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Microstructure observations of *Lemna aequinoctialis* ecotype

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Abstract: **A duckweed from a pond close to Yamuna River in Delhi NCR was collected and its characteristics were studied. The genomic DNA of the frond was used as a template in PCR to amplify the DNA barcodes of duckweed family (i.e., four plastid genes, Maturase K, Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, RNA Polymerase Beta subunit, RNA Polymerase C and three intergenic spacers, namely, trnH-psbA, atpF-atpH and PsbK-PsbL). Homology analysis of PCR products led to the identification of the frond as an ecotype of *Lemna aequinoctialis*. The abaxial surface of *Lemna aequinoctialis* displayed distinctive wax globules by Scanning electron microscopy. Such wax globules have never been reported from angiosperms yet. Intercellular details disclosed by Transmission Electron Microscopy showed extensive deposition of starch granules and lipid droplets in the cytoplasm. The chloroplasts were observed to have extensive lamellae in close association with plastoglobules in the mesophyll cells. The surface of *Lemna aequinoctialis* ecotype has unique wax globules on the adaxial surface and numerous stomatal apertures on the abaxial surface.**

Keywords: ***Lemna aequanetolis,*** **Scanning Electron Microscopy, Transmission Electron microscopy.**

I. BACKGROUND

Duckweeds have been a botanical marvel as they are one of the smallest monocots. Earlier these angiosperms were called "the Darwinian-Wallace Demon" because of their reproductive capabilities, fitness and the capability to live forever (Kutschera, 2015). However, now it is clear that the duckweeds cannot grow indefinitely due to the environmental restrictions (Liu, 2021) and thus are not Darwinian demons. Duckweeds divide by fragmentation such that in proportion to their body mass they surpass rest of the Angiosperms in their speed of proliferation. The clonal growth is responsible for their capability of covering the surface of ponds and slow-moving streams (Kutschera, 2015; Lemon, 2000). The benefits of Fig. 1 PCR amplification of DNA barcoding markers duckweeds are numerous like they are a good source of food for cattle as they accumulate biomass much more than the field crops (Krishna, 2008) that too by utilizing wastewater (Oron, 1994). They are considered beneficial crop for bioethanol production also (Cui, 2010; Porath, 1979). They have been utilized as a gene expression system due to their stability (Vunsch, 2007). *L.minor* has been used in the phytoremediation of wastewater from environmental pollutants (Ekperusi, 2019). Duckweeds have also made their way in genetic engineering because of the speed of their growth. The recent development is the usage of duckweeds as bioreactors (Yang, 2021).

Geographic isolation plays a major role in speciation of duckweeds because they divide vegetatively and cannot transfer genes from one organism to another. Taxonomical recognition of *Lemna* has been tough due to their worldwide distribution, high phenotypic plasticity, reduced size and dearth of morphological

characteristics. The organs are difficult to recognize but duckweeds have an intermediate shoot system with modified leaves and stems with adventitious roots (Les, 1997). The morphological basis of identifying *Spirodela* and *Landolita* is based on the number of roots. Duckweeds belong to Lemnaceae that comprise five genera and 34 species all together (Landolt, 1986; Lemon, 2000; Les, 1999). Among these are several ecotypes which differ in their physiological characteristics. The morphological characteristics are plastic and are influenced by the surrounding conditions of *Lemna* (Heller, 1996). As a result, taxonomy depends heavily upon differences in DNA sequences. RAPD, AFLP, and DNA barcode have been employed to delineate the phylogenetic connections of duckweeds (Les, 2002; Martirosyan, 2008). Many DNA markers have been sought to recognize the universal markers for interspecific and intraspecific identification of Lemnaceae (Bog, 2015). Genus *Lemna* has four sections and 13 species, namely, *L.disperma, L.gibba, L.japonica Landolt, L.minor, L.obscura, L.turionifera, L.trisulca, L. aequinoctialis, L. perpusilla, L. tenera, L. minuta, L. valdiviana, L. yungensis* (Wang, 2010). Still despite the advent of DNA markers and barcoding techniques the recognition of *Lemna* species remains extremely difficult even for the specialists.

In the present study we report the isolation of a duckweed frond from a pond-like collection of Yamuna river water in Delhi-NCR. We utilized the duckweed DNA barcodes to recognize the isolated duckweed and found it to be the closest relative of *Lemna aequinoctialis.* The surface details of the frond showed presence of numerous stomata on the abaxial surface while wax droplets were extensively distributed on the adaxial surface. The isolate showed epibiotic relationship with peritrich ciliates which are very selective in their choice of basibiont. As duckweeds are sessile and cannot actively disperse ciliates thus, we conclude that the abaxial surface of *Lemna aequinoctialis* offers benefits for the survival of peritrich ciliates in the aquatic habitat. TEM disclosed numerous air vacuoles, chloroplasts without grana, substantial accumulation of starch and plastoglobules.

I. RESULTS

A. Gene Amplification of duckweed Barcodes

DNA barcoding primers elaborated in table 1 were proposed by CBOL (Consortium for the Barcode of Life) plant-working group to discriminate land species that were later refined to suit the duckweeds by Wang et al (Cross, 2017). These included four plastid coding genes (*rpoB, rpoC1, rbcL* and *matK*) and three noncoding spacers (*atpF-atpH, psbK-psbI* and *trnH-psbA*) based on the Lemna minor chloroplast genome sequence. The genomic DNA of the sample at hand showed amplification at 580 bp for Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (*Rbcl*), 406 bp for RNA

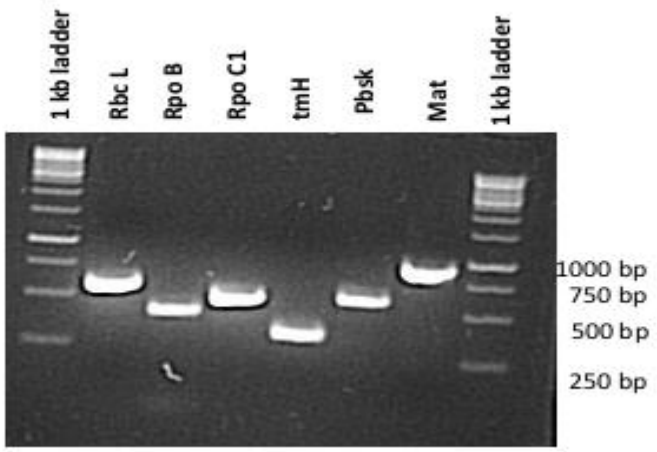


Fig. 1 PCR amplification of DNA barcoding markers. Lane 1 and 7 show 1 kb ladder for reference. Lane 2 shows amplification of *Rbcl*, lane 3 shows amplification of *RpoB*, lane 4 shows amplification of *RpoC1*, lane 5 shows amplification of *tmh*, lane 6 shows amplification of *Pbsk.*

Polymerase Beta subunit (*RpoB*), 509 bp for RNA Polymerase C (*RpoC1*), 300 bp for *trnH-psbA* intergenic spacer, 544 bp for psbK-psbI and 862 bp for Maturase K (*matK*) (figure1). The genomic DNA did not show any amplification for the *atpF-atpH* non-coding intergenic region, which is possible as the Universal primers might not be apt for the amplification or the genomic arrangement of *atpF* and at pH might be different from the previous knowledge and this can be confirmed in a later study by whole genome sequencing.

B. Bioinformatics Analysis of the sequenced duckweed DNA Barcodes

Results as depicted in Figure 2 clearly indicate that *Lemna aequinoctialis* strains alias Lesser Duckweed are the closest relatives for all samples. Previous literature there are at least five genera (*Spirodela, Landoltia, Lemna, Wolffiella,* and *Wolffia*) and more than 37 species known to belong to duckweeds. Amongst all, only one of the duckweeds, *Spirodela polyrhiza, alias* Greater Duckweed, has the genome sequence available. Genus *Lemna* has seven species and based on the estimated 1C-values they all are known to have huge genome size variation ranging from 323 Mbp in *Lemna valdiviana* to 760 Mbp *Lemna aequinoctialis*. Using several gene markers, we were able to characterize the duckweed as *Lemna aequinoctialis*, however, we believe that sequencing the whole genome of these lesser duckweeds would help in understanding their physiology, metabolism, and evolution. Considering the funding predicaments, we were not able to sequence the whole genome for *Lemna aequinoctialis* in this present study.

Query gene name and function	Seq Primer	Closest organism	Accession	Query Coverage	E value	% Identity
Maturase K (matK)	Forward	*Lemna aequinoctialis* strain LC02	KP017667.1	100%	0	99.84%
		Lemna aequinoctialis strain LC01	KP017666.1	100%	0	99.84%
	Reverse	*Lemna aequinoctialis* strain LY	KY009686.1	100%	0	100.00%
		Lemna aequinoctialis strain LC24	KP017688.1	100%	0	100.00%
PsbK-PsbI intergenic spacer	Forward	*Lemna aequinoctialis* strain 6612	GU454311.1	98%	0	99.55%
		Lemna perpusilla strain 8539	KJ136047.1	89%	0	96.77%
	Reverse	*Lemna aequinoctialis* strain 6612	GU454311.1	100%	0	99.53%
		Lemna perpusilla strain 8539	KJ136047.1	89%	8.00E-175	96.59%
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL)	Forward	*Lemna minor* isolate CH332	LC219111.1	100%	0	100.00%
		Lemna aequinoctialis strain 6612	GU454408.1	100%	0	100.00%
	Reverse	*Lemna minor* isolate CH332	LC219111.1	100%	0	100.00%
		Lemna minor voucher PS0367MT01	GQ436374.1	100%	0	100.00%
RNA polymerase beta subunit (rpoB)	Forward	*Lemna aequinoctialis* strain LC05	KP017719.1	100%	4.00E-178	100.00%
		Lemna aequinoctialis strain LC01	KP017715.1	100%	4.00E-178	100.00%
	Reverse	*Lemna aequinoctialis* strain LC05	KP017719.1	100%	2.00E-180	100.00%
		Lemna aequinoctialis strain LC01	KP017715.1	100%	2.00E-180	100.00%
RNA polymerase C (rpoC1)	Forward	*Lemna minor voucher* PS0367MT01	GQ435979.1	100%	0	100.00%
		Lemna aequinoctialis strain 6612	GU453954.1	92%	0	100.00%
	Reverse	*Lemna aequinoctialis* strain 6612	GU453954.1	100%	0	100.00%
		Lemna minor voucher PS0367MT01	GQ435979.1	100%	0	100.00%
trnH-psbA intergenic spacer	Forward	*Lemna aequinoctialis*	HG963808.1	100%	2.00E-06	100.00%
		Wolffiella lingulata strain 7289	JN160604.1	100%	2.00E-06	100.00%
	Reverse	*Lemna aequinoctialis* strain 6612	GU454500.1	100%	3.00E-100	94.67%
		Lemna aequinoctialis strain 7126	GU454502.1	100%	6.00E-97	93.85%

Fig. 2 Sequence homology-based characterization of duckweeds: PCR-amplified DNA using forward and reverse primers were sequenced and further subjected to BLASTn against NCBI nucleotide database. Only two topmost hits are shown here. The red shade represents homologs in *Lemna aequinoctialis*. NCBI accession, query coverage, evalue and % identity is also provided.

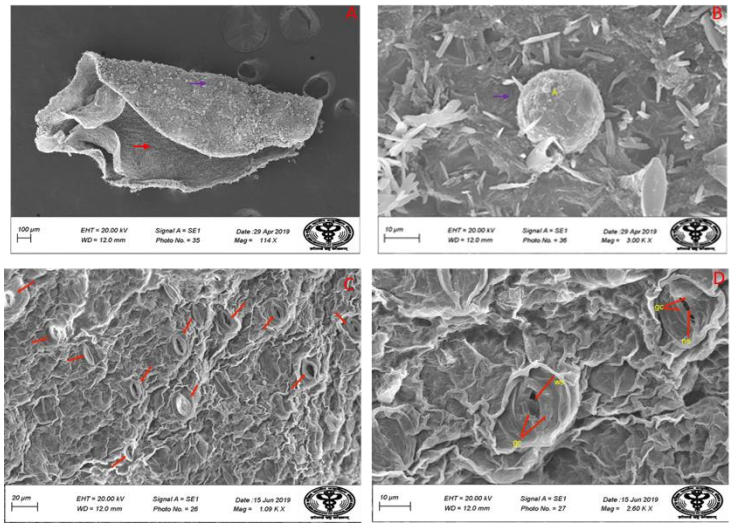
C. *Microscopic Examination*

Morphology of a typical *Lemna aequinoctialis* : It is a boat-like frond with a single rhizoid attached at the ventral surface of the frond. The morphology of *Lemna aequinoctialis* visualized with foldscope and compound microscope looked like a green oval structure in multiple stages of the frond development. A single rhizoid was found attached to the center of the abaxial/ ventral surface of the frond. The presence of a single rhizoid confirms that the species at hand is an ecotype of *Lemna aequinoctialis*. Other duckweeds like *Spirodela* sprout more than one rhizoid and Wolffia lacks rhizoids. The morphological features of the rhizoid were observed with the help of a compound microscope. The rhizoids were also visualized to be displaying multiple rods of cells and each rod ended independently so the end of the rhizoid was uneven. The length of the rhizoid was about 0.5 cm calculated using a ruler. Other than nutrient uptake the rhizoids are supposed to be playing a role in the dispersal of duckweeds as they entangle with the fur of other animals. The rhizoids of adjacent fronds entangle with each other to make a close-knit colony (Borisjuk, 2018). Vegetative reproduction could be observed by a compound microscope. A young frond showed a daughter bud (blue arrow) which was temporarily attached to the mother frond like a dumbbell structure A frond enlarges in size and shows further budding, a newly detached bud could be seen in lateral view.

D. *Scanning Electron Microscopy of Lemna aequinoctialis*

The size of an old frond was found to be 2.04 mm by Scanning Electron Microscopy. The average size of the collected *Lemna aequinoctialis* was found to be 1.82 mm by SEM. The duckweed surface has an adaxial surface towards the air and an abaxial surface facing water. The abaxial surface was irregular bearing distinct circular structures (purple arrows) spread uniformly (fig. 3A) across the frond, but the polygonal epidermal cells could not be visualized like they were seen in *Spirodela* (Pan, 1979) and *Wolffia* (Barthlott, 1998). It was observed that there were rows of stomata (red arrows) arranged parallel to one another on the adaxial surface (fig. 3 A), the number of stomata were more than those observed in *Wolffia* and *Spirodela*. The adaxial surface was covered with a heavy wax layer indented periodically with stomata (fig. 3C) which were spaced close together (red arrows), here many were wide open stomatal apertures (ws) and some were closed or had a narrow opening (ns). Figure 3B shows the close up of the wg/ wax globules found on the abaxial surface. We followed the terminology of the wax classification proposed by *Barthlott* el al. (Laird, 2019), to recognize the epicuticular wax. Fine wax crystalloids (term as per *Barthlott* el al., 1998) could be seen spread all over the dense adaxial surface in between the stomatal apertures (fig 3C). Guard cells surrounded the stomatal aperture, while subsidiary cells were absent (fig 3C). Some stomatal apertures were wide

Fig. 3 Representative cryo-Scanning Electron Micrographs, A. An intact frond showing adaxial surface with stomata (red arrows), the



abaxial surface showing circular wax globules/ wg (purple arrows). B. 20µM scale bar: Close-up of the adaxial surface showing numerous stomata indenting the wax layer on the surface. Stomata could be seen with closed, open and wide-open aperture. C. 10µM scale bar: the abaxial surface of the leaf showed wax globule (wg) of 0.29 micron, plates and rosettes. D. 10µM scale bar: Stomata could be observed closely, gc signify the guard cells surrounding both the narrow stomatal aperture (ns) and the wide stomatal aperture (ws).

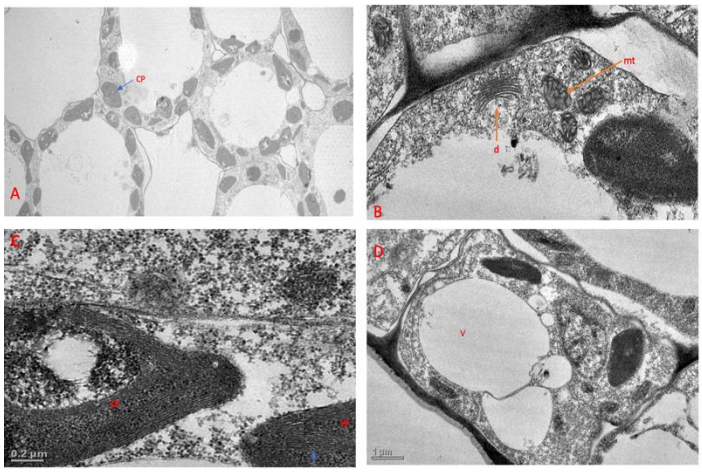
open (ws) while others were narrow (ns) as can be seen in fig 3C. The abaxial surface was also observed and was found to be

lacking stomata. We followed the terminology of the wax classification proposed by *Barthlott* el al (Jones, 2021). The abaxial surface was distinctly covered with spherical (purple arrow, fig 3A) wax crystals or wax globules (wg). Wax globules could not be seen on the abaxial surface of *Spirodela* which instead showed a net of wax spread over the cells (Pan, 1979). The size of one wg on the abaxial surface was 24.99 µ m in correspondence to the scale in SEM. The classification by *Barthlott* el al. was created after studying 13000 species, still we could not find a record of wax globules (wg), or similar structure displayed by any other Angiosperm in their study. As per the terminology proposed by *Barthlott* el al. plates (p) could be seen distributed between the wg, these plates organized into rosette (r) at several places on the abaxial surface (Fig. 3D). In *Spirodela* ecotypes these crystalloids were only sparsely spread across the abaxial surface. In our study we found density of these white crystalloids on the dense abaxial surface of *Lemna aequinoctialis*.

E. Microscopic Examination of Lemna aequinoctialis

TEM was conducted on the isolated fronds of the *Lemna* species. The structure was well suited to a floating plant on a water surface because the vacuoles occupy the maximum space in the plant's internal morphology (fig. 4A), the vegetative frond consisted of chlorenchymatous cells with chloroplast (CP) being the most abundant structure (fig. 4A). Figure 4B). The

Fig. 4 Representative Transmission Electron Micrographs of *Lemna aequinoctialis* leaves. A. At 0.2 µM scale bar Chloroplast (CP) were visible in chlorenchymatous cells, B. At 0.2 µM scale bar, dictyosomes



were observed (g) and mitochondria (mt) were observed at the periphery of the cell., C. Ultrastructural features of a chloroplast could be seen at 0.2 µM scale bar. Blue arrows pinpoint > 3 thylakoid stromal lamellae. D. At 1 µM scale bar vacuoles were observed.

dictyosomes (d) was obvious with cis and trans cisternae which indicated that the plant was actively making cellular proteins. Numerous mitochondria (mt) were visible indicating that the cell was metabolically active. In figure 4C, chloroplasts stroma lamellae were observed but the lamellae were not organized into grana, thylakoid structures were seen, and ribosomes were distributed throughout the cell. The intercellular spaces demonstrate different stages of wall splitting. The cytoplasm shows numerous ribosomes. Vacuoles (v) are visible in figure 4D.

II. DISCUSSION

Like *Arabidopsis thaliana*, duckweeds are being considered as model systems for studying ecology and evolution because of their short lifespan, morphological simplicity, cosmopolitan distribution, availability of DNA barcodes for species identification, and feasibility of multigenerational studies (Crawford, 2006). Therefore, knowledge about the basic anatomy of their system is key to understand all the applied studies and fundamental research. Scientists have recently employed X-ray computed microtomography for studying the anatomy and morphology of the duckweeds (Sree, 2015). But such techniques are neither universally available nor affordable and demand expertise. We utilized simple microscopic tools like foldscope, compound microscope and electron microscope to study the isolated duckweed which was identified by us to be the closest relative of *Lemna aequinoctialis*. Duckweeds show several sympatric species as a result the variance among genomes of local species are minimal. Therefore, duckweeds are considered model systems for studying evolution and speciation in plants (Rozema, 2002). It is known that the duckweed genomes display geographical distinctions therefore reporting species from different areas is particularly important. Especially in the light of the fact that despite asexual reproduction, duckweeds show immense genotypic variations. DNA barcodes of plastid genomes are usually utilized for duckweed taxonomic identification. Please add the atp discussion here.

Lemna aequinoctialis displayed distinct surface characters from *Wolffia microscopica* (Chen, 2013) and *Spirodela polyrhiza* (Pan, 1979) plant even when we fixed them in similar manner as was done by previous researchers. The polygonal cells reported on the surface of *Wolffia* and *Spirodela* could not be visualized despite similar fixing chemicals. Number of stomata were also more numerous than those reported for Wolffia and *Spirodela* species. *Lemna aequinoctialis* had a thick cuticle with heavy wax coating as opposed to weak cuticle reported in *Wolffia microscopica*. Plant cuticle plays central role in protecting plants from environmental stress. Since duckweeds float on water thus conservation of water cannot be the objective of a thick cuticle, rather protection from UV radiations could be one of the functions of the duckweed cuticle. Since we collected the sample in April from a pond with a secluded collection of Yamuna river in Delhi NCR, when Delhi receives scorching direct sunlight, we believe that the thick layer of wax on the adaxial between the stomata have protective role against UV irradiation in the field. Some phenolic components in the cuticle have been known to have UV-attenuating properties (Long, 2003; Domínguez, 2011). Wax cuticles are known to scatter light to protect against harmful UV irradiation (Chen, 2013). Moreover, the thick wax covering may

help to fight environmental stress and water and air-borne diseases.

Leaf surface cuticle has been studied for terrestrial plants because it protects the plants from dehydration. Taxon specificity has been discovered for leaf cuticle components in several groups of plants, like grasses are rich in beta diketones. Species-specific variations are also found for basic biochemical components like alkanes, aldehydes, esters, etc. (Jetter, 2006; Koch, 2006) . Adaptive role of leaf waxes has been discussed for terrestrial plants (Koch, 2006). Many plant species show substantial layer of wax composed of triterpenes and aliphatic acids on their leaves. These layers differ as per the geographical region therefore, it is obvious that the leaf cuticle undergoes evolutionary variation to adjust to the environmental conditions. Many researchers have reported fatty acids and primary alcohols to be the main components of the duckweed cuticles, a recent study reported fatty acids to form more than 95% of the cutin (Pan, 1979), but due to fund restrictions cutin wax compositional analysis is beyond the scope of our study. We found distinct wax globules on intact abaxial surface of *Lemna aequinoctialis*. The plant wax globules are known to be composed of fatty alcohols because the fatty alcohols can form surface crystals. We speculate that a thick wax coverage on the abaxial surface protects the duckweeds from infection and deeper attachment by other aquatic organisms. *Barthlott* et al. also considered the cuticle wax important for the ecological interactions of plants with the environment. They studied the epicuticular wax of 14000

Table I. Primers for the seven DNA barcoding markers

species of angiosperms and classified a total of 23 wax projections as crystalloids, platelets, tubules, etc., as per their structure. Still, the wax globules or circular crystalloids reported by us on the abaxial surface of *Lemna aequinoctialis* were not documented in their extensive analysis. *Barthlott* el al. adhered great systematic significance to these wax structures due to their specific diversity. We conclude that the duckweed wax morphology needs to be studied to document the diversity of ecotypes or species. The classification of wax structures can become an important measure along with genotypes to classify duckweed ecotypes.

III. CONCLUSIONS

In this study we isolated a duckweed and identified it to be an ecotype of *Lemna aequinoctialis*. The frond was studied via Scanning Electron Microscopy to uncover unique features like wax globules on adaxial surface and numerous stomatal apertures on the abaxial surface. These features had a distinct pattern from other duckweed species. The Transmission Electron Microscopy uncovered numerous features common to duckweeds.

A. Methods

1) Source of plant and Sample collection

Duckweed fronds were collected from an undisturbed pond close to Yamuna River in Delhi NCR with the help of a bucket and a strainer. Homogenous sample was obtained with the help of forceps and washed in distilled water in a petridish to remove any attached plankton. As the duckweeds are not endangered therefore specific ethical clearance was not required. The remaining water sample was returned to the same pond and was not discarded into the water drainage system of Delhi. Homogenous sample was obtained with the help of forceps and washed in distilled water in a petridish to remove any attached plankton.

S.No	Primer Sequence	Primer name
1.	5’-TTAGCATTTGTTTGGCAAG-3’	**psbK-psbI (F)**
2.	5’-AAAGTTTGAGAGTAAGCAT-3’	**psbK-psbI (R)**
3.	5’-ACTCGCACACACTCCCTTTCC-3’	**atpF-atpH (F)**
4.	5’-GCTTTTATGGAAGCTTTAACAAT-3’	**atpF-atpH (R)**
5.	5’-GTTATGCACGAACGTAATGCTC-3’	**tmH-psbA (F)**
6.	5’-CGCGCGTGGTGGATTCACAATCC-3’	**tmH-psbA (R)**
7.	5’-CGTACTGTACTTTTATGTTTACGAG-3’	**matK (F)**
8.	5’-ATCCGGTCCATCTAGAAATATTGGTTC-3’	**matK (R)**
9.	5’-ATGCAGCGTCAAGCAGTTCC-3’	***rpoB* (F)**
10.	5’-TCGGATGTGAAAAGAAGTATA-3’	***rpoB* (R)**
11.	5’-GGAAAAGAGGGAAGATTCCG-3’	***rpoC1*(F)**
12.	5’-CAATTAGCATATCTTGAGTTGG-3’	***rpoC1*(R)**
13.	5’-GTAAAATCAAGTCCACCACG-3’	***rbcL*(F)**
14.	5’-ATGTCACCACAAACAGAGACTAAAGC-3’	***rbcL*(R)**

2) DNA Isolation

The *Lemna* fronds were carefully separated to homogeneity and washed for 5 to 10 times to remove any other sticking zooplanktons and phytoplanktons. The Sample was crushed using liquid nitrogen in a mortar and pestle, further processed using the DNA isolation kit (Qiagen, Catalog no:69106). Plant tissue was firstly disrupted mechanically followed by lysis using lysis buffer. Further it was kept for incubation. RNAse A in the buffer digested the RNA present in the sample. After lysis proteins and polysaccharides were salt precipitated. Cell debris and precipitates were removed by centrifugation. Ethanol and binding buffer were added to cleared lysate to promote binding of DNA to membrane of column. Sample was then centrifuged, DNA bound to the membrane while other polysaccharides and debris pass through the column by two wash steps. The DNA was eluted by water or low salt buffer. The genomic DNA was saved at -80°C until further use.

3) PCR amplification

The genomic DNA isolated from the duckweed fronds was utilized as the template DNA to amplify the target genes by polymerase chain reaction. The primers outlined in table 1 were utilized in PCR to amplify genes from duckweed family or the seven barcoded DNA markers, namely, Maturase K, PsbK-PsbL intergenic spacer, Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, RNA Polymerase Beta subunit, RNA Polymerase C, trnH-psbA intergenic spacer, and atpF-atpH intergenic spacer (Wang et al., 2010). The conditions utilized for the Polymerase chain reaction were as described by Wang et al. (Wang et al., 2010). Reaction mixture for each reaction contained the genomic DNA for duckweed, 5 Units of Taq DNA polymerase, Taq DNA Polymerase buffer, dNTPs and respective forward and reverse primers as per table 1.

4) DNA isolation and DNA sequencing

The seven bands were individually excised and eluted by standard method utilizing the kit. The seven respective DNA aliquots were sent for sequencing with their respective primers for dideoxy-DNA sequencing on an ABI 3730 XL 96-capillary array DNA analyzer using Life Technologies BigDye terminator version 3.1 at the South Delhi University DNA Sequencing Facility.

5) Bioinformatics Analysis

Sequences were extracted by reading the chromatogram files using FinchTV 1.4.0 (http://www.geospiza.com/Products/finchtv.shtml). DNA sequences of all samples were with good quality scores. To find the closest homologs and to verify their identity, all sequences were subjected to BLASTn 2.9.0 (Citation: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2803857/) standalone program against non-redundant (NR) NCBI nucleotide database with an e-value cutoff of $1E^{-5}$ and 35% identity.

B. Microscopic examination

The sample was prepared to be visualized with foldscope and compound microscope to observe the morphology of the fronds.

1) Scanning Electron Microscopy

The whole fronds of the duckweed were fixed in the Karnovsky's Fixative or a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The fixed sample was stored in sodium phosphate buffer at 4 ˚C. This was followed by secondary fixation Osmium tetroxide (OsO4). The surface of the fronds was visualized by Scanning Electron Microscope (ZEISS Evo 18) at the All India Institute of Medical Sciences (AIIMS), Delhi, India.

2) Transmission Electron Microscopy

The sections were cut using RMC PowerTome PC. It was stained using a saturated solution prepared by adding excess uranyl acetate to 10 ml of filtered 50% ethanol in a centrifuge tube which was shaken vigorously for 2 min and excess uranyl acetate was spun down. After that, it was stained using aqueous lead citrate solution (prepared using half of a pellet of sodium hydroxide to 12 ml of double distilled water in a centrifuge tube (pH 12-13) which was shaken well until it dissolved. 50 mg of lead citrate was added and mixed well for 2 min and centrifuged). Small droplet of lead citrate was pipetted on a piece of parafilm kept in a petridish and was place the grid (300 mesh × 62 µm pitch, copper) with its section side facing down onto the stain. It was stained for 5 to 10 mins and each grid was briefly washed in 0.02 M sodium hydroxide and then twice in double distilled water. Grids were then dried and the sections were observed under TEM. The processed sections were visualized by TECNAI G20 HR-TEM 200kV Company (THERMO SCIENTIFIC) at the All India Institute of Medical Sciences, Delhi, India.

ABBREVIATIONS

DNA, Dioxyribonucleic acid; ns, Narrow stomatal aperture; SEM, Scanning Electron Microscope; TEM, Transmission

Electron Microscope; wg, wax globules; ws, wide open stomatal aperture.

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