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Phylogenetic Insights and First Record of *Auriculoscypha anacardiicola* on *Holigarna arnottiana* from the Northern Western Ghats, India

Kunhiraman C. Rajeshkumar[1*, 2], Shital Desai[3, 4], Parayelil A. Ansil[1], Abhishek A. Rane[4], Kuniyil Harikrishnan[1], Sruthi O. Paraparath[1], Samantha C. Karunarathna[5], Nakarin Suwannarach[6,7], Ajay Kumar Gautam[8]

[1]*Biodiversity and Palaeobiology (Fungi) Gr., MACS Agharkar Research Institute, G. G. Agarkar Road, Pune - 411 004, Maharashtra, India.*

[2]*Faculty of Science, Savitribai Phule Pune University, Ganeshkhind, Pune - 411 007, Maharashtra, India.*

[3]*Discipline of Botany, School of Biological Sciences and Biotechnology, Goa University, Taleigao Plateau - 403 206, Goa, India.*

[4]*Tropical Biodiversity Exploration Foundation, Vanoshi Forest Homestay, Kudase village, Sindhudurg - 416 511, Maharashtra, India.*

[5]*Center for Yunnan Plateau Biological Resources Protection and Utilization, College of Biological Resource and Food Engineering, Qujing Normal University, Qujing, Yunnan - 655 011, P.R. China.*

[6]*Center of Excellence in Microbial Diversity and Sustainable Utilization, Chiang Mai University, Chiang Mai - 50200, Thailand.*

[7]*Office of Research Administration, Chiang Mai University, Chiang Mai - 50200, Thailand.*

[8]*Patanjali Herbal Research Department, Patanjali Research Institute, Haridwar - 249 405, Uttarakhand, India.*

**Corresponding author Email: rajeshfungi@gmail.com*

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ABSTRACT

This study records *Auriculoscypha anacardiicola* on a new anacardiaceous host, *Holigarna arnottiana* for the first time from the Northern Western Ghats, Maharashtra, India. This enigmatic fungus has a triple tropical level symbiosis between fungus-insect-plants and is only found in the Western Ghats and adjacent habitats in India. *Auriculoscypha anacardiicola* is a basidiomycetous fungus associated with coccid scale insect, *Neogreenia zeylanica* and anacardiaceous trees, *Anacardium occidentale* and *Mangifera indica*. This fungus was previously documented from the Malabar region of Kerala, India. The phylogeny based on the internal transcribed spacer region (ITS) placed the basidiomycete from the new host allied to the existing strains in the *Auriculoscypha* clade of the order *Septobasidiales*, allied to the genus *Septobasidium*. This study extends the geographic occurrence of this genus from the south to the northern Western Ghats, Maharashtra.

Keywords: Coccid insect, New Host Record, Phylogeny, Phytoparasitism, *Septobasidiales*, Taxonomy

INTRODUCTION

Auriculoscypha Reid & Manimohan (Reid and Manimohan, 1985) is a monotypic genus belonging to order *Septobasidiales*. Its species *A. anacardiicola* is known to form obligate association with coccid insects found on the bark of trees belonging to Anacardiaceae (Reid and Manimohan, 1985; Lalitha and Leelavathy, 1990; Lalitha, 1992). The relationship of coccid insect with the plant hosts represents a sort of indirect phytoparasitism since the insect depends on the plant for nutrition. The genus *Auriculoscypha* was initially reluctantly assigned to the order *Auriculariales*, although the authors did acknowledge its affinities to the order *Septobasidiales* (Reid and Manimohan, 1985). Subsequently upon the discovery of an obligate insect relationship and the presence of a yeast phase in the life cycle of the fungus, the systematic position of the fungus in order *Septobasidiales* was confirmed (Lalitha and Leelavathy, 1990; Lalitha *et al.*, 1994). In the later treatment of the fungus by Bandoni (1995) and Swann *et al.* (2001), it was treated under order *Septobasidiales* which was phylogenetically backed by Kumar *et al.* (2007). *Neogreenia zeylanica* is a little-known soft-scale coccid

belonging to *Margarodidae* which forms symbiotic association with *Auriculoscypha,* which are originally reported as a minor pest of cashew and mango trees originally from Sri Lanka.

So far, the fungus has been reported only from Kerala, Karnataka and Goa states, mostly from the bark of *Anacardium occidentale* and occasionally on *Mangifera indica* belonging to Anacardiaceae and very rarely spotted on other plants such as Euphorbiaceae (Reid and Manimohan, 1985; Lalitha, 1992). The present study aims to identify an unusual record of *Auriculoscypha*-like basidiomycete on *Holigarna arnottiana* through morphology and molecular sequencing using ITS from the northern Western Ghats of Maharashtra, India.

MATERIALS AND METHODS

Sample collection and Morphological study

The samples were collected during 2024 from the northern Western Ghats regions of Sindhdurg District, Maharashtra state, India. Minimalistic sampling was followed for collection to preserve the *in-situ* diversity of the fungus (Rathnayaka *et al.,* 2024). The samples were air-dried and stored in brown paper bags for further studies. For molecular studies, fresh basidiocarps were stored at 4°C after returning to the laboratory to avoid cross-contamination from fast-growing saprotrophic fungi. Morphology was studied using a binocular stereomicroscope (Olympus SZX16 with Digi-CAM, Japan). Sections were made using a razor blade and mounted separately in lactic acid-cotton blue and 10% KOH for microscopy. Microscopic observations were noted using Carl Zeiss Axio imager A2 (Zeiss, Germany). Key morphological characteristics were evaluated for species-level identification following Reid and Manimohan (1985). The specimens were deposited in the Ajrekar Mycological Herbarium (AMH) at MACS Agharkar Research Institute, Pune, India.

Molecular sequencing and Phylogeny

DNA extraction and PCR were performed using the Sigma RED Extract-N-AmpTM Seed PCR Kit, following the manufacturer's instructions, in a thermocycler ProFlexTM PCR system (Applied Biosystems, Foster City, USA). Primers used for amplifying ITS region were ITS4 and ITS5 following the cycling parameters given by White *et al.* (1990). The PCR products were purified with FavorPrep PCR Purification Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan) and sequenced with the same primers using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reactions were run on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems).

For phylogenetic analyses, the NCBI GenBank nucleotide sequence database was searched using MegaBLAST (Morgulis *et al.*, 2008) to identify the closest matching sequences in the database. An updated phylogeny of *Auriculoscypha* was assembled following Kumar *et al.* (2007), with the addition of other relevant ITS sequences of the genus retrieved from GenBank. The sequence was aligned, trimmed and manually edited in MEGA v. 11.0.11 (Tamura *et al.*, 2021) using MUSCLE, and the phylogeny tool AliView v. 1.28 (Larson, 2014) was used to transfer the alignment file into PHYLIP format. The alignment file is deposited in FigShare (https://figshare.com/articles/dataset/Auricyloscypha_ITS_align/27080812?file=49345159).

Phylogenetic analyses were performed using maximum likelihood (ML) in IQ-TREE v. 2.1.3 (Trifinopoulos *et al.*, 2016) and RAxML v. 8.1.11 (Stamatakis, 2006; Stamatakis *et al.*, 2008). Nodal support was evaluated using 1000 ultrafast bootstrap (UFboot) pseudo-replicates in IQ-TREE and 1000 rapid bootstrap pseudo-replicates in RAxML. Model selection was made using the 'auto' option available in IQ-TREE, which chose 'TPM2+F+I+G4' as the best-fitting model. The 'GTR G+I' model was determined a priori for RAxML analysis. The model test was performed using MrModeltest2 v. 2.4 (Nylander, 2004) according to the Akaike Information Criterion (AIC) for selecting nucleotide substitution models for Bayesian posterior probability (PP) analysis performed using MrBayes v. 3.2.7 (Ronquist *et al.*, 2012). 'GTR+I+G' was found to be the best-fitting model. For phylogenetic trees generated using IQ-TREE, only clades with UFboot BS≥96% were considered supported, and for RAxML, BS≥70% were considered supported. Posterior probabilities (PP) were estimated, allowing unlinked parameter estimation and independent rate variation. Trees were sampled

using a variant of the Markov Chain Monte Carlo (MCMC) method. For inferring the PP of genus *Auriculoscypha* and related species, phylogenetic trees were sampled every 1000th generation in 50,00,000 generations from running 4 simultaneous Markov chains. The first 25% of trees containing the burn-in phase of the analyses were discarded. The remaining trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. Only clades with PP≥0.97 in a Bayesian framework were considered supported. Phylogenetic trees were visualized using the program FigTree 1.4.0. (Rambaut, 2014). DNA sequences that were newly generated in this study were deposited in GenBank.

RESULTS

Auriculoscypha anacardiicola D.A. Reid & Manim., *Trans. Br. Mycol. Soc.* **85(3)**: 532 (1985)

(**Figure 1 and 2**)

*Basidiocarp: s*tipitate-cupulate, with central, dorsally attached cylindrical stipe; woody or leathery, non-gelatinous, cup-shaped or saucer-shaped pileus (3–25 mm). *Disc:* 6 mm diam; color white to creamy with grey extending from the center outwards, becoming either slate-grey, grey with ochraceous tint, or cinnamon-brown with grey tint; smooth to pruinose, often deeply umbilicate. Outer surface radially wrinkled towards the margin and densely zonate or somewhat sulcate, minutely felty tomentose but often glabrescent towards the stipe, color whitish at the margin and ochre to dark brown or even black. *Stipe:* 2–12 ×1.5–3.5 mm, terete, horny, smooth to knobby, minutely pruinose or glabrous, arising from a small tubercle containing mostly a single or rarely 2–3 crawlers (juveniles) of coccid insect which remain partially or fully embedded in the bark. *Tubercles:* ovo-ellipsoid to sub-globose, 2–5 mm in diam., surface smooth, walls woody, cavity internally lined by waxy material secreted by the coccid. *Flesh:* up to 1 mm in thickness, golden-brown, cottony-fibrous, without cuticular layer, non-gelatinous. *Hyphae of context:* 4.4–6.6 µm wide, hyaline near the margin, becoming pale brown to brown, septate, clamp connections not observed, scarcely branched. *Surface tomentum:* brown to pale brown or even formed of subhyaline hyphae measuring 4.5–6.6 µm in width, elongated, rarely branched, with obtuse rounded apex, narrow with secondary septation towards the tip. *Conidiophores:* branched, thick-walled, paler towards the tip with pointed apex. *Conidia:* hyaline thin-walled, suballantoid to ellipsoid, enteroblastic, amerosporous,4.5–9.5 × 2.5–3.5 µm. *Hymenium:* a catahymenium. *Hyphidia:* narrow, unbranched, septate, 2–3 µm wide, with thickened wall which gets thin towards the apex. *Basidia:* scattered, 70–90 × 10–15 um, thin-walled getting slightly thickened towards the base, hyaline, clavate to circinate, 1–2 septate, with only two fertile segments, lacking swollen probasidium. *Basidiospores:* 22–43 × 9–10 (–12) um, thin-walled, hyaline, cylindric to allantoid, septa transverse to muriform.

Specimens examined: INDIA. Maharashtra: Sindhdurg District, Kasal Village, elev. 66m, 15.99379°N, 73.68263°E, 04 Sep. 2024, Shital Desai, Abhishek A. Rane & Rajeshkumar K.C. (AMH 10772). INDIA, Maharashtra: Sindhdurg District, Kudase, elev. 34 m, 15.72251°N, 73.9792°E, 23 Jul. 2024, Shital Desai, Abhishek A. Rane & Rajeshkumar K. C. (AMH 10772).

The ITS sequence obtained from specimen in this study was deposited in GenBank under the number PQ350396. Based on a MegaBLAST search of NCBIs GenBank nucleotide database, the closest hits using ITS were of *A. anacardiicola* voucher MIN:AK 274 [NR119581; identities = 496/504 (98%), gaps = 0/504 (0%)] and *A. anacardiicola* voucher DUKE:DAH(309-A1) [DQ241470; identities = 472/501 (94%), gaps = 10/501 (1%)]. The sequence data of *A. anacardiicola* was analyzed with other available sequences in the genus *Auriculoscypha* in NCBI to determine the identity and placement of the species (**Figure 3**). The tree was rooted with *Malassezia furfur*.

For ITS, the analyzed dataset comprised a total of 33 sequences with 794 positions. The matrix had 656 distinct alignment patterns, with 5.88% undetermined characters or gaps. Estimated base frequencies were: A = 0.288026, C = 0.197130, G = 0.180833, T = 0.334011; substitution rates AC = 1.473623, AG = 2.961598, AT = 1.668521, CG= 0.786324, CT = 3.322360, GT = 1.000000; gamma distribution shape parameter (α) = 0.928023. The best-scoring IQ-TREE tree and

RAxML tree had a final likelihood value of– 10183.706 and –10178.461988, respectively. The ML tree obtained with IQ-TREE, RAxML, and the tree obtained from the PP analysis were topologically congruent and hence, the phylogenetic tree obtained using IQ-TREE is presented (**Figure 3**), with superimposed support values from the RAxML and Bayesian posterior probability analysis. *Auriculoscypha anacardiicola* formed a clade within *Septobasidiales*, allied to the clade, including *Septobasidium buriti.*

Figure 1: ***Auriculoscypha anacardiicola*** (AMH 10772). **A**, *Holigarna arnotiana* trunk; **b,** Fruiting branch of the tree; **c**, Fungal fruiting bodies on the bark; **d**–**f**, Basidiocarps of *A. anacardiicola*; **g**, Brownish black basal tubercle; **h**–**j**, Section of basal tubercle showing waxy coating and adult female of the coccid *Neogreenia zeylanica*; **Scale bars:** c = 20 mm; d and e = 10 mm; f = 5 mm; g = 2 mm; h–j = 1 mm.

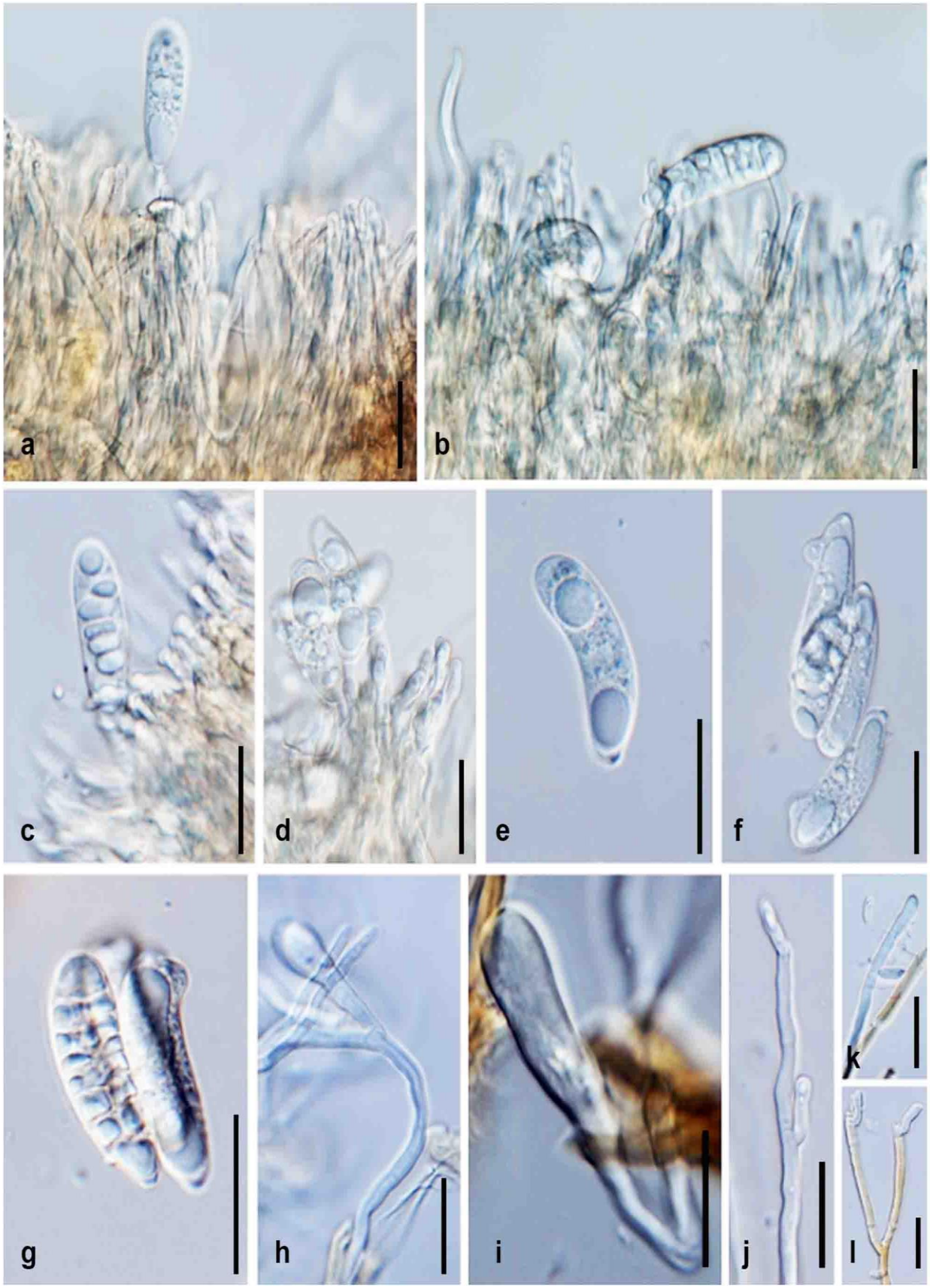

Figure 2: Micromorphological structures of ***Auriculoscypha anacardiicola*** (AMH 10772). **a**–**d**, Hymenium with developing and mature Basidiospores; **e–g**, Young and mature Basidiospores; **h and i,** Immature basidium with hypha; **j–l**, Marginal mycelia with ellipsoid amerosporous conidia. **Scale bars:** a–l= 20 µm.

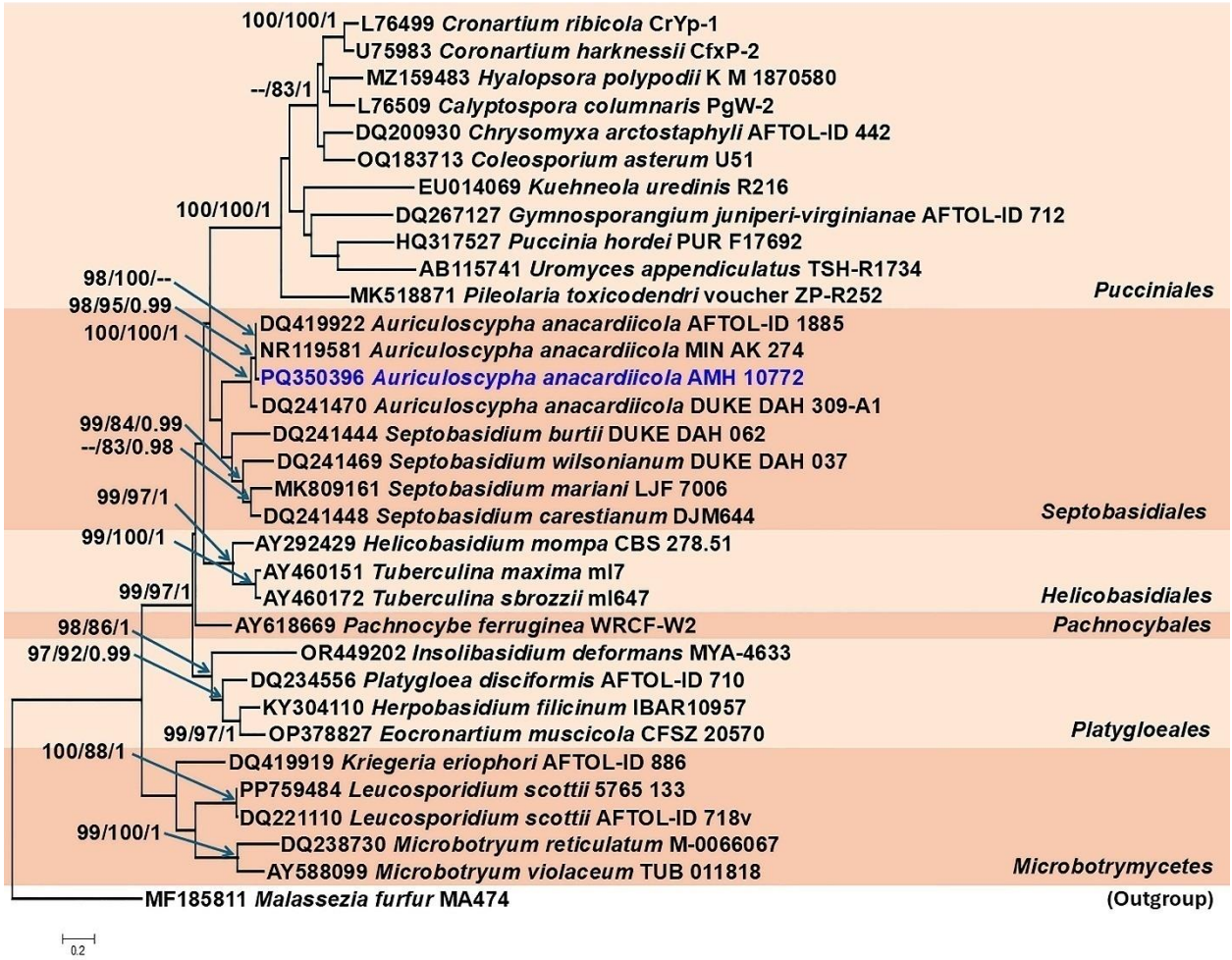

Figure 3: Phylogenetic tree generated from Maximum Likelihood (ML) analyses based on ITS sequence. Branch support values from 1000 non-parametric bootstraps for RAxML (R-BS) and IQ-TREE (UFboot-BS) and posterior probability (PP) from the Bayesian analysis are shown at the nodes (Ufboot-BS≥95% / R-BS≥70% / PP≥0.97). Low support values are not represented. The tree is rooted with *Malassezia furfur* MA474 (MF185811). The sequences generated for *Auriculoscypha anacardiicola* in this study are highlighted in blue.

DISCUSSION

The present study forms the first report of *A. anacardiicola* from *Holigarna arnottiana*, which belongs to the family Anacardiaceae. The present study is the report of *A. anacardiicola* from the Maharashtra region of the northern Western Ghats. The significance of this study is that the genus *Auriculoscypha* is only reported so far from the Southern Western Ghats and it's a rare fungus having limited geographic occurrence and unique in its association. The association of *A. anacardiicola* is consistently found on Anacardiaceous host especially on cashew and mango plants and single report on *Macaranga peltata*. The unique morphology (stipitate-cupulate, woody or leathery, dorsally attached cylindrical stipe) of the strain found on *Holigarna* (AMH 10772) is more branched compared to the earlier reported accessions on other hosts. We have noted up to 3 cupulate pileus arising from a single stipe that was consistent and that lead to the further molecular study of this strain. Based on our molecular study, the strain from *Holigarna* (AMH 10772) is closely allied to the erstwhile reported strains allied to the genus *Septobasidium*. Further assessment and comparison of the new accession of *Auriculoscypha* with members of Septobasidiaceae, was noted and the uniqueness of this phyto-parasitic insect symbionts (Swannetal, 2001) was reinstated in this study. As earlier reported in Lalitha (1992), the insects parasitized by *Auriculoscypha* on *Holigarna* host, tubercle was formed and arrested the movement, metamorphosis and reproduction of the insect.

While assessing the micromorphlogy of the strain in this study, we have noted that the conidial formation from the margins of the basidiocarp that was dubious (Reid and Manimohan, 1985) in previous studies but

our study established these facts and recorded the enteroblastic conidiogenesis and amerosporous, ellipsoid morphology. This study emphasizes the consistent occurrence of *Auriculoscypha* on Anacadaceous hosts that is endemic to the Western Ghats of India. Endemism in fungal species of India is yet to be studied in detail and contributions to the red data book of fungi from India is also highly limited due to 'data deficiency'. Our work further extends the geographic distribution of this species to the northern Western Ghats, Maharashtra. *Holigarna* occurs mostly in the evergreen forest of Western Ghats region of Indian subcontinent (also in Bangladesh and Indo-China). Similarly, *Mangifera indica* was originated in India and Myanmar. Whereas *Anacardium occidentale* (cashew) originated in the Cerrados of Central Brazil. Portuguese missionaries introduced it to India in the late 16th century, where it proliferated at low elevations near the coastline. *A. anacardiicola* thrive well on these Anacardaceous hosts but never been reported in highly explored habitats for fungi in Brazil, Myanmar, Bangladesh or China that establish the restricted distribution of the taxon limited to the Western Ghats and its natural occurrence on indigenous hosts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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