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Morpho-molecular assessment of *Acetabularia jalakanyakae* Sp. Nov. (Dasycladales, Chlorophyta) - a new species from Andaman and Nicobar Islands, India

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Acetabularia (Dasycladales) is an extant genus of a single-celled green alga. There are four species of this genus reported from India, three reported from Andaman and Nicobar Islands. For this study, *Acetabularia* isolate was collected from a rocky intertidal habitat in the Andaman and Nicobar Islands. Light microscopy and Scanning Electron Microscopy were used for the morphological characterization. The distinct traits of caps of the thalli were prioritized because, traditionally, species delimitations in *Acetabularia* mainly were based on cap morphology. Our isolate showed morphological similarity with *Acetabularia crenulata*. However, the number of hairs in the inner ring of lobes of caps and the stalk length were observed to be different from *A. crenulata* and other closely related species. The phylogenetic tree constructed for partial 18S rDNA using the Maximum Likelihood (ML) method revealed the evolutionary affinity of this new species with *Acetabularia dentata*. Based on morphological and molecular synapomorphy, a new species of *Acetabularia*, *Acetabularia jalakanyakae* is formally proposed herein, and the further implications of this species discovery are discussed.

[**Keywords**: 18S rDNA, Maximum Likelihood (ML) method, New species, Phylogenetic tree, Scanning electron microscopy, Seaweed]

Introduction

Acetabularia is a unicellular green algal genus belonging to the family Polyphysaceae (formerly Acetabulariaceae) of the order Dasycladales. Species of Polyphysaceae are found in both subtropical[1] and tropical waters[2]. The present diversity of Dasycladales includes 38 species belonging to 10 genera and are divided into two families Dasycladaceae and Polyphysaceae, which can be considered as 'living fossils[1]. Molecular phylogenetic studies supported the Polyphysaceae derived from the Dasycladaceae[1,3,4].

This alga is dioecious and has a height of 0.5 to 10 cm. It comprised of three anatomical parts: at the bottom – a rhizoid with a set of short roots, in the middle – a long stalk, and at the top – an umbrella of branches that further fused into a cap. It is also called "Mermaid's Wineglass" because of its beautiful umbrella-shaped cap[5]. The life cycle of *Acetabularia* is complex and needs approximately 115 days to complete[6]. Species in *Acetabularia* genus possess giant plastid genomes with size up to ca. 2000 Kb, formed due to a secondary increase in the number of tandem repeats[7]. It also resembles higher plants in terms of chloroplast and protein synthesis mechanism[8].

The cap morphology is widely used in taxonomic studies because it is species-specific[9-12]. The umbrelloid ring of the cap consists of two zones: the inner, corona superior, a combination of rays, and an inner ring of lobes and is present in all species of *Acetabularia* and the outer zone, corona inferior, which is absent in some species. Various authors have described the complex morphogenesis of the cap structure, *viz.* Graf zu Solms-Laubachin[13] (*Acetabularia acetabulum)*, Valet[14] (*A. dentata* and *Polyphysa parvula*) by using light microscopy and Minowa & Shihira-Ishikawa[9] (*A. calyculum*) using Scanning Electron Microscope (SEM). Sawitzky *et al.*[10] concluded that the morphology of cap formation followed the same pattern in three species: *Acetabularia acetabulum*, *Acetabularia kilneri*, and *Polyphysa peniculus* by using SEM.

The 18S rDNA dataset has been extensively used for molecular studies in *Acetabularia*. Olsen *et al*.[15] made use of this locus to reveal that both *Acetabularia* and *Polyphysa* are not monophyletic. Later, Berger *et al*.[1] included *Acicularia schenckii* (as *Acetabularia schenckii*) and *Polyphysa peniculus* (as *Acetabularia peniculus*) under the genus *Acetabularia* based on the cap morphology and 18S

rDNA sequences. Further, Barattolo *et al.*[16] described a new species of Polyphysacean alga *Acetabularia moldavica* with a synapomorphic feature of the presence of both inferior and superior coronae.

The genus *Acetabularia* has 13 species, currently accepted taxonomically[17]. Of the only four *Acetabularia* species reported from India to date, three are from Andaman and Nicobar Islands. These are *Acetabularia calyculus*[18,19], *Acetabularia crenulata*[18], and *Acetabularia acetabulum*[19], while the species reported from the mainland Indian coast is *Acetabularia parvula*[20]. The present study attempted to identify and characterize a new *Acetabularia* isolate from Andaman and Nicobar Islands using morphological and molecular methods.

Materials and Methodology

Taxon sampling and morphological assessment

The unidentified species was collected from Port Blair, Andaman and Nicobar Islands, India, on 5th May 2019. The collected algal thalli were transported to the laboratory in a ziplock polythene bag at 4 – 10 °C. These were cleaned and stored at -80 °C for further studies. Representative specimens were pressed and deposited in the herbarium of Agharkar Research Institute, Pune and Central University of Punjab, Bathinda, India.

Photographs were taken from both upper and lower surfaces by a standard camera with a macro lens. A slide was prepared by taking the cap portion and fixing it to a glass slide using glycerol and visualized under a light microscope (CX24, Olympus Japan). Images were taken at 60X, 80X, and 100X magnifications. For SEM analysis small individual caps (1 to 2 mm diameter) were subjected to double fixation; 4 % glutaraldehyde in 0.1 Molar potassium phosphate buffer (pH 7.4) for pre-fixation and an acetone dehydration series (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 100 % acetone in water). Following the drying of the samples, analyses were performed to study the ultrastructure of the cap and to observe the outer and inner rings of lobes with a scanning electron microscope (SEM Carl Zeiss, Germany).

Molecular analysis

DNA isolation was performed by HiPurA Algal Genomic DNA Extraction Kit (HiMedia Laboratories Pvt. Ltd., Mumbai). The cap region was used for the extraction of DNA. The PCR reaction mixture of 20 µl was prepared, which consist of 2 µl of 10X buffer (Applied Biosystems, India), 2 µl of $MgCl_2$ (25 mM), 2 µl of 2 mM dNTP (Imperial Life Sciences, India), 0.2 µl of *Taq* DNA polymerase (Imperial Life Sciences, India), 2 µl of forward and reverse primers, 4 µl of template DNA and rest Milli-Q water. Forward primer 1547F (5′-AATGCCTAGT-AGGTTCCGGTCATCAG-3′)[1] was used in combination with reverse primer ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′)[21]. The ExoSAP-IT PCR clean-up kit (USB Corporation, Cleveland, OH, USA) was used to remove unattached primers and nucleotides from the amplified sequence. For this, ExoSAP-IT (2 µl) and PCR product (5 µl) were incubated for 15 minutes at 37 °C and denatured for 30 minutes at 80 °C in a PCR machine.

Purified PCR amplicons were subjected to bi-directional sequencing PCR using ABI BigDye Terminator Cycle Sequencing Ready® Reaction Kit v3.1 (Applied Biosystems, Foster City, CA,USA)[22]. Sequencing reactions were purified using the traditional ethanol/EDTA precipitation method[23]. The dried samples were suspended again in 15 µl of Hi-Di™ Formamide and vortexed for 30 minutes and were then transferred to a sequencing plate for capillary gel electrophoresis (Applied Biosystems 3730xl Genetic Analyzer, Foster City, CA, USA). The sequence analysis and contig assembly were performed using Geneious® prime v2020.0.4 (Biomatters Limited, New Zealand available at https://www.geneious.com). For sequence homology search BLASTn (www.blast.ncbi.nlm.nih.gov) was used. The newly generated sequence was deposited in the NCBI GenBank database.

Phylogenetic analysis

Multiple sequence alignment (MSA) was carried out using the 18S rDNA sequence of the isolate and other sequences of related taxa retrieved from the NCBI database (Table S1). These sequences were aligned by the MUSCLE algorithm[24] in MEGA X. The best-fitting nucleotide substitution models were tested using the ML Model Test in MEGA X[25], and the best model with the lowest Bayesian Information Criterion (BIC) score[26] was selected, followed by pairwise distance calculation. Phylogenetic analysis was conducted using Maximum Likelihood (ML) method[27] in MEGA X, and substitution bias was modelled by Kimura 2-parameter model with Gamma distribution. A total of 1000 bootstrap replicates were examined under the ML criterion to estimate interior branch support[28]. In the ML phylogenetic analysis, 27 nucleotide sequences were involved, and

Oltmannsiellopsis viridis (FN562431 and D86495) is used as an out-group. Pairwise distance matrix corrected using Kimura 2 Parameter with Gamma distribution model was constructed with the closest species using MEGA X to study differences between them. Using this alignment, RNAalifold 2.4.18 software (http://rna.tbi.univie.ac.at/) was used to construct Minimum Free Energy (MFE) secondary structure to locate the mismatches and indels in the secondary structure.

Morphological comparison

The morphological features of all closely related *Acetabularia* species based on descriptions available from the literature[1] were compared with the isolate of current study (Table 1), including stalk length, cap diameter, number of cap rays, their morphology and the number of hairs per lobe.

Results

Morphological observations

The specimen is composed of an unbranched cylindrical stalk and rhizoidal holdfast. Out of the total 300 observations, one sample has along, branched stalk with two caps attached at the same stalk. The long stalk ranged between 20 – 40 mm in length. A circular, umbrella-shaped cap appeared at the top (Fig. 1A), with a diameter of 5 – 13 mm (Fig. 1B), which consists of an inner ring (corona superior) and an outer ring (corona inferior). The cap is made up of 40 – 60 merged cap rays (Fig. 2A). Bifurcations formed due to the rooting of lobes in the ring of coronal inferior (Fig. 2C – E) and pointed structure formed at the outer end of the cap rays (Fig. 2E, F). The corona superior consists of a ring of 40 – 60 lobes at the centre (Fig. 3C). Each lobe comprises six or seven hairs, which remarkably distinguish the species from others (Fig. 3F, G). Altogether, 11 numbers of caps were examined and counted the number of hairs in each of them (Fig. 4). Nine samples had seven hairs per cap, while the rest two had 6 numbers of hairs. The developing cap showed a swollen lobe inside and early partition at inner and outer rings (Fig. 3H). The morphological characteristics of isolate of present study with related species are presented in Table 1.

Phylogenetic analysis

The BLASTn hits showed nearly or less than 97 % identity with other *Acetabularia* species. The closest species was *Acetabularia dentata* having a 96.88 % identity (Table 2) with the isolate of present study.

Phylogenetic relationships inferred from Maximum Likelihood (ML) method using the 18S rDNA

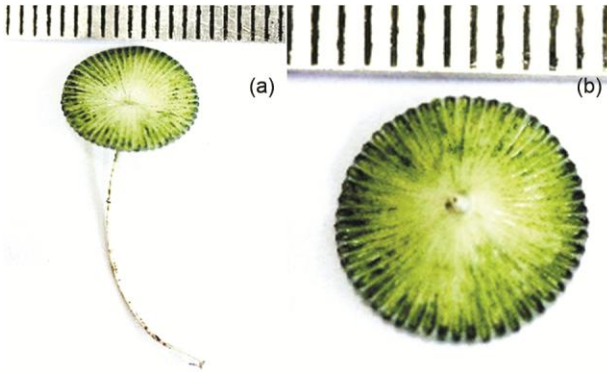

Fig. 1 — *Acetabularia jalakanyakae* Sp. Nov. collected from Andaman and Nicobar Islands. (A) Whole sample; and (B) Lower surface of the cap. Scale represents 1 mm distance between two bars

Table 1 — Comparison of morphological characteristics of the Indian isolate with other closely related species (Berger *et al.*[1]).

Morphological characteristics	*Acetabulariaja lakanyakae* Sp. Nov.	*Acetabularia dentata* Solms-Laubach	*Acetabularia caliculus* J. V. Lamouroux	*Acetabularia kilneri* J. Agardh	*Acetabularia major* Martens	*Acetabularia acetabulum* (Linnaeus) P. C. Silva	*Acetabularia crenulata* J. V. Lamouroux
Length of stalk (mm)	20-40	10-20	25-40	45-100	60-200	30-60	25-100
Cap diameter (mm)	5-13	2-3 (rarely up to 4.5)	4-6	10-14	10-20	5-12	7-15
Number of cap rays and their morphology	40-60 fused with pointed ends.	25-40 having the shape of a long triangle with a distinct sharp apiculum.	25-35 curved upwards having emarginated or truncate apices.	48-60 with blunt ends, bearing a distinct spine and notches at their inner walls.	60-80 slender with truncated or rounded apices and notches at their inner walls.	55-90 laterally joined with smooth, blunt ends.	30-80 completely fused with pointed ends.
Number of hairs (per lobe) in Corona superior	7	2 to 4	2 to 3	4 to 6	6 to 9	4 to 5	2

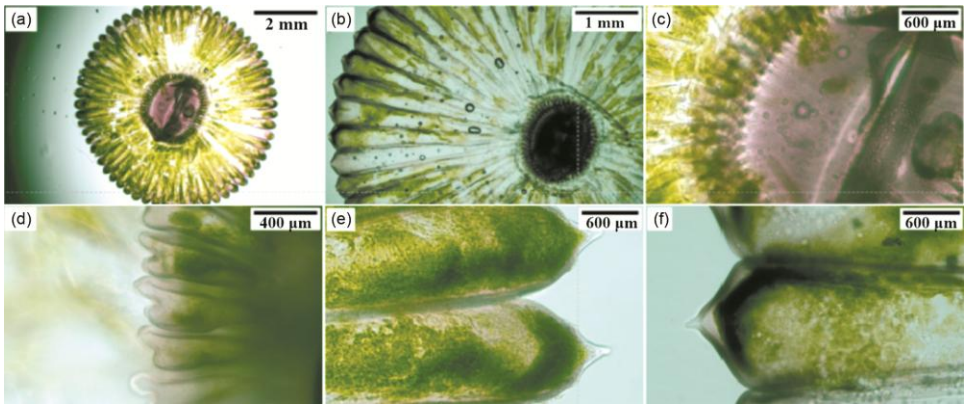

Fig. 2 — Light microscopic images of the sample. (A) Whole cap; (B) Cap rays; (C) Outer ring of the lobes; (D) Magnified image of the lobe; and (E & F) Pointed tip of the rays. Scale bar given on the upper right side

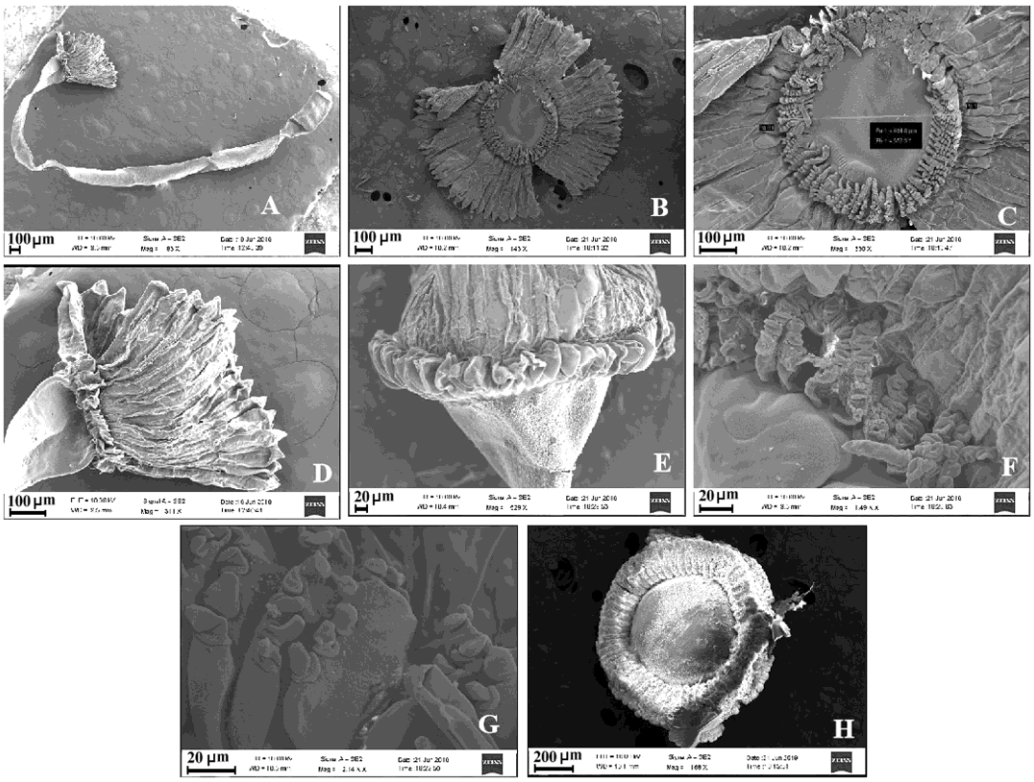

Fig. 3 — SEM images of the sample. (A) Whole image of the sample; (B & C) Top view of the cap; (D) Side view of the cap; (E) Outer ring of lobes; (F) Inner ring of lobes; (G) Hairs in the lobe; and (H) Cap in the early-stage. Scale bar given on the lower left side

sequences of Dasycladales (Fig. 5) resulted in a moderately resolved phylogram with four clades. Indian isolate formed a monophyletic clade with *Acetabularia dentata* (Z33468) and *Acetabularia major* (Z33462). Within the monophyletic clade, the pair-wise distance of *A. jalakanyakae* showed 0.00000 with *A. dentata*, 0.04918 with *A. major*, 0.0392156863 with *A. kilneri*, 0.02885 with *A. caliculus*, and 0.10726 with *A. ryukyuensis*. Pair-wise alignment with *A. dentata* revealed that there were four mismatches (at positions 1679, 1731, 1732, and 1733) and one indel (at 1680). Minimum Free Energy (MFE) Secondary Structure of the pair-wise alignment revealed that all four mismatches and one indel are located in the loop regions of the molecule (Fig. 6). In the phylogram reported in this study, *Acetabularia* and *Parvocaulis* were monophyletic, with a clade that had isolates from both of these genera.

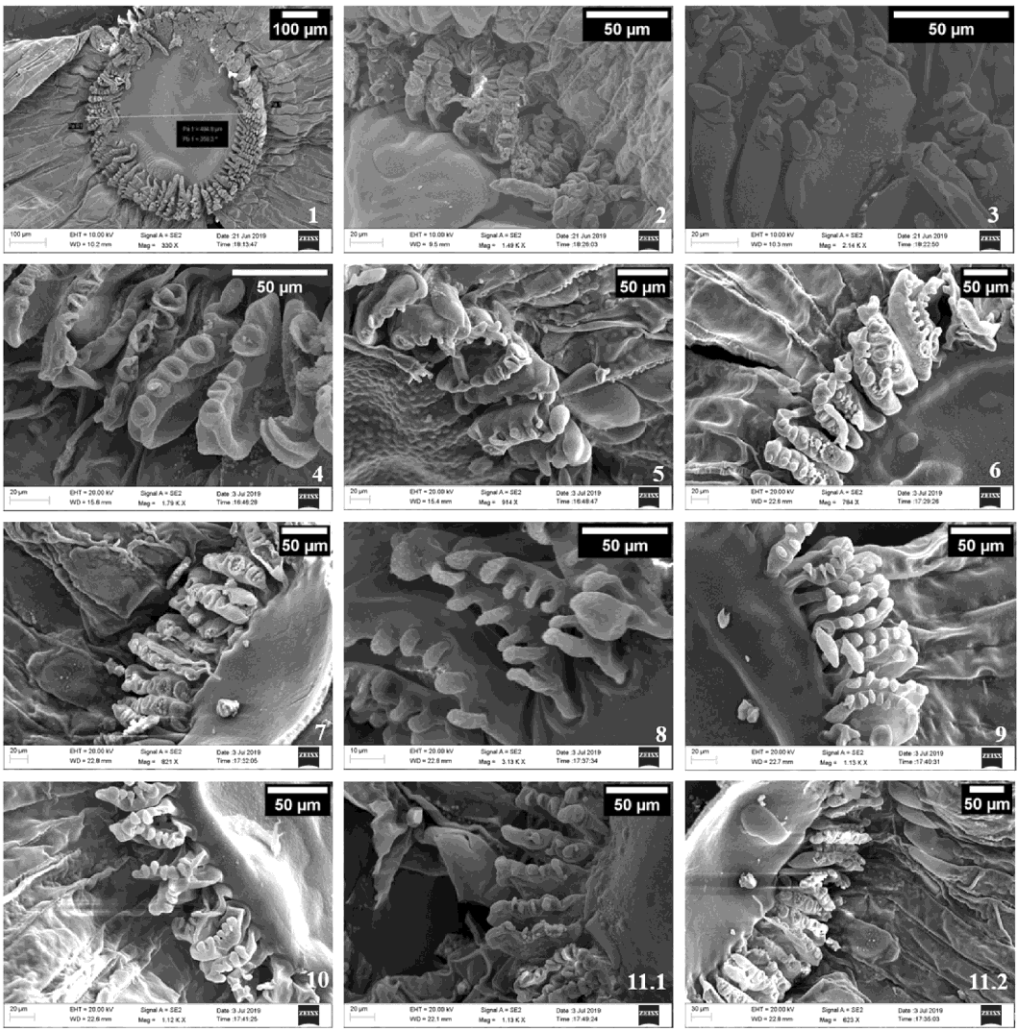

Fig. 4 — SEM images of cap structures of 11 different samples. Samples 4 and 7 with 6 numbers of hairs and all other samples with 7 numbers of hairs. Scale bar given on the upper right side

Table 2 — Top 5 BLASTn hits

S. No.	Species	GenBank Accession number	% Identity
1	*Acetabularia dentata*	Z33468	96.88 %
2	*Acetabularia caliculus*	AY165780	96.25 %
3	*Acetabularia kilneri*	AY165778	96.25 %
4	*Acetabularia major*	Z33462	96.25 %
5	*Acetabularia acetabulum*	AY165775	94.38 %

Discussion

The sample of *Acetabularia jalakanyakae* Sp. Nov. reported in current study shows unique morphological characters when compared to other related species. It has a stalk length of 20 – 40 mm, which is similar to *Acetabularia calyculus*. The cap diameter ranged between 5 – 12 mm, whereas it is only 4 – 6 mm for *Acetabularia calyculus.* Cap rays with a pointed edge is a crucial features of the new species, which is shared with the *Acetabularia crenulata*. Upon investigating, the number of hairs in each lobe of corona superior of *A. crenulata* has only 2 hairs, whereas *A. jalakanyakae* possesses 6 or 7 hairs.

Based on the morphological and molecular synapomorphies, a new species of *Acetabularia* is described here. The new species is evolutionarily and morphologically distinct from the closely related species of *Acetabularia i.e. A. dentata* and *A. major*. Therefore, a new species of *Acetabularia* (Polyphysaceae) as per the Phylogenetic Species Concept[29] is proposed herewith.

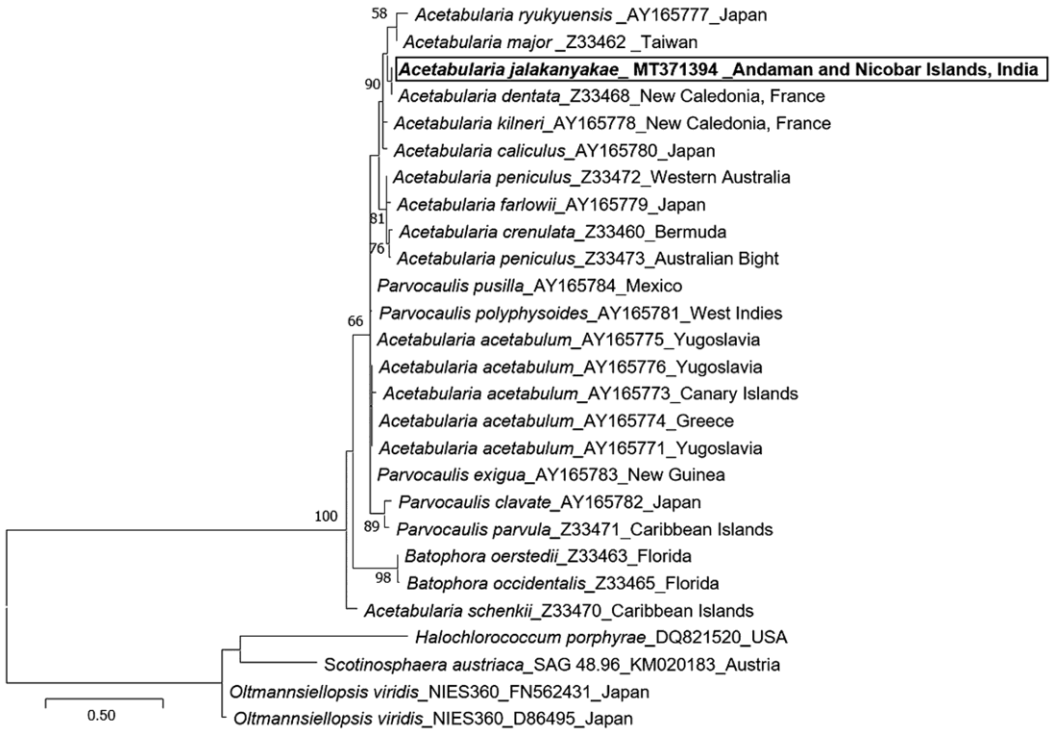

Fig. 5 — Maximum likelihood (ML) phylogram based on 18S rDNA sequences using the Kimura 2-Parameter model of molecular evolution in MEGA X. Numbers near nodes represents the ML bootstrap proportion. The newly sequenced *Acetabularia jalakanyakae* is marked in bold. The tree with the highest log likelihood (-866.21) is shown. The analysis involved 27 nucleotide sequences. All positions having gaps and missing data have been eliminated. Scale bar given on the bottom is in the units of average nucleotide substitutions per site

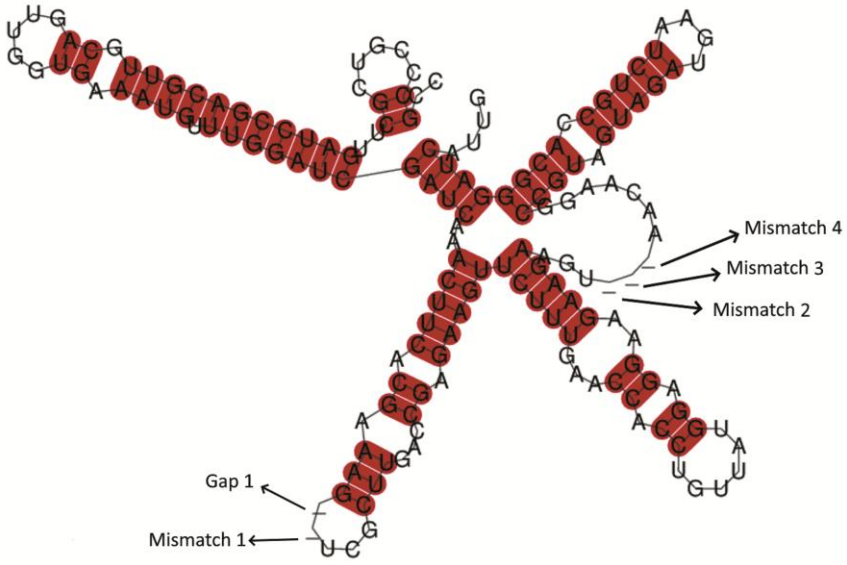

Fig. 6 — Minimum free energy (-37.90 kcal/mol) secondary structure of the pair-wise alignment between *Acetabularia dentata* and *Acetabularia jalakanyakae* constructed with RNAalifold 2.4.18. Conserved sites (complimentary base pairing) are highlighted in red, while gap and mismatches are annotated separately

***Acetabularia jalakanyakae* Sp. Nov. (Fig. 1)**

Description

The main body is comprised into three regions: a basal part with a rhizoidal holdfast, a middle region with a long stalk, and the topmost upper part with an umbrella-shaped circular cap. It consists of an unbranched cylindrical stalk. Stalk length is 20 – 40 mm with a cap diameter of 5 – 13 mm. The cap is completely

fused, having 40 – 60 cap rays. The outer end of each ray forms a pointed structure. The outer ring (Corona inferior) consists of slender lobes with bifurcation. The inner ring (Corona superior) has 40 – 60 lobes without bifurcations. The inner ring comprised of 6 or 7 hairs per lobe and shows protrusions inside. The developing cap shows a swollen lobe inside and early partition at inner and outer rings.

Holotype

Collected from intertidal rocks at Port Blair, Andaman and Nicobar Islands, India. Herbarium voucher is deposited at Agharkar Research Institute, Pune, India, (Index Herbarium code: AHMA), under voucher no. AHMA - 32437. The DNA sequence of 18S rDNA partial region of the isolate generated and deposited at GenBank under accession #MT371394.

Isotype

Isotype deposited at the Central University of Punjab, Bathinda (Index Herbarium code: CUPB), under voucher no. CUPB-ACT-2019-1.

Etymology

Specific epithet which is feminine noun in genitive means 'mermaid' in Sanskrit to refer the aesthesis of the cap that resembles mermaid's umbrella.

Limitations

The study analysed only a few algal samples from one site in Andaman Islands. More efforts to study the species biogeography would have added value to this report. The study did not analyse the life cycle patterns as the investigation did not involve any algal culturing. As life cycle and ontogeny are highly informative, this would have significantly improved the manuscript. Additionally, the length of generated sequence is only 234 bp and all four mismatches with the closest hit (*A. dentata*) were all ambiguities (N or R). However, strength of morphological synapomorphies stands valid for the description of new species.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at http://nopr.niscair.res.in/jinfo/ijms/IJMS_50(09)701-708_SupplData.pdf

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Conflict of Interest

The authors declare that no conflict of interest.

Author Contributions

FB and RK conceived the idea of the manuscript. Sample was collected by FB. KCS and MA performed the experiments, analyzed the data, and drafted the manuscript. KG edited and reviewed the manuscript. FB proof checked and finalized the manuscript. KCS and MA contributed equally to this work.

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