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Morphological study and Molecular Phylogenetic analysis of Class Litostomatea (Phylum: Ciliophora)

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ABSTRACT:

Two Litostomatean ciliates *Didinium nasutum* and *Lacrymaria* sp. were collected from India’s largest fresh water lake, Kolleru, India. They were investigated by means of live observation, feulgen staining and SSU rRNA encoding gene sequencing. Monophyly of the class Litostomatea was tested with 38 sequences which including the two sequences obtained from this study for the first time from India and 36 sequences from NCBI. SSU rRNA based phylogenetic tree was estimated to understand the taxonomical position of the two isolates, apparently the monophyly of the class Litostomatea and their sub classes were tested. The results indicate that the class Litostomatea is monophyletic and the sub class Haptoria is unresolved. The *Lacrymaria* sp. obtained here is potentially new or the species which was described earlier only by means of morphological methods with no molecular evidences.

Key words: SSU rRNA– Monophyly– Haptoria– Litostomatea.

1. INTRODUCTION:

The Taxonomy of the class Litostomatea has traditionally been a story of uncertainty as these organisms exhibits diversity in species ranging from aerobic free– living predators to anaerobic endocommensals. Lynn proposed two subclasses i.e. Haptoria and Trichostomatia. However, a third subclass, Rhyncostomatia, was recently established by Vďačný et al. (2011) and resolve the status of Dileptus and Tracheleus species. The general classification of class Litostomatea consists of three subclasses and can be summarized as follows: 1) Trichostomatia 2) Haptoria (Lacrymariida, Haptorida, Didiniida, Pleurostomatida, Spathidiida) and 3) Rhyncostomatia (Dileptiida and Tracheliida). Rhyncostomatia and Trichostomatia had been proven to be monophyletic by Vďačný et al. (2011) however, the ambiguity on the monophyly of the class Haptoria is still unresolved.

Although lot of molecular work has been done in the last two decades, still there were lot of morphotypes needed to be supported with molecular data. The Litostomateans in terms of morphological and molecular data has been under– sampled and the molecular work has been progressively increasing in the past decade. But from tropical countries like India, there were negligible molecular sequences available online on Litostomateans and the several taxa needed to be characterized to help unknot the uncertainty inside the Haptorian’s evolution. The main objectives of this study are as follows: 1) to characterize novel or never surveyed Litostomateans ciliates from India 2) To test the hypothesis of monophyletic evolutionary pattern of Litostomatea and its subclasses. In this present study as a first step towards Litostomateans from India, two Haptorian ciliates *Didinium nasutum* and *Lacrymaria* sp. were characterized by both morphological and molecular methods. This study provided molecular support to *Didinium nasutum* for the first time from Indian Litostomateans and also added an interesting uncharacterized Lacrymaria sp. sequence to the GenBank and provided hints on potentially novel or uncharacterized species from India. The results recovered the monophyly of class Litostomatea with high bootstrapping values of 100% however, the monophyly of subclass Haptoria is still unresolved in this study.

2. MATERIALS AND METHODS:

2.1. Methods for Morphological species identification and Taxon sampling and culturing:

Sampling was carried out in India’s largest freshwater lake “Kolleru” (Andhra Pradesh, India) (16°36' 48.4"N & 081°18'32.7"E) where sediment samples together with some aliquot of surface water were collected, using sterile 50 ml Falcon tubes. Monoclonal cultures were tried to establish for several times but ended up losing the material hence the taxa that were processed for experiments and described here are true populations which were maintained in its original sample in the laboratory for a period of 1 month from the date of sampling by feeding with appropriate preys. Paramecium polycaryum, Paramecium aurelia as a prey for *Didinium nasutum* however the species attained resting cyst form after few weeks whereas in case of Lacrymaria it was kept in the original environmental sample to let the species predated on the small ciliates e.g. scuticociliates). Several failed attempts on Lacrymaria sp. were done with scuticolciliates as a feed originated from the same sample. It resulted in keeping the Lacrymaria alive for a period of 20 days but no signs of division were seen and apparently lost the species. (Fig-1)

2.2. Live observations:

Preliminary morphological identification of live specimens followed by Feulgen nuclear staining and morphometric analysis were performed following the method. However, due to consistent cyst forming ability much morphometric data from live specimens of *Didinium nasutum* was not possible to collect however, Feulgen staining was performed to reveal the composition of nuclear apparatus. Pictures from life and stained specimens were taken and record the data. Where as in case of Lacrymaria sp. was survived in the original sample for a few days and later it was disappeared.

2.3. DNA extraction and storage:

Approximately 90 cells of *Didinium nasutum* cells and 25 Lacrymaria sp. were washed and fixed in 70% ethanol for Total genomic extraction in their respective eppendorfs by following the method.

2.4. PCR, Sequencing and Molecular Phylogeny:

The almost full–length SSU rRNA gene for both *Didinium nasutum* and *Lacrymaria* sp. were amplified, sequenced, assembled, eventually molecular phylogenetic analysis was performed by following the methods. 186 The PCR was successful in the first attempt for *Didinium nasutum* (16 ng/ µl) to process for sequencing whereas in case of Lacrymaria sp. the PCR reaction in the first attempt did not yield the right PCR amplification product of the target sequence hence additionally a semi nested PCR was performed and subsequently achieved the right quantity (14 ng/µl) which was sufficient enough to get the target sequence.

Later PHYML 5.3.2 software was used to produce maximum likelihood tree (Guindon & Gascuel, 2003). A total of 38 taxa was used for phylogenetic analysis, including the two newly sequenced species (highlighted in red & Bold text) and the outgroup sequences (sequences in number of four from Oligohymenophorea, three from Spirotrichea, and four from Armophorea). Other than the two new sequences used in the study, the remaining sequences were selected from online data server (GenBank–NCBI). Lengths of sequences were trimmed according to shortest ones obtaining characters matrix of 1719 for both *Didinium nasutum* and for *Lacrymaria sp*. Tree was built on the modified character matrix employing the GTR+I+G (4 discrete categories) model as indicated by AIC parameter, calculated by Model Test. All the nodes were recovered with a calculated bootstrap values (100 pseudo replicates) for performing Maximum Likelihood analysis.

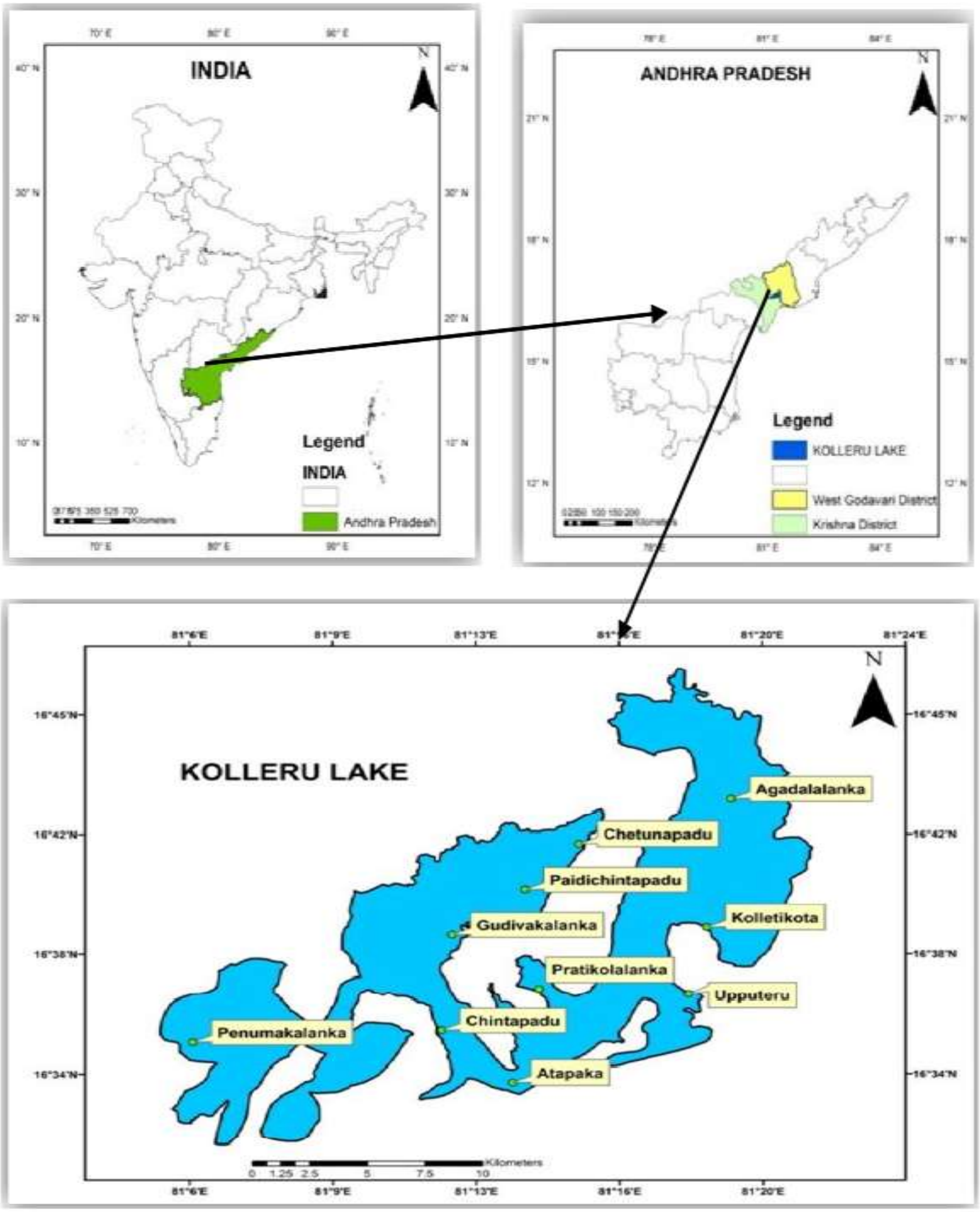

Figure:1- Study Area

3. Results:

Class : Litostomatea, Small & Lynn 1981

Subclass : Haptoria Corliss, 1974

Order : Haptorida Corliss, 1974

Family : Didiniidae Poche, 1913

Genus : *Didinium* (O.F. Müller, 1773) Stein, 1859.

3.1. Didinium nasutum (Fig 2A–C)

Synonyms:

Chytridium, Ecclissa, Rinella.

Diagnosis:

Body is transparent or beige colored, barrel shaped with the somatic cilia restricted to two girdles, 1 equatorial and other 1 is just behind the conical oral area. Oral cone protruded out from the anterior region and bundles of toxicysts are present inside the cone and scattered in the cytoplasm. Typical live and Feulgen-stained specimen showed horse–shoe shaped macronucleus and its associated micronucleus.

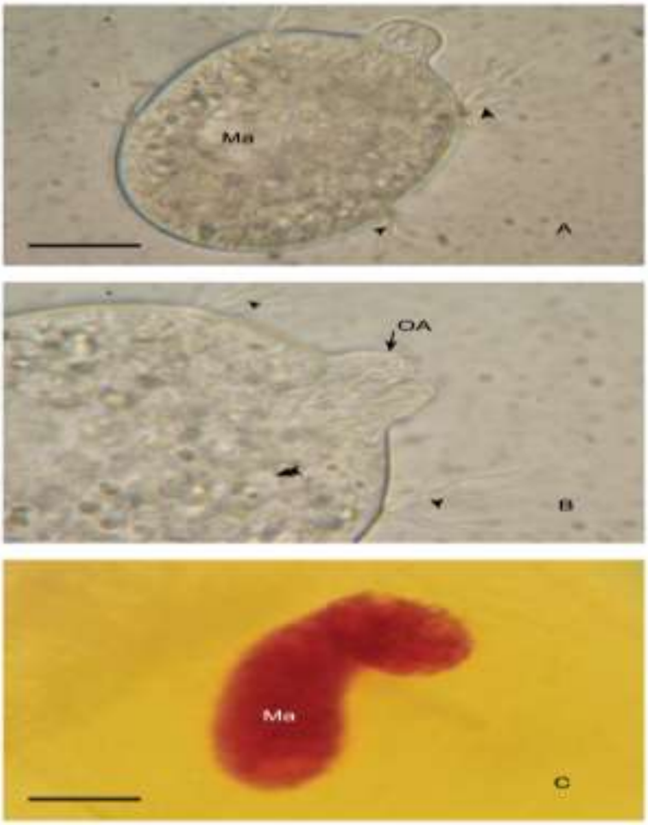

Figure:2- Photomicrographs of *Didinium nasutum* from life A, B and after Feulgen staining (C),
(A) Slightly pressed typical individual showing oral cone and nuclear apparatus and ciliary bands,
(B) Details of anterior and posterior ciliary band and oral cone ejecting toxicysts,
(C) Details of semi-circular macronucleus.
Abbreviations: OA, oral apparatus, Ma, macronucleus, ciliary band (arrow heads), cytoplasmic inclusions (double arrow heads). Bars stand for 20 µm.

Single contractive vacuole located at the posterior end with one pore. Voracious predator feeds on Paramecium sp., other ciliates ranging from small to larger specimens and rotifers. Rapid swimmer moving in all directions to the body axis.

Type locality

Didinium nasutum was collected from India’s largest freshwater lake, Kolleru (16°43'11.2"N, 081°19'36.1"E), Andhra Pradesh, India

Type material

A slide with the Feulgen stained *Didinium nasutum* specimen (indicated with a black circle of ink on the coverslip) and some Holotype specimens has been deposited in the collection of the largest freshwater lake, Kolleru.

Gene sequence:

18S rDNA sequences of *Didinium nasutum* have been deposited in NCBI GenBank database with the accession numbers (KY855551).

Description of *Didinium nasutum*:

Cells in vivo were oval or barrel shaped and measures about 118–140; the posterior end of the cell is rounded and the anterior end of the cell is slightly protruded out as conical projection called oral cone which is covering the cytostome. The oral cone is composed of numerous bundles of toxicysts. The length and width of the oral cone was measured about 9x10.4 µm. A distinct horse–shoe shaped or semicircular disc like macronucleus occupied the middle portion of the body with a small micronucleus attached to it. A pulsating small contractile vacuole was located at the posterior portion (3/4) of the cell. Body is encircled by two dense ciliary bands i.e. anterior ciliary band and posterior ciliary band respectively (Fig. 2.A**).**

Molecular characterization and Phylogeny:

Molecular analysis on *Didinium nasutum* species isolated in this study showed 99.9 % identity with another *Didinium nasutum* sequence (U57771) available from NCBI GenBank. The results had clearly showed that the *Didinium nasutum* forms sister clade with another *Didinium nasutum*, U57771; clustered to Enchelyodon sp., U80313 and Homalozoon vemiculare, L26447; as a single clade with in the subclass Haptoria (Fig. 2-C).

The more detailed description will be presented in the following discussion part.

Class : Litostomatea Small & Lynn, 1981
Subclass **:** Haptoria Corliss,1974
Order **:** Haptorida Corliss, 1974
Family :Lacrymariidae
Genus **:** Lacrymaria Bory, 1826.

Lacrymaria sp.

Synonyms none

Diagnosis:

Body is often flask–shaped with the anterior end like bulb with dense cilia, anterior end is highly contractile with the neck like region bearing the oral region, neck connected to posterior broad end carrying cellular components.

Type locality

Lacrymaria sp. was collected from India’s largest freshwater lake, Kolleru (16°43'10.2"N 081°19'35.2"E), Andhra Pradesh, India.

Type material:

none (lost the ciliate in few days)

Gene sequence:

18S rDNA sequences of L*acrymaria* sp. have been deposited in NCBI GenBank database with the accession numbers (KY855552).

Description of *Lacrymaria* sp.

Lacrymaria sp. measured about 196 x 24µm (measured when fully extended), the body appeared transparent to slightly beige color. The body has been appeared as a “vase” shape with small "head" like structure at the anterior end of a long slender "neck" which continued as a broad posterior base where the cell organelles rests. This species is highly contractile especially at the neck region which was waving rapidly in all directions to capture the prey however it swam slow to moderate speed rotating about main body axis. The live specimens usually have a single granular ellipsoid shaped macronucleus measured about 32x23 µm with two nodes like appearance with a distinct groove in the centre and a single micronucleus was located in the vicinity of the groove. Cytoplasm carried lot of mineral crystals, small conspicuous and round shaped inclusions, two types of extrusomes or toxicysts scattered all over the body and appeared in tow shapes as follow: long rod and bow shaped respectively.

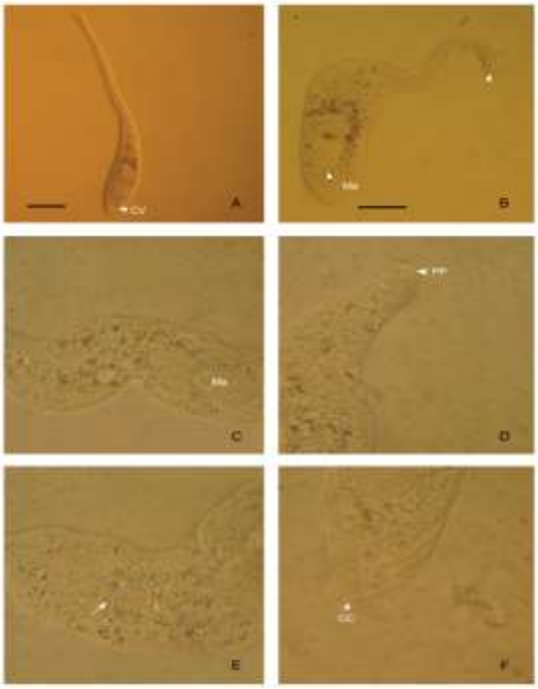

Figure:3- Photomicrographs of *Lacrymaria sp*. from life (A-F).

(A) Dorsal view of elongated specimen showing nuclear apparatus and contractile vacuole;
(B) Dorsal view of representative specimen showing variation in shape
(C) Cytoplasm showing nuclear apparatus;
(D) Detailed view of pharyngeal plug;
(E) Details of cytoplasm showing extrusomes;
(F) Details of posterior portion showing caudal cirri. Abbreviations, PP, Pharyngeal plug: Ma-Macronucleus, Extrusomes (Arrow) CC, Caudal cirri. Bars stand for 30 µm.

Its entire cell body is covered with cilia arranged in spirals. it has two contractile vacuoles, one at the middle portion in the proximity of the macronucleus and the second one at the sub–terminal end of body. The posterior end is slightly pointed. The anterior region carried head like structure with the oral apparatus carrying distinct pharyngeal plug. Margins of pharyngeal plug bear longer cilia when compared to somatic cilia of whole body Lacrymaria fed primarily on smaller organisms such as other small scuticociliates, but may sometimes taps out the larger ciliates (Fig.3).

Molecular characterization and Phylogeny of *Lacrymaria:*

Lacrymaria sp. isolated in this study showed identities values with the previously published sequences as follows: 100% with Lacrymariidae sequence (LN869967) and 98% with *Lacrymaria marina* (DQ777746) available in the GenBank. However, it differs by 37 mismatches and 5 gaps which is a huge difference to interpret as" Lacrymaria marina" indeed the sequence presented in this study can be ascribed as new isolate or a sequence which was never ascribed to one of the morphotype published by subsequent authors without any molecular data. Phylogenetic tree clearly showed that the species isolated in this study was clustered with Lacrymaria marina with a high bootstrapping of 98% and embedded inside the order Haptoria.

Molecular Phylogeny of the class Litostomatea:

In the first subset of phylogenetic analysis, species of the subphylum Intramacronucleata (Oligohymenophorea, Spirotrichea and Armophorea) were used as outgroups and phylogenetic tree was estimated by using 38 sequences in which two newly sequenced SSU rRNA gene of *Didinium nasutum* and *Lacrymaria* sp. in the present study were used and the remaining 36 sequences were selected from NCBI GenBank (refer table 1). The ML tree is shown in fig-3.

Is the class Litostomatea monophyletic?

Results had clearly showed that the presently sequenced *Didinium nasutum* sequence grouped together (100% bootstrapping value) with the sequence of *Didinium nasutum*, U57771. Together they clustered with Enchelyodon sp., U80313 and Homalozoon vemiculare, L26447 forming a monophyletic clade, although not well supported by statistical values. Moreover, Chaenea teres, EF486860 and Chaenea vorax, DQ190461 branched off basally inside the Haptorida clade which, in turn, formed a sister clade with the order Pleurostomatida.

Is Haptorida monophyletic?

The monophyly of the order Pleurostomatida is strongly supported as follows: all the Loxophyllum species (Loxophyllum rostratum, DQ411864; Loxophyllum jini, EF123708) clustered into a monophyletic clade that forms a sister group to Loxophyllum utricularie (L26448) whereas all the Lithodidae family members like (Litonotus paracygnus, EU242509; Litonotus paracygnus, DQ190464) selected occupying the basal position with in this clade which in turn grouped with a Amphileptidae members (Amphileptus aeschtae, EU242510; Hemiophrys procera, AY102175).

The newly sequenced Lacrymaria sp. grouped with Lacrymaria marina (DQ777746) (98% of bootstrap) and together clustered with Phialina salinarum (strong bootstrapping support 99%) forming a monophyletic clade and branched separately from other haptorians and occupying the basal position in the Haptorida. Arcuospathidium muscorum, DQ411859 clustered with Enchelys polynucleate, DQ411861 and together they formed a strongly supported clade with Spathidium stammeri, DQ411862. The two sequences of Epispathidium (Epispathidium papilliferum, DQ411858 and Epispathidium papilliferum, DQ411857) formed the sister clade of the subclass Trichostomatia.

The Dileptus sequences (DQ487195& AF029764) did not cluster together with other Spathidae family member and they also branched away from Didinidae family, suggesting that Dileptus should be shifted to the subclass Rhyncostomatia (Vďačný et al., 2011). The subclass Trichostomatia forms a strongly supported clade, including the following sequences: Balantidium coli AF029763, Triadinum caudatum FM201782, Bandia smalesae AF298822, and Macropodinium yalabense AF042486.

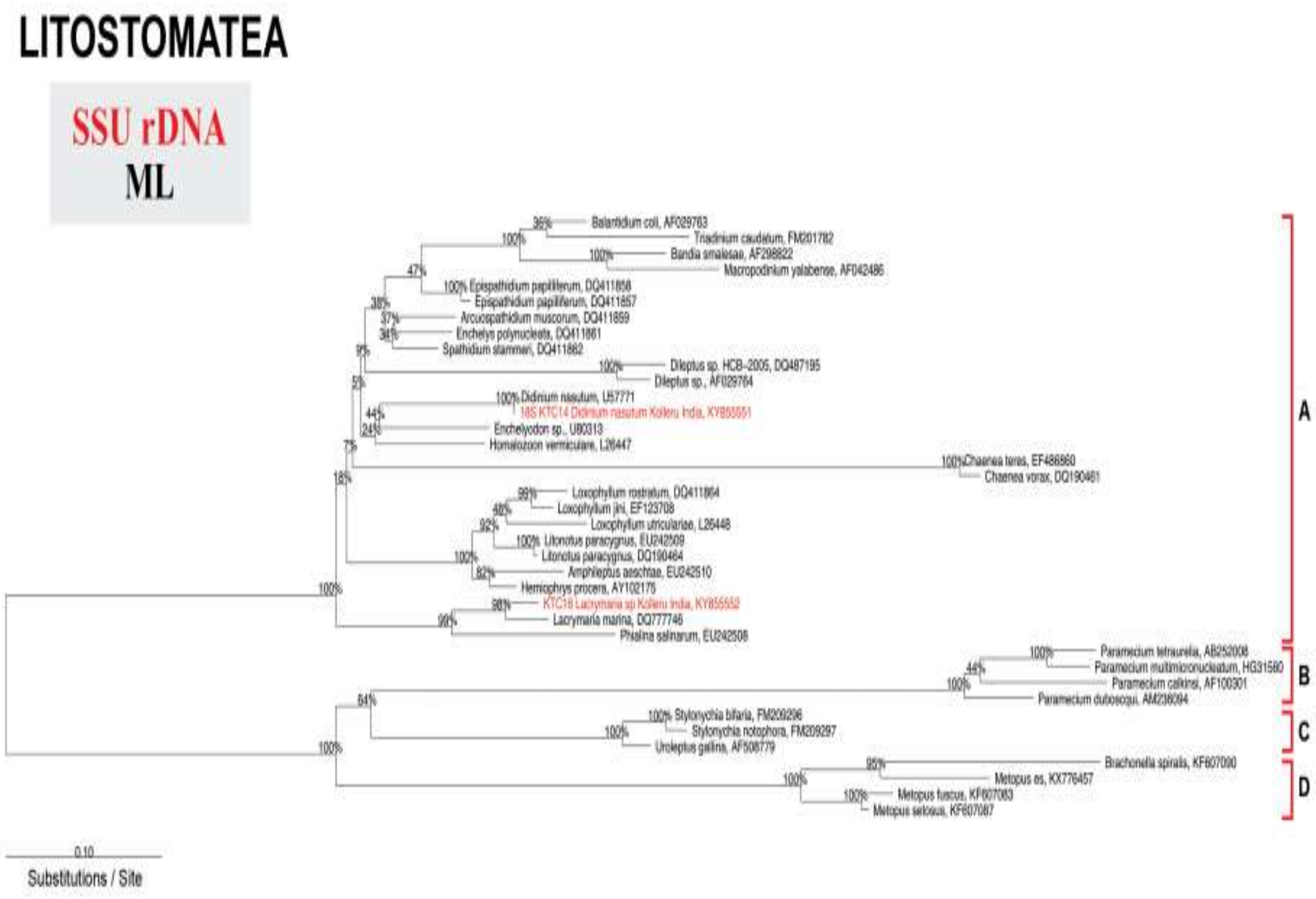

Figure: - 4 Maximum-Likelihood (ML) tree of the class *Listomatea* inferred from the small subunit ribosomal RNA (SSU rRNA) gene sequences. Numbers at the nodes represent the bootstrap values of ML (values above 70% are shown). Sequences obtained in the present work are shown in bold text highlighted with red colour.

3.4. Discussion

Considerations on *Didinium nasutum*:

The Haptorian predatory ciliate *Didinium nasutum* was reported for the first time as Vorticella nasuta by Müller (1773). Later it was nomenclated as *Didinium nasutum* by Stein (1859) and placed in the order Peritricha. Bütschli removed these taxa from Peritricha, and placed in the order Holotricha (Bronn, 1889). The order Haptorida was created by Corliss and transferred the *Didinium nasutum* within the subclass Haptoria (Corliss, 1974). The class Litostomatea was later created and placed the taxa under this group (Small & Lynn, 1981).

On the other hand, Balbiani made an attempt to describe the morphology and biology (Balbiani, 1973). In the beginning of 20th century, the feeding behavior details on trichocysts were described (Mast, 1909). Later the morphological trait i.e. two zone of cilia was raised as a key to identify this ciliate (Hausman, 1921). It was followed by the first Taxonomical revision (Kahl, 1930). In the 1960's Indian authors were also had contributed to the morphological description (Mahajan, K. K., 1969; Naidu, 1965).

Afterwards, for the first time the species was described by means of silver impregnation and scanning electron microscopy (Wessenberg & Antipa 1968). The predation method in detail was described Wessenberg & Antipa 1970). In 1977, studies on the preservation of this ciliate (McGrath et al., 1977) was described. Meanwhile in the speciesomic scheme produced by Corliss the ciliate was placed inside the family Didinidae (Corliss, 1977) produced. Foissner (1984) described the species by means of both live observations and silver impregnation. In 1986, this species had been described briefly inside the taxonomical monograph (Dragesco & Dragesco–Kernéis, 1986). In 1988, it has also recorded inside the protozoological catalogue by Foissner, et al. (1988). The ultrathin sections were produced to reveal the chromatin of the nucleus by Raikov et al. (1995). In 1996, this species was also reported in the Himalayan region of northern pole of India Gupta & Shukla (1996). Foissner et al. (1999) redescribed this species. The details about the resting cysts of *Didinium nasutum* were explained as a survival strategy by Verni & Rosati (2011). This was also retrieved from marine biotype by Kalavati & Raman (2008) and from sewage treatment plants by Bindu et al. (2013). Chromatin Organization of macronucleus was determined (Popenko et al., 2015); feeding behavior on paramecium species was discussed in the trophic state (Kh & Mansimova, 2017). The morphology of this predatory ciliate studied here is in agreement with the previous studies (Foissner et al., 1999; Kalavati & Raman, 2008).

Considerations on the morphology of *Lacrymaria* sp.

The morphometry obtained here was quite handy to realize the genus. However, it was not sufficient enough to attribute to any species published as there were no staining techniques performed hence depended on the molecular methods to characterize.

Considerations on Molecular Phylogeny of the Class Litostomatea:

The fate of the sequences obtained in this present study as well of the monophyly of the class Litostomatea has been tested with the ML method. The ML tree resulted in clustering of the sequences of interest with all the litostomateans sequences under a monophyletic group with a strong bootstrapping support of 100% and the remaining Intramacronucleata sequences were clustered in their respective groups as outgroups as expected. The monophyly of the Litostomatea is consistent with the previous molecular studies (F. Gao *et al.,* 2016; S. Gao et al., 2008; Strüder– Kypke et al., 2006; Vďačný *et al*., 2011a; Vd'ačný, Bourland, Orsi, Epstein, & Foissner, 2012; Vďačný. P., 2011b) (Yan Y *et al*., 2019).

Sub class Haptoria:

The monophyly of the subclass Haptoria has not been resolved and remained paraphyletic (F. Gao et al., 2016; S. Gao et al., 2008; Strüder–Kypke *et al.*, 2006; Zhang et al., 2012). Our analysis had been aimed to test the position of the newly sequences from India and to check their respective positions in the tree and subsequently aimed to interpret the monophyly of the subclass Haptoria. The topology of the trees inferred from 18S rRNA sequence data of *Didinium nasutum* and *Lacrymaria* sp., had been indicated that they clustered inside the subclass Haptoria. The tree looked paraphyletic too in our tests with the following interpretations in agreement with the previous studies.

Considerations on *Dileptus* sp. within the subclass *Haptoria:*

The two isolates of *Dileptus* species were branched deeply outside the spathidae members inside the Haptoria with high mutation rates in agreement (Strüder–Kypke et al., 2006) and leaves ambiguity on the status inside the Haptorian members too. Nevertheless, the taxonomical ambiguity of *Dileptus* has been recently resolved (Vďačný et al., 2011) and classified them under the third subclass Rhyncostomatia within the Litostomatea.

Sub class Trichostomatia:

Subclass Trichostomatia forms a strong clade with the Balantidium coli clustered as a sister species to Triadinum caudatum and together they grouped with Bandia smalesae, (Zheng W. *et al*., 2021) and Macropodinium yalabense. This clade in turn grouped with the Epispathidium papilliferum species belongs to family spathidae of Subclass Haptoria which eventually resulted in the ambiguity on the monophyly of Haptoria.

Order Pleurostomatida:

Six sequences from NCBI GenBank were use in this study from the order Pleurostomatida to check the monophyly and the results strongly supported the monophyly of the order Pleurostomatida is as follows: All the Litonotidea species clustered with Amphileptidae members.

3.5. Concluding remarks and Evolutionary considerations:

Based on the present study form India, the author for now could not provide any conclusive evidence for the monophyly of Haptoria, with members of Haptoria grouping with Trichostomatia and also analyzed that Litostomateans in terms of molecular data is still under–sampled and suffered including this study.

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