Studies in Fungi 3(1): 49–58 (2018) www.studiesinfungi.org ISSN 2465-4973
Article

Doi 10.5943/sif/3/1/7

Morphotaxonomic and phylogenetic analysis of *Saprolegnia ferax* from India

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Singh PN, Singh SK, Lagashetti AC, Ackah FK 2018 – Morphotaxonomic and phylogenetic analysis of *Saprolegnia ferax* from India. Studies in Fungi 3(1), 49–58, Doi 10.5943/sif/3/1/7

Abstract

Saprolegnia ferax is isolated from polluted water sample collected from Mula River, Pune, Maharashtra, India. The isolated taxon is illustrated and compared with the morphotaxonomy based first record of Indian *S. ferax* isolated by Wani et al. (2017). The present study deals with the re-description of the isolated taxon *S. ferax* based on detail morphological features, sequence analysis and phylogeny of ITS and LSU regions of rDNA.

Key words – ITS and LSU rDNA – Morphotaxonomy – Oomycete – *Saprolegnia*

Introduction

The genus *Saprolegnia* belongs to the order Saprolegniales (Oomycota) and has diploid life cycle with both asexual and sexual reproduction (a zoosporic, oogonial, oosphere and antheridial stage). The type species of the genus *Saprolegnia* was established as *Saprolegnia molluscorum* (Nees 1823). Later on, the status of type species was revised as *Saprolegnia ferax* by Kützing (1843). Species belonging to the genus *Saprolegnia* are worldwide in distribution and could be easily encountered across terrestrial and aquatic ecosystems (Johnson et al. 2002, Phillips et al. 2008). The members of this genus are known to be saprophytes, parasites and under some circumstances opportunistic pathogens as well. The majority of the species are responsible for causing diseases on aquatic animals (Blaustein et al. 1994, Fernández-Benéitez et al. 2008, Berger et al. 2009, Ruthig 2009, Perotti et al. 2013, Rezinciuc et al. 2014, Sandoval-Sierra et al. 2014). There are reports suggesting that some species of *Saprolegnia* causes high mortality rate in wildlife and aquaculture populations (van West 2006, van den Berg et al. 2013). Currently, Index Fungorum database (http://www.indexfungorum.org/) reveals that the genus is comprised of about 77 species.

The first Indian record of *S. ferax* was documented by Wani et al. (2017) from water bodies of Pachmarhi, Hoshangabad, India. The taxonomy of the previous Indian collection of *Saprolengia ferax* by Wani et al. (2017) was mainly based on the meagre morphological descriptions and the keys provided by Coker & Mathew (1937), Hamid (1942), Johnson (1956), Coker (1923, 1979), Srivastava & Srivastava (1977), Chowdhry & Agarwal (1980), Misra (1982), Agarwal & Hasija (1986), Khulbe (2001). However, the lack of robust taxonomy for this genus frequently leads to misidentification of species. During past two decades, the morphological observations are now being supplemented with molecular characteristics (DNA sequencing and phylogenetic analysis) to

Submitted 10 April 2018, Accepted 24 April 2018, Published 2 May 2018
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avoid ambiguity in identification of species.

In continuation of our fungal biodiversity exploration, documentation and conservation, several microfungi have been collected, identified and recently reported from Western Ghats regions in India (Singh et al. 2015, Singh & Singh 2016, Singh et al. 2017). As a part of fungal biodiversity exploration, the polluted river water sample was collected for the isolation and documentation of aquatic oomycetous fungi. The aim of the present work is to characterize and re-describe the Indian record of *Saprolegnia ferax* based on morphology and phylogenetic analysis of the ITS and LSU rDNA region followed with suitable practice of culture conservation.

Materials & Methods

Collection

The water sample was collected from stagnant, polluted Mula River, Pune, Maharashtra, India including dead and decaying organic matter in a plastic bottle.

Isolates and morphology

The collected water sample was poured in sterilized glass water trough. Different baiting materials like cockroaches, ants, houseflies, mosquitoes were collected and sterilized by autoclaving. The sterilized baiting materials were then added to the glass trough and allowed for incubation at room temperature. After 3–4 days, the baits were colonized by whitish cottony filamentous fungal growth. In stereoscopic observation, numerous zoosporangia were found to be emerging from filamentous hyphae. To obtain the pure culture, the growing fungal filaments were taken with the help of fine forceps into sterile ampoules containing sterile distilled water and a pinch of antibiotic to avoid bacterial contamination and washed several times with sterile distilled water. Finally, it was plated on potato carrot agar (PCA) medium. After 1–2 weeks of incubation at 25^{o} C, pure isolated colonies were obtained.

Slides prepared in lacto phenol and cotton blue mount from incubated baiting samples and pure colonies growing in PCA plate confirmed the presence of *Saprolegnia* morphologically. Microphotographs of various morphological structures were taken with Carl Ziess AXIO-10 microscope. The pure culture in metabolically inactive state, preserved in absolute ethanol (Sandoval-Sierra & Diéguez-Uribeondo 2015), and deposited in NFCCI (National Fungal Culture Collection of India). Dried culture voucher (Wu et al. 2004) was deposited in the Agharkar Research Institute (AMH).

DNA Extraction

The fungal genomic DNA was extracted following the protocol of Aamir et al. (2015). Briefly, 7 days old fungal mass was placed in a 2 ml tube containing a ceramic pestle, 60–80 mg sterile glass beads (425–600 µM, Sigma) and lysis buffer (100 mM Tris HCl [pH8.0], 50 mM EDTA, 3% SDS). Homogenization of fungal mass was done twice in a FastPrep®-24 tissue homogenizer (MP Biomedicals, USA) at 6 M/S for 60 sec. The resulting fungal tissue homogenate was centrifuge at 13,000 rpm for 10 mins. and supernatant was transferred to a fresh micro centrifuge tube. To the supernatant, 2 µl of RNase A (10 mg/ml) was added and incubated at 37 °C for 15 mins. After the RNase A treatment, equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) was added and mixed well, followed by centrifugation at 13,000 rpm for 10 mins. The upper aqueous layer was taken in a fresh micro centrifuge tube and then the DNA was precipitated with 100% ethanol. The DNA pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 mins. The DNA pellets were air dried and dissolved in 1x TE buffer.

PCR and sequencing

The internal transcribed spacer ITS and LSU regions of rDNA were amplified from fungal genomic DNA by PCR using the primers ITS4 & ITS5 and LR0R and LR7 respectively (White et al. 1990). The PCR products were purified with FavorPrepTM PCR Purification Kit (Favorgen

Biotech Corporation, Taiwan). Sequencing of the PCR products was accomplished with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), using the amplification primers. The cycle sequencing products were run on an ABI Avant 3100 automated DNA sequencer (Applied Biosystems, USA). The manually edited sequence of ITS and LSU of rDNA of our fungal isolate was deposited in the NCBI nucleotide sequence database (Gene Bank Acc. No. ITS- MF470377 and LSU- MF470378).

Phylogenetic analyses

The sequences of the ITS and LSU – rDNA of the isolated *S. ferax* (NFCCI 4174) were subjected to BLASTn sequence homology searches. On the basis of the BLASTn search results, genetically related species were chosen for the construction of the phylogenetic trees (separately for ITS and LSU-rDNA), which included different species of *Saprolegnia* (Table 1). The *Achlya catenulata* Pires-Zottar., A.L. Jesus, Marano & J.I. Souza was chosen as an out-group. Multiple sequence alignment was performed using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) and the phylogenetic analysis was performed by using the Maximum Likelihood method based on Tamura 3-parameter model (Tamura 1992) for ITS sequences and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) for LSU sequences in MEGA 7 (Kumar et al. 2016). One-thousand bootstrap replicates were analyzed to obtain nodal support values.

Table 1 Isolate origin and GenBank accession number of the isolates used in this study.

Sr. No.	Culture	Strain No.	GenBank Accession No.	
			ITS	LSU
1	***Saprolegnia ferax***	**NFCCI 4174**	**MF470377**	**MF470378**
2	*Saprolegnia aenigmatica*	NeSS1	KP941579	-
3	*Saprolegnia aenigmatica*	NeSS2	KP941580	-
4	*Saprolegnia aenigmatica*	PelSS	KP941578	-
5	*Saprolegnia aenigmatica*	RJBCC0031	KR872875	-
6	*Saprolegnia aenigmatica*	RJBCC0023	KR872867	KX555486
7	*Saprolegnia anisospora*	CBS110060	HQ643974	-
8	*Saprolegnia anisospora*	SAP1148	JX418015	KX555487
9	*Scoliolegnia asterophora**	CBS53167	HQ643975	HQ665250
10	*Scoliolegnia asterophora**	SAP1296	KF718178	KX555488
11	*Saprolegnia australis*	EZ1	KX214620	-
12	*Saprolegnia australis*	SAP0286	KF717961	KX555489
13	*Saprolegnia delica*	SAP0550	KM095840	-
14	*Saprolegnia delica*	SAP9030	KM095845	-
15	*Saprolegnia delica*	SAP0202	-	KX555490
16	*Saprolegnia delica*	CBS 345.62	-	HQ665214
17	*Saprolegnia delica*	ABDN_73	KF420266	-
18	*Saprolegnia delica*	ABDN_31	KF420226	-
19	*Saprolegnia diclina*	SAP0244	AM228849	KX555491
20	*Saprolegnia diclina*	734F1	KP189445	-
21	*Saprolegnia diclina*	ATCC 90215	AY455775	-
22	*Saprolegnia diclina*	CBS53667	HQ643978	HQ665254
23	*Saprolegnia eccentrica*	SAP1288	KF718140	KX555492
24	*Saprolegnia eccentrica*	CBS55167	HQ643983	HQ665260
25	*Saprolegnia eccentrica*	CBS21135	HQ643981	HQ665151
26	*Saprolegnia eccentrica*	CBS19938	HQ643982	HQ665147
27	*Saprolegnia ferax*	SAP0255	KF717884	KX555493
28	*Saprolegnia ferax*	CBS30537	HQ643987	HQ665199
29	*Saprolegnia ferax*	CBS17342	HQ643984	HQ665142

Table 1 Continued.

Sr. No.	Culture	Strain No.	GenBank Accession No.	
			ITS	LSU
30	*Saprolegnia furcata*	SAP1294	KF718143	KX555494
31	*Saprolegnia litoralis*	SAP1486	KF718048	KX555495
32	*Saprolegnia litoralis*	CBS53567	HQ643991	HQ665253
33	*Saprolegnia megasperma*	SAP1287	KF718186	KX555496
34	*Saprolegnia megasperma*	CBS53267	HQ643993	HQ665251
35	*Saprolegnia monilifera*	CBS55867	HQ643996	HQ665270
36	*Saprolegnia monoica*	CBS59967	HQ643998	HQ665280
37	*Saprolegnia monoica*	CBS53967	HQ643999	HQ665255
38	*Saprolegnia parasitica*	SAP0210	AM228825	KX555497
39	*Saprolegnia parasitica*	CBS 113187	-	HQ665074
40	*Saprolegnia parasitica*	VI05337	KC994646	-
41	*Saprolegnia racemosa*	RJBCC0001	KR872845	KX555498
42	*Saprolegnia racemosa*	RJBCC0013	KR872857	-
43	*Saprolegnia subterranea*	SAP1293	KF718124	KX555499
44	*Saprolegnia subterranea*	CBS113343	HQ644009	HQ665078
45	*Saprolegnia terrestris*	SAP1327	KF718138	KX555500
46	*Saprolegnia terrestris*	CBS110063	HQ644011	HQ665061
47	*Saprolegnia truncata*	CCIBt 3988	KT213556	-
48	*Saprolegnia turfosa*	SAP1279	KF718190	KX555502
49	*Saprolegnia turfosa*	CBS32735	HQ644012	HQ665210
50	*Saprolegnia turfosa*	CBS31381	HQ644013	HQ665203
51	*Saprolegnia bulbosa*	-	AY267011	-
52	*Saprolegnia bulbosa*	ch1B	KF225574	-
53	*Saprolegnia hypogyna*	CBS86972	HQ643989	HQ665304
54	*Saprolegnia hypogyna*	ABDN_11	KF420214	-
55	*Saprolegnia semihypogyna*	-	AY647194	-
56	*Protoachlya paradoxa*	SAP1792	KX555485	KX555503
57	*Achlya catenulata*	CCIBt 4029	KP006455	KP006449

* *Scoliolegnia asterophora*: Previously it was named as *Saprolegnia asterophora.*

Results

Saprolegnia ferax (Gruith.) Kütz., Phycol. general: 157 (1843) Figs 1–4

Material examined – INDIA, Maharashtra, Pune, (18° 31' N, 73° 55' E), from polluted Mula River water, 30 November 2016, coll. P.N. Singh, Agharkar Research Institute (AMH-9901) (dried fungal culture). GenBank accession Number ITS–MF470377 rDNA & LSU–MF470378.

Sexual morph: Oogonial and Antheridial. Asexual morph: Zoosporic.

Colonies on potato carrot agar (PCA) at 25 ºC, reaching 61 × 61 mm in diam after 7 days, colourless, watery with shiny surface, margin irregular. Hyphae stout, smooth, branched, hyaline, 12.5–93 µm thick. Gymmae abundant, simple to branched, 16–87.2 × 10.5–18.5 µm, cylindrical, lomentous, hyaline, intercalary to lateral. Zoosporangium abundant, clavate to cylindrical or filamentous, simple to branched, sometimes catenate, hyaline, 11.5–1000 × 13.5–55 µm. Zoospores oval to subglobose, flagellate, hyaline, 12.5–17.5 × 10.5–13.5 µm. Flagellum thin, club-shaped, smooth walled, hyaline, up to 10 µm long. Encysted zoospores globose to oval, hyaline, 9.5–27.5 × 10–22 µm. Oogonia abundant, stalked, globose to sub-globose, smooth walled, hyaline, 13–57 µm in diameter. Oogonial stalks of various lengths and widths; usually simple. Antheridia variable in shape and size, present on few oogonia only, monoclinous to diclinous. Oospores globose, 2.2–19 µm, smooth walled, hyaline, centric, 1–15 in number.

Sequences comparisons and phylogenetic analysis

The ITS and LSU sequences from multiple isolates of different species of *Saprolegnia* (24 species), *Achlya*, *Scoliolegnia* and *Protochlya* available in GenBank and also those which appeared in BLAST search were analyzed. Our taxon was found to be clustered with *Saprolegnia ferax* with moderately supported boot-strap value in both ITS and LSU based phylogenetic trees.

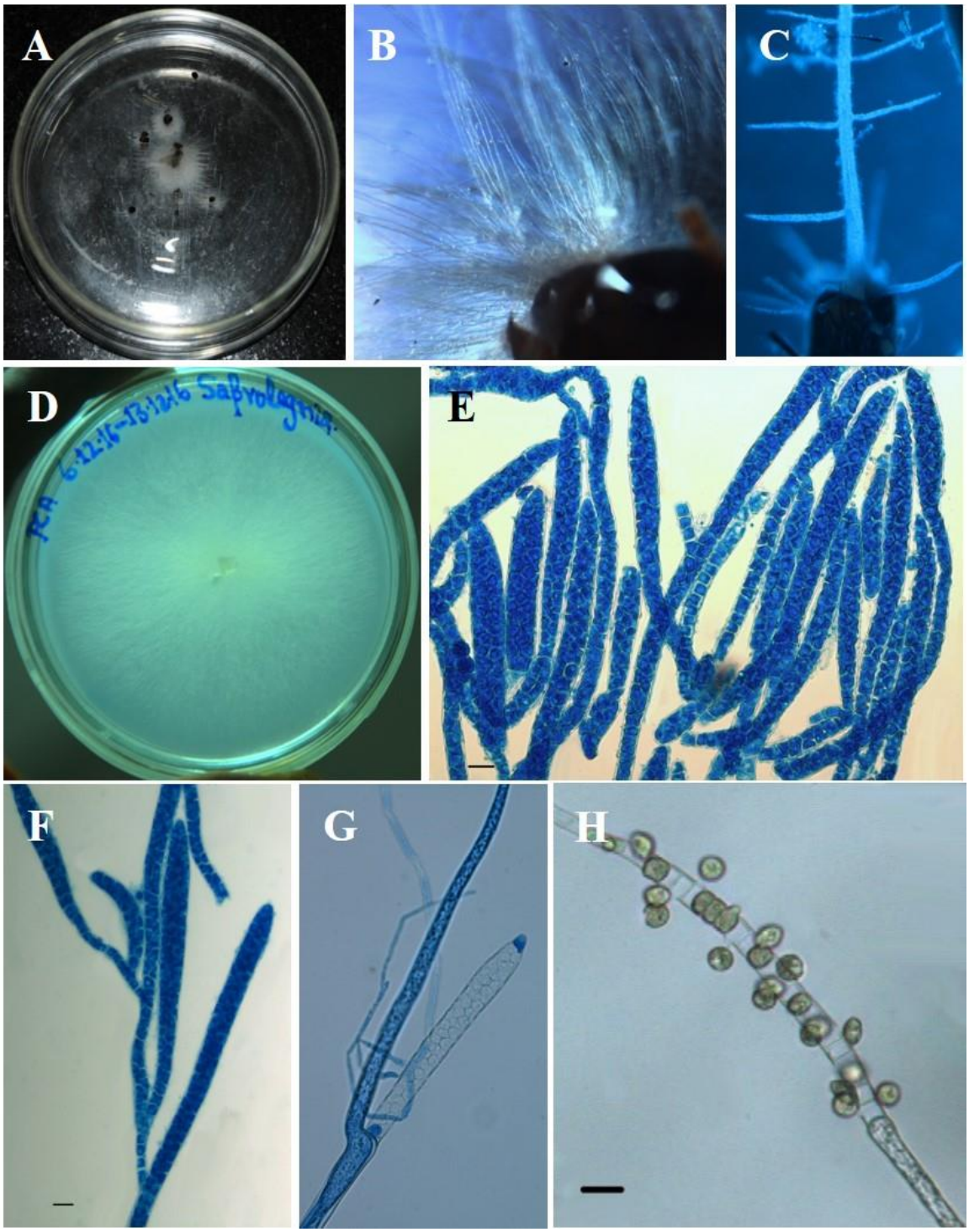

Fig. 1 – *Saprolegnia ferax* (NFCCI 4174). A Colonies on incubated natural substrate showing cottony growth. B Stereoscopic view of numerous hyphae emerging from incubated natural bait (Insect). C Enlarged stereoscopic view of stout hyphae emerging from incubated bait. D Colony characteristics (front view on Potato carrot agar). E Numerous zoosporangia. F Branched, cylindrical and clavate, hyaline zoosporangia. G Cylindrical, solitary zoosporangium. H Simple filamentous zoosporangium with dispersed encysted zoospores. Scale bars: E–H = 20 μm.

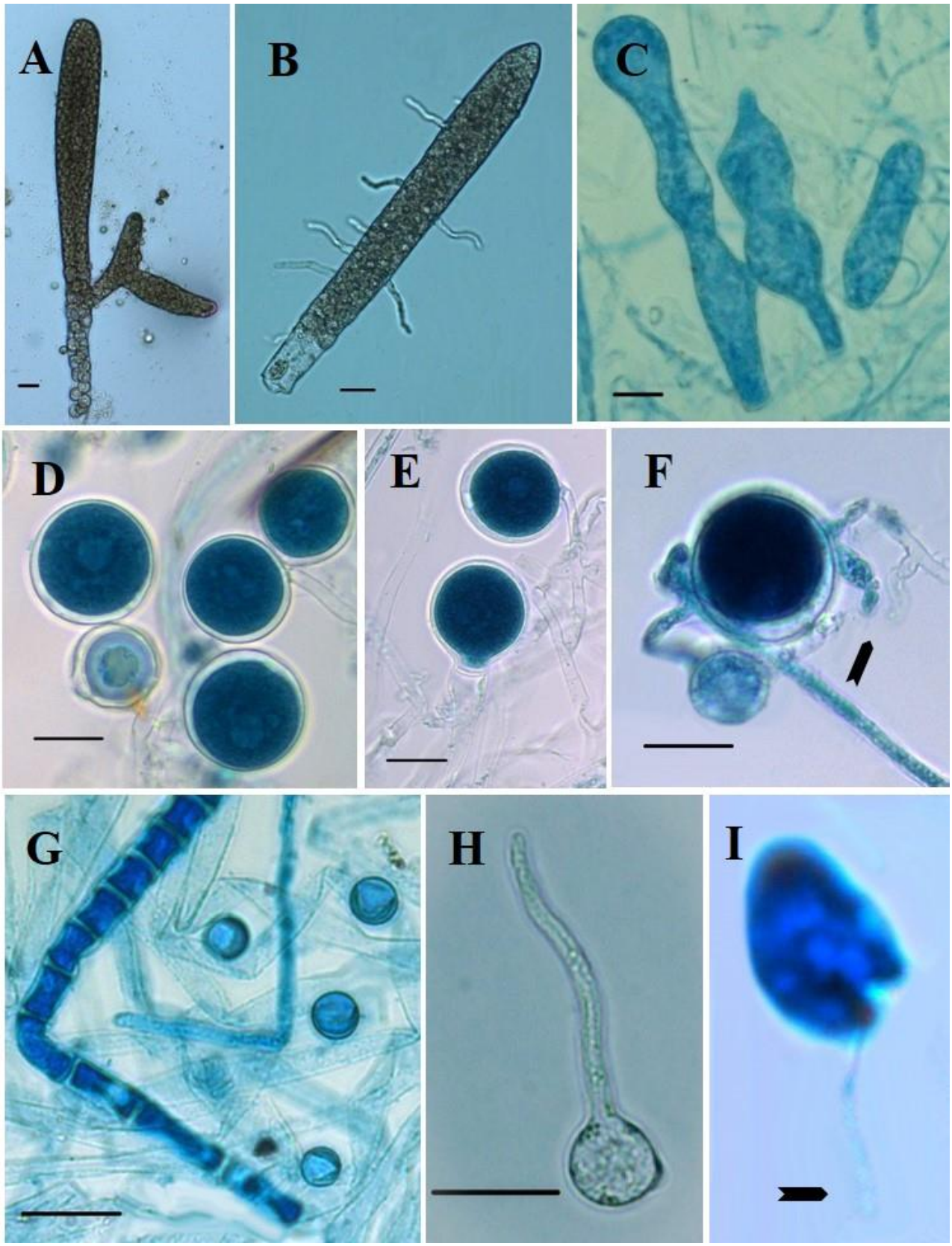

Fig. 2 – *Saprolegnia ferax* (NFCCI 4174). A A branched zoosporangium. B Encysted zoospores within sporangium producing germ tubes. C Hyaline gymmae. D Numerous oogonia with single to many oospores. E Stalked oogonia. F Oogonia and antheridia (showing arrow). G Vegetative hyphae and globose encysted zoospores (aplanospores). H A germinating zoospore producing germ tube. I A zoospore with hyaline clavate flagellum (showing arrow). Scale bars: A–H = 20 μm.

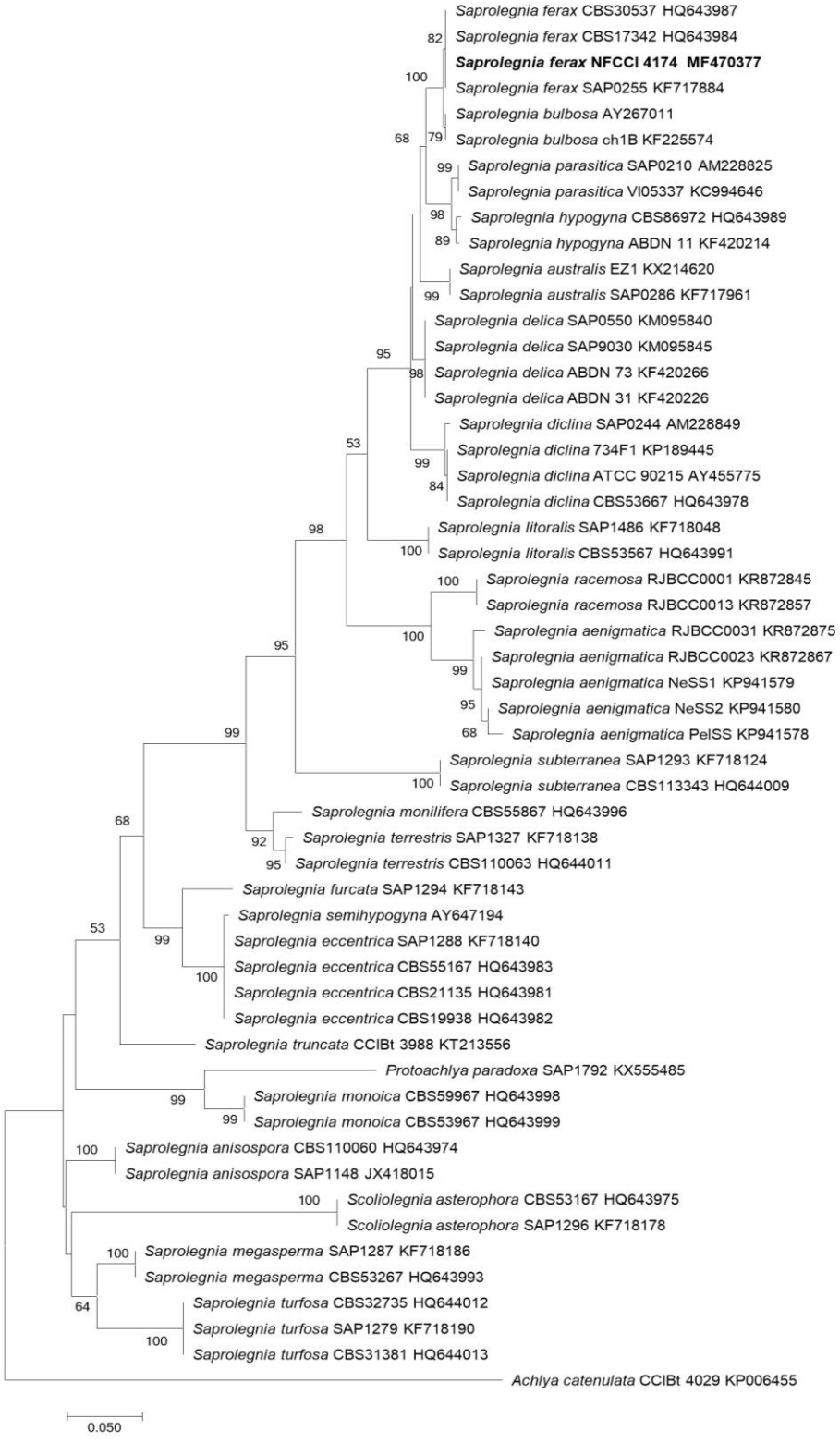

Fig. 3 – Phylogram generated from ITS-rDNA sequences: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood (-4643.4249) is shown. The present taxon from India (*Saprolegnia ferax*) having GenBank Accession Number MF470377 is shown. The *Achlya catenulata* was considered as the out group. Evolutionary analysis was conducted in MEGA7 (Kumar et al. 2016).
* *Scoliolegnia asterophora*-Previously it was named as *Saprolegnia asterophora*.

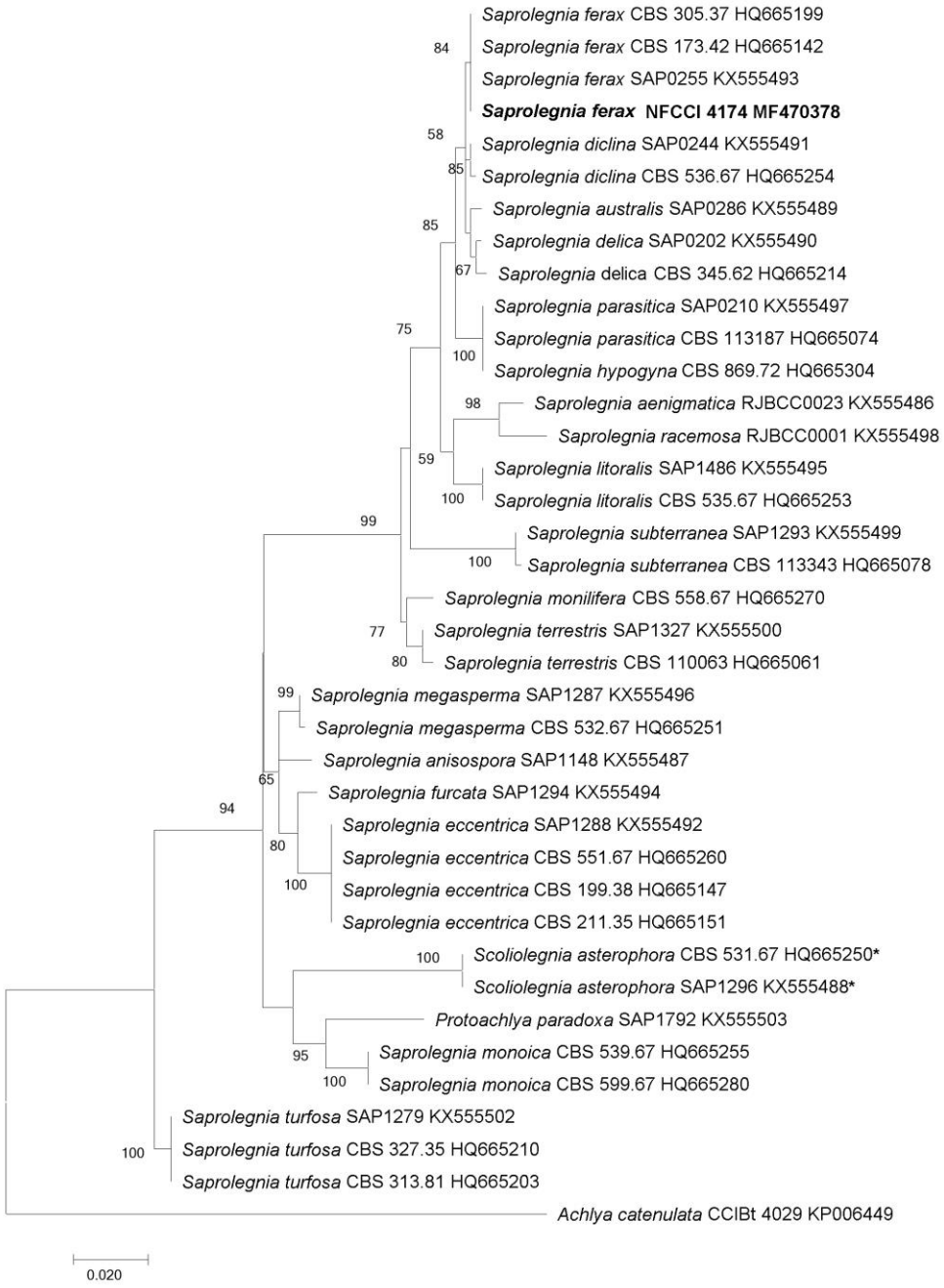

Fig. 4 – Phylogram generated from partial LSU-rDNA sequences: The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-2515.9044) is shown. The present taxon (*Saprolegnia ferax*) having GenBank Accession Number MF470378 is shown. The *Achlya catenulata* was considered as the out group. Evolutionary analysis was conducted in MEGA7 (Kumar et al. 2016).
* *Scoliolegnia asterophora*-previously named as *Saprolegnia asterophora*.

Discussion

Saprolegnia ferax was described as a new record from India (Wani et al. 2017) on the basis of morphotaxonomic characters only.

However, the present isolate shows morphological variations as compare to previously described *S. ferax* (Wani et al. 2017). The length and width of zoosporangium in the present collection is larger and highly branched as compared to the earlier description (11.5–1000 × 13.5–55 µm vs. 125–475 µm × 23.4–53.12 µm). Moreover, the encysted zoospores width is quite larger than that of previously recorded Indian *S. ferax* (12.5–17.5 × 10.5–13.5 µm vs. 9.37–12.5 µm diam.). Therefore, in this study we have re-described the present taxon on the basis of detailed morphological studies combined with phylogenetic analysis of ITS and LSU region of rDNA.

Acknowledgements

We thank Director, MACS' Agharkar Research Institute, Pune for providing necessary facilities to carry out the research work.

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