequences. *Prog. Nat. Sci.,* **19**, 931-945 (2009).

Lopez-Calderon, J., R. Riosmena-Rodríguez, J.M. Rodríguez-Baron, J. Carrión-Cortez, J. Torre, A. Meling-López, G. Hinojosa-Arango, G. Hernández-Carmona and J. García-Hernández: Outstanding appearance of *Ruppia maritima* along Baja California Sur, México and its influence in trophic networks. *Mar. Biodivers.,* **40**, 293–300 (2010).

Lucas, C., T. Thangaradjou and J. Papenbrock: Development of a DNA barcoding system for seagrasses: Successful but not simple. *Plos ONE*, **7**, e29987 (2012).

Mannino, A.M., M. Menendez, B. Obrador, A. Sfriso and L. Triest: The genus *Ruppia* L. (Ruppiaceae) in the Mediterranean region: An overview. *Aquat. Bot.,* **124**, 1–9 (2015).

Martínez-Garrido, J., J.C. Creed, S. Martins, C.H. Almada and E.A. Serrão: First record of *Ruppia maritima* in west Africa supported by morphological description and phylogenetic classification. *Bot. Mar.,* **60**, 583-589 (2017).

Misra, S., A.K. Srivastava, S. Verma, S. Pandey, S.S. Bargali, T.S. Rana and K.N. Nair: Phenetic and genetic diversity in Indian Luffa (Cucurbitaceae) inferred from morphometric, ISSR and DAMD markers. *Genet. Resour. Crop Evol.,* **64**, 995-1010 (2017).

McCann, C.: Notes on the genus *Ruppia* (Ruppiaceae), *J. Bombay Nat. Hist. Soc.,* **45**, 396-402 (1945).

Nei, M. and S. Kumar: Molecular Evolution and Phylogenetics. Oxford University Press, New York (2000).

Pattnaik, A.K., P.C. Panda and G. Rastogi: Survey, characterization, ecology, and management of macrophytes in Chilika Lagoon. In: Ecology, Conservation, and Restoration of Chilika Lagoon, India (Eds.: C. Finlayson, G. Rastogi, D. Mishra, D and A. Pattnaik). Springer, pp. 415-438 (2020).

Ramamurthy, K., N.P. Balakrishnan, K. Ravikumar and R. Ganesan: Seagrasses of Coromandel coast, India. Flora of India - Series 4, B.S.I. Publication, Coimbatore, India, 80 pages (1992).

Rodrigues, R.S., S.B. Savant and T.G. Jagtap: A note on *Ruppia* species (Ruppiaceae) from abandoned saltpan in the Gulf of Kutch, India. *Indian J. Mar. Sci.,* **38**, 457–460 (2009).

Roy, S., Tyagi, A., Shukla, V., Kumar, A., Singh, U.M., Chaudhary, L.B., Datt, B., Bag, S.K., Singh, P.K., Nair, N.K. and T. Husain: Universal plant DNA barcode loci may not work in complex groups: A case study with Indian Berberis species. *PloS ONE*, **5**, p.e13674 (2010).

Saitou, N. and M. Nei: The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* **4**, 406–425 (1987).

Short, F.T., T. Carruthers, W. Dennison and M. Waycott: Global seagrass distribution and diversity: A bioregional model. *J. Exp. Mar. Biol. Ecol.,* **350**, 3–20 (2007).

Stanford, A.M., R. Harden and C.R. Parks: Phylogeny and biogeography of Juglans (Juglandaceae) based on matK and ITS sequence data. *Amer. J. Bot.,* **87**, 872–882 (2000).

Storchova, H., R. Hrdlickova, J. Chrtek, M. Tetera and D. Fitze: An improved method of DNA isolation from plants collected in the field and conserved in saturated NaCl/CTAB solution. *Taxon*, **49**, 79–84 (2000).

Sundaravadivelu, R., P. Shanmugam, A.K. Patnaik and P.K. Suresh: Migration of Chilika Lake Mouth. In: Proceedings of the Fourth International Conference in Ocean Engineering (ICOE2018) Springer, Singapore, pp. 369-380 (2019).

Tomlinson, P.B.: Helobiae (Alismatidae). In: Anatomy of the Monocotyledons (Ed.: C.R. Metcalfe). Clarendon Press, Oxford, 559 pages (1982).

Triest, L. and T. Sierens: Is the genetic structure of Mediterranean Ruppia shaped by bird-mediated dispersal or sea currents? *Aquat. Bot.,* **104**, 176-184 (2013).

Triest, L. and T. Sierens: Seagrass radiation after Messinian salinity crisis reflected by strong genetic structuring and out-of-Africa scenario (Ruppiaceae). *PLoS ONE*, **9**, e104264 (2014).

von Cräutlein, M., H. Korpelainen, M. Pietiläinen and J. Rikkinen: DNA barcoding: A tool for improved taxon identification and detection of species diversity. *Biodivers. Conserv.,* **20**, 373-389 (2011).

White, T.J., T. Bruns, S. Lee and J. Taylor: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (Eds.: M.A. Innis, D.H. Gelfand, J.J. Sminsky and T.J. White). Academic Press, San Diego, pp. 315–324 (1990).