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Phylogenetic relationships of *Auriculoscypha* based on ultrastructural and molecular studies

T. K. Arun KUMAR[a,b,*], *Gail J. CELIO*[a], *P. Brandon MATHENY*[b], *David J. McLAUGHLIN*[a], *David S. HIBBETT*[b], *P. MANIMOHAN*[c]

[a]*Department of Plant Biology, University of Minnesota, 1445 Gortner Avenue, St Paul, MN 55108-1095, USA*
[b]*Department of Biology, Clark University, 950 Main Street, Worcester, MA 01610, USA*
[c]*Department of Botany, University of Calicut, Kerala 673635, India*

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ABSTRACT

The phylogeny of *Auriculoscypha anacardiicola*, an associate of scale insects in India, is investigated using subcellular characters and MP and Bayesian analyses of combined nuLSU-rDNA, nuSSU-rDNA and 5.8S rDNA sequence data. It has simple septa with a pulley-wheel-shaped pore plug, which is diagnostic of phytoparasitic members of the *Pucciniomycetes*, and hyphal wall break on branching, a phenomenon unique to some simple septate heterobasidiomycetes. The septal ultrastructure of *A. anacardiicola* is similar to that of the genus *Septobasidium*. The close relationship to *Septobasidium* is also confirmed by rDNA sequence analyses. The polyphyletic nature of the order *Platygloeales*, noted in earlier studies, is evident from the present molecular analysis as well. The placement of *Auriculoscypha* in the *Platygloeales* can no longer be justified and both ultrastructural and molecular evidence strongly support the placement of *Auriculoscypha* in the *Septobasidiales*.

Introduction

Auriculoscypha is a basidiomycete currently known only from southwest India where it is seen only on the bark of anacardiaceous trees in obligate association with a coccid (Reid & Manimohan 1985; Lalitha & Leelavathy 1990; Lalitha 1992). The fungus obtains its nutrition from the coccid, which in turn feeds on trees, showing an indirect form of phytoparasitism. The basidiomata are pendant, cupulate with a central dorsally attached stipe and of a woody non-gelatinous texture. The fungus grows from a small tubercle just below the bark inside which one to three coccids are held captive.

Auriculoscypha was erected as a monotypic genus by Reid & Manimohan (1985) with *A. anacardiicola* as the type species. It remains a monotypic genus. Although it was assigned to the order *Auriculariales*, Reid & Manimohan (1985) acknowledged its affinities to the order *Septobasidiales* and suggested it could be a connecting link between the two orders. *Auriculoscypha* is a genus without any obvious relationship with other genera of *Auriculariales s. lat.* (Reid & Manimohan 1985), although its pendant-cupulate basidiomata and transversely septate basidia are reminiscent of the genus *Auricularia*. The combination of the non-gelatinous brown fibrous context, thick-walled brown septate hyphae devoid of clamp-connections, circinate basidia with two fertile segments, and the large spores that eventually become septate suggested to Reid & Manimohan (1985) a possible connection with *Septobasidiales*. At the time, an insect association was not known for *Auriculoscypha*, and

* Corresponding author. Present address: Department of Botany, University of Calicut, Kerala 673635, India.
E-mail address: arunkumartk3@rediffmail.com
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hence Reid & Manimohan were reluctant to place it in the *Septobasidiales*.

Evidence of an obligate relationship with a coccid was obtained by Lalitha & Leelavathy (1990), who suggested a transfer of *Auriculoscypha* from *Auriculariales* to *Septobasidiales*. This was followed by the observation by Lalitha *et al.* (1994) of a yeast phase in the life cycle of *Auriculoscypha*, which supported its re-disposition in *Septobasidiales*. Based on these observations, Bandoni (1995) and Swann *et al.* (2001) placed *Auriculoscypha* in *Septobasidiales*. Interestingly, the eighth (Hawksworth *et al.* 1995) and ninth (Kirk *et al.* 2001) editions of the *Dictionary of the Fungi* place *Auriculoscypha* in *Platygloeaceae* without giving any reason for such a designation. *Auriculoscypha* clearly has a convoluted systematic history and its phylogenetic relationships remain uncertain.

In the meantime, the concept of a monophyletic group of organisms included in the subphylum *Pucciniomycotina* (Bauer *et al.* 2006; syn. *Urediniomycetes*) has emerged primarily based on biochemical and ultrastructural characters, comprising the rust fungi, the *Septobasidiales*, and some species that previously were considered as smuts, jelly fungi, deuteromycetes or ascomycetes (Swann & Taylor 1995; Swann *et al.* 2001; Aime *et al.* 2006). Emphasis on ultrastructural data resulted in the dismemberment of the order *Auriculariales*, which now is restricted to species having septal pore swellings and a complex septal pore apparatus (Wells & Bandoni 2001). In addition, ultrastructural and biochemical information have made it clear that the members of the order *Platygloeales* are not monophyletic (Swann *et al.* 2001).

With this backdrop, we decided to examine the ultrastructural and molecular characteristics of *Auriculoscypha*. Ultrastructural characteristics associated with the septal pore apparatus have been used extensively in the phylogenetic classification of basidiomycetes (Bandoni 1984; Suh & Sugiyama 1993; Bauer & Oberwinkler 1994; McLaughlin *et al.* 1995; Nishida *et al.* 1995; Swann *et al.* 1999). Similarly, rDNA sequences have proved to be very useful in elucidating fungal phylogeny in the *Pucciniomycotina* (Nishida *et al.* 1995; Berres *et al.* 1995; Maier *et al.* 2003; Sampaio *et al.* 2003; Wingfield *et al.* 2004; Aime *et al.* 2007) though relationships within the *Septobasidiales* and *Platygloeales* are poorly known (Swann *et al.* 2001). The present study aims to integrate evidence from the septal ultrastructure and nuclear ribosomal DNA sequences in order to understand the relationships of *Auriculoscypha*.

Materials and methods

Fresh basidiomata of *Auriculoscypha anacardiicola* were collected from the Calicut University Campus, Kerala State, India, during June 2005 and pure cultures were initiated from spore deposits in tap water agar (TWA). On TWA, the germinating basidiospores produced a yeast stage that eventually became mycelial. Isolates were maintained on 2 % malt extract agar (MEA) at room temperature. Isolate AK274 was used in the present investigations. The basidiome from the spore-print of which the isolate AK274 was derived and the culture are deposited at the University of Minnesota Herbarium (MIN) and Mycological Culture Collection, respectively.

Electron microscopy

One-week-old cultures of *Auriculoscypha anacardiicola* were fixed by flooding petri plates with 2 % (v/v) gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for about 15 min at room temperature. Pieces of mycelial mat up to 3 × 3 mm in area were cut from the actively growing regions of the colonies using a razor blade. Specimens were fixed in fresh gluteraldehyde solution for 1 h and rinsed in three changes of 0.1 M sodium cacodylate buffer for 15 min each. The samples were then post-fixed in 1 % (v/v) osmium tetroxide in the same buffer for 4 h at room temperature, rinsed with glass-distilled water three times for 15 min each, and stained overnight with 0.5 % (v/v) aqueous uranyl acetate at room temperature in the dark. After three 10-min rinses in water, specimens were dehydrated in a graded acetone series (25 % for 2 h, 50 %, 75 %, 95 % for 30 min, and 100 % for 1 h), and infiltrated with Quetol 651 resin (Ted Pella, Inc., Redding, CA). Half of the samples were dish embedded (Mims *et al.* 2003), while the other half were flat embedded (following Kleven & McLaughlin 1989). Resin was polymerized at 74 °C for 24–48 h.

Blocks were sectioned on a Reichert–Jung ultramicrotome using a diamond knife. Thin (96–100 nm) sections were collected on Formvar-coated single-slot copper grids and were dried on carbon-coated Formvar bridges using the procedure of Rowley & Moran (1975). Sections were stained with 3 % uranyl acetate followed by triple lead stain (Sato 1968), and examined with a Philips CM-12 transmission electron microscope operating at 60 kV.

DNA extraction, amplification and sequencing

DNA was extracted from ground mycelium of isolate AK274 and CBS101449 (*Kriegeria eriophori*, culture obtained from CBS Culture Collection) using a EZNA Fungal DNA Kit (Omega Bio-tek, Doraville, Georgia). The nuLSU, nuSSU, and ITS regions, including the 5.8S rRNA gene, were amplified by PCR performed on a MJ Research PTC-200 thermo-cycler. The primers LROR and LR7 (Vilgalys & Hester 1990) were used for amplifying the nuLSU. Primers PNS1, NS41, NS19b and NS8 (White *et al.* 1990; O'Donnell *et al.* 1998) were used for the nuSSU regions. ITS regions were amplified using the primers ITS1F and ITS4 (White *et al.* 1990; Gardes & Bruns 1993). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Sequencing reactions were set up using a Big-dye 3.1 dye terminator chemistry (Applied Biosystems, Foster City, CA) and sequenced at the DNA sequencing facility in the laboratory of Rytas Vilgalys (Biology Department, Duke University). The sequencing primers used for nuLSU regions were LROR, LR7, LR3, LR3R and LR5 (Vilgalys & Hester 1990, http://www.biology.duke.edu/fungi/mycolab/primers), for nuSSU regions the primers PNSI, NS41, NS19b, NS19bc, NS6, NS51, NS8 (White *et al.* 1990; O'Donnell *et al.* 1998) and ITS1F and ITS4 for the ITS regions (White *et al.* 1990; Gardes & Bruns 1993, http://www.biology.duke.edu/fungi/mycolab/primers). Sequence chromatograms were compiled using software Sequencher 4.1 (GeneCodes, Ann Arbor, MI). Sequences newly generated for this study have been deposited at GenBank (www.ncbi.nlm.nih.gov) (DQ419918, DQ419919, DQ419920, DQ419921, DQ419922).

Phylogenetic analysis

The alignment included 37 taxa with two species of *Malassezia* (*Ustilaginomycetes*) used as the outgroup. Representatives were chosen from the *Microbotryomycetes* and the five recognized orders of *Pucciniomycetes* as proposed by Bauer *et al.* 2006 and the AFTOL classification project (http://www.clarku.edu/faculty/dhibbett/AFTOL.htm). Sequences of taxa, other than the newly generated ones, were taken from the AFTOL database (http://ocid.nacse.org/research/aftol/data.php) and the missing rDNA sequences for certain taxa were supplemented with those available from GenBank (Table 1). Sequences for the nuLSU region were missing for two taxa, six taxa lacked nuSSU data, and the 5.8S region sequences were unavailable for 16 taxa. For *Helicobasidium corticioides* and *Septobasidium burtii*, only the nuSSU sequences were available. Sequences for the 25S and 18S regions of *Septobasidium* sp. were taken from those deposited in GenBank as *Septobasidium* sp. (AY254182) and *Septobasidium canescens* (AY123320), respectively. The sequence data for the nuLSU, nuSSU, and 5.8S regions were aligned separately in McClade 4.0 (Maddison & Maddison 2000). Ambiguously aligned characters were excluded, and gaps were scored as missing data. The individual alignments were later combined in an interleaved format. The combined dataset is available from TreeBASE (http://www.treebase.org/tree-base/) with study and matrix accession numbers, S1611 and M2894, respectively.

MP and Bayesian (Huelsenbeck *et al.* 2001) methods were used for phylogenetic inference. MP was performed with PAUP 4.0b10 (Swofford 2002) using the heuristic search option with tree bisection–reconnection (TBR) branch swapping algorithm, with 20 replicates using random step-wise addition, holding one tree at each step, saving all minimal length trees and setting maxtrees unrestricted. All characters were given equal weight. BS analysis (Felsenstein 1985) was carried out to assess the relative branch support with the full heuristic search option on 1K replicates (BS parameters were the same as employed in MP tree searches) and retaining only groups with frequencies more than 50 %. Bayesian phylogenetic inference was done using the software MrBayes 3.1.1 (Ronquist & Huelsenbeck 2003) with a general-time reversible (GTR) model, including a proportion of invariable sites and

Table 1 – Sampled taxa with GenBank sequence accession numbers

Taxon	25S	18S	5.8S
Auriculoscypha anacardiicola	DQ419920	DQ419921	DQ419922
Chrysomyxa arctostaphyli	AY700192	AY657009	DQ200930
Coleosporium asterum	AF522164	AY123286	–
Cronartium ribicola	DQ354560	M94338	L76499
Endocronartium harknessii	AY522175	M94339	U75983
Eocronartium muscicola	AF014825	AY123323	–
Gymnosporangium juniperi-virginianae	AY629316	AY123289	DQ267127
Helicobasidium corticioides	–	U75303	–
H. mompa	AY254178	U77064	AY292429
H. purpureum	AY222049	–	AY460132
Herpobasidium filicinum	AY512850	–	–
Hyalopsora polypodii	AY512852	AB011015	–
Insolibasidium deformans	AF646099	AY123292	–
Jola cf. *javensis*	DQ416207	DQ416206	–
J. hookerianum	DQ416208	–	–
Kriegeria eriophori	AY745728	DQ419918	DQ419919
Kuehneola uredinis	AY745696	DQ092919	–
Leucosporidium scottii	AY646098	AY707092	DQ221110
Malassezia furfur	AY745725	AY083223 GB	AY743635
M. pachydermatis	AY745724	DQ457640	AY387142
Microbotryum reticulatum	AY213003	U79566	–
M. violaceum	AY512864	U77062	AY588099
Pachnocybe ferruginea	L2028	–	AY618669
Pileolaria toxicodendri	AY745699	DQ092921	–
Platygloea disciformis	AY629314	DQ234563	DQ234556
P. pustulata	AY512871	DQ198786	–
Puccinia hordei	DQ354527	AY125412	AY187089
Pucciniastrum goeppertianum	AF522180	AY123305	L76509
Rhodotorula hordea	AY631901	AY657013	DQ234557
Septobasidium burtii	–	AY123322	–
S. carestianum	L20289	DQ198787	–
Septobasidium sp.	AY254182	AY123320	–
Thanatophytum crocorum	AY885168	D85648	AY460132
Triphragmium ulmariae	AF426219	AY125402	–
Tuberculina maxima	AY222044	–	AY460151
T. sbrozzii	AY222045	–	AY460172
Uromyces appendiculatus	AY745704	DQ354510	AB115741

gamma distributed substitution rates at the remaining sites. Multiple independent analyses were run from random starting trees for 2M generations, with trees saved every 100 generations, using four chains and a burn-in of 2K trees. Likelihood scores were plotted against the number of generations to assess stationarity of the Metropolis-coupled MCMC sampling process. These curves indicated that a choice of a 10 % burn-in was justified. Thirty-six thousand trees from the 40K trees sampled were used to calculate the PPs. PP values above or equal to 0.95 were considered significant.

Results

Ultrastructural observations

Hyphae of *Auriculoscypha anacardiicola* have a consistently thick double wall (combined lateral wall thickness ranging from 140–640 nm) composed of electron-dense fibrillar material (Fig 1A). A thin diffused zone of fibrillar material is also seen on the outer wall surface, which may be the gelatinous, adhesive substance that gives the culture mycelium its sticky nature. Hyphal branch initiation involves disruption of the outer wall layer. The sequence of events associated with lateral branch formation and outer wall rupture are evident from the micrographs taken at different developmental stages (Fig 1A–C). Almost round electron-sparse areas initially appear in the outer wall layer beneath the outer diffuse zone before branch initiation (Fig 1A–B). As the branch initial develops, these electron-sparse regions gradually increase in area leading to complete dissolution of the electron-dense fibrillar portion of the wall. The developing branch emerges through this ruptured part of the outer wall. New branch walls are formed from and are continuous with the inner wall layers of the old hyphae, leaving a distinct collar-like disruption mark around the base of the new branch (Fig 1C).

A study of seven septa with partial or full serial sections revealed the structure of the septal pore apparatus. The septa are simple, continuous with the lateral wall, and become slightly thinner toward the middle. This is followed by a sudden swelling and an abrupt constriction resembling a beak-like structure at the pore (Fig 1A–D). The pore is occluded by a pulley-wheel-shaped electron-dense matrix surrounded by a zone of ribosome exclusion (Fig 1D–F). The average pore diameter is 40 nm. Septal pore caps are absent. Large microbodies (approximately 140–300 nm in diameter) are consistently observed surrounding the zone of exclusion (Fig 1D–E). Except at the two zones of exclusion, numerous ribosomes, probable lipid globules or storage products, appearing as large electron-dense bodies, and vacuoles are present throughout the cell (Fig 1A–F). The electron-dense bodies are also present around the zone of exclusion (Fig 1D–F).

Molecular analysis

Our molecular analyses yielded phylogenetic trees indicating the relative placement of the genus *Auriculoscypha* within the closely related taxa. The parsimony analysis yielded a single tree (2028 steps, CI = 0.577). Trees generated from MP and MrBayes analyses were extremely similar in topology, and hence only the Bayesian tree is shown (Fig 2). These analyses identified five clades supported by high BS and PP values. *A. anacardiicola* was positioned closer to the *Septobasidium* species with 100 % BS and PP value (1.0) and its placement in *Septobasidiales* is supported by all analyses. The whole *Septobasidiales* clade also receives a high PP score. *Platygloea pustulata* is positioned closer to the *Septobasidiales* rather than among the *Platygloeales*. Of the 37 taxa included in the study, 12 taxa belonged to the *Pucciniales* (syn. *Uredinales*), six in *Platygloeales* and four were grouped in *Septobasidiales*. *Helicobasidium purpureum*, *H. mompa*, *Tuberculina sbrozzii*, *T. maxima*, and *Thanatophytum crocorum* clustered together as a distinct clade, the *Helicobasidiales* R. Bauer *et al.* (2006). *Pachnocybe ferruginea* is the type species of *Pachnocybe* and the only representative of the *Pachnocybales* sampled here.

Discussion

Ultrastructural evidence strongly supports the placement of *Auriculoscypha anacardiicola* in the *Septobasidiales*. It has simple septa with a pulley-wheel-shaped pore plug, which, according to Swann *et al.* (2001), is diagnostic of phytoparasitic *Pucciniomycetes* (Fig 2). The septal ultrastructure of *Auriculoscypha* is somewhat similar to the septal pore apparatus in the genus *Septobasidium* observed by Dykstra (1974). Unfortunately, only limited information is available on septal structure of *Septobasidium* from Dykstra's (1974) work, which is the only published account on the topic. Although five species of *Septobasidium* were considered in that study (Dykstra 1974), the septal pore information is inadequate and only *S. burtii* could really be compared. In *S. burtii* a single very low magnification micrograph suggests that a pulley-wheel plug may be present (Dykstra 1974: fig. 7). In *Septobasidium* no zone of exclusion around the septal pores and no bordering microbodies were observed, but one micrograph (Dykstra 1974: fig. 5, lower right) suggests a possible microbody. A recent study by Bauer *et al.* (2006) gives structural data on the septal pore apparatus of *Septobasidium carestianum*, revealing a pulley-wheel-shaped pore plug. The single micrograph from this study again does not provide details on the microbodies and zone of ribosome exclusion but confirms the pulley-wheel-pore plug in *Septobasidium*, a feature that it shares with *Auriculoscypha*, thus supporting the link between the two genera. The rupture of the outer hyphal cell wall layer at branching is characteristic of several clades of the *Pucciniomycotina* (Fig 2), including rusts, some *Agaricostilbomycetes* and several *Pucciniomycetes* members such as *Jola*, *Eocronartium*, *Pachnocybe*, and *Helicobasidium* (Swann *et al.* 2001), but has not yet been observed in *Septobasidium*. Wall breakage in *Auriculoscypha* probably involves enzymatic degradation of the outer wall layer.

The close relationship to *Septobasidium* is also confirmed by rDNA sequence analyses (Fig 2). The polyphyletic nature of *Platygloeales*, noted in earlier studies (e.g., Swann *et al.* 2001), is evident from the present molecular analysis as well. The two species of *Platygloea* included in the study settle in two different clades. Moore (1990) had initially erected the order *Platygloeales* to incorporate the phylogenetically varied simple-septate auricularioid taxa. Based on molecular phylogenetic analyses, Weiss *et al.* (2004) first used the order in

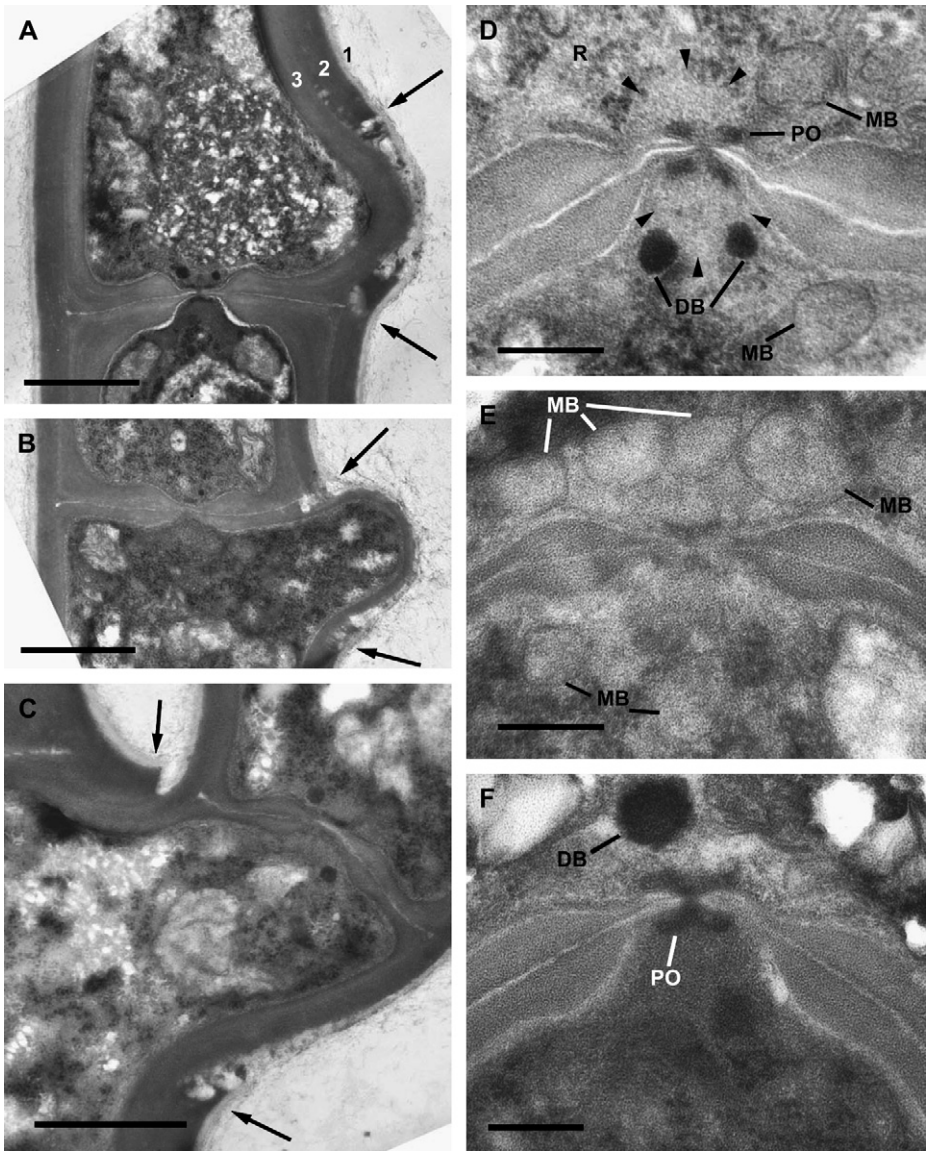

Fig 1 – Hyphal wall breakage at branching and septal pore apparatus of *Auriculoscypha anacardiicola*. (A–C) New branch emergence and associated wall breakage. Bars = 1 μm. (D–F) Simple septa and subcellular structures in and around the septal pore complex. Bars = 0.25 μm. (A) Inner (3) and outer (2) hyphal wall layers covered by a diffused zone (1). Wall dissolution begins with the appearance of electron-light areas in the outer wall layer (between arrows). (B) Growth of new branch through the dissolved wall (arrows). (C) New branch walls formed from and continuous with the inner wall layers of the parent hypha leaving a distinct collar-like disruption mark (arrows) around the base of the new branch. (D) Septum with wall swellings that abruptly taper towards the pore, pore plug (PO), zone of organelle exclusion (indicated by arrowheads), ribosomes (R), microbodies (MB) and electron-dense globules (DB). (E) Large microbodies surrounding the zone of ribosome exclusion. (F) Pulley-wheel-shaped plug (PO) occluding the septal pore and electron-dense globule (DB).

a new sense, to represent the lineage comprising *Platygloea disciformis* and its relatives. The order *Platygloeales s. strict.* is a monophyletic group containing *P. disciformis* and six other genera listed in Bauer *et al.* (2006). The results of the present investigation reveal that the placement of *Auriculoscypha* in *Platygloeales* as in the *Dictionary of the Fungi* (Hawksworth *et al.* 1995; Kirk *et al.* 2001) can no longer be justified.

Although the position of *Auriculoscypha* within *Septobasidiales* is now clear, its precise relationships within the family *Septobasidiaceae* are not evident from the present study. The family *Septobasidiaceae* is a monophyletic group containing five genera, all of which are phytoparasitic insect symbionts (Swann *et al.* 2001). All species are associated with scale insects on living plants and have simple septal pores, auricularioid basidia, characteristic haustoria, and lack clamp connections. *Auriculoscypha* differs from all other genera of the family in having woody, stipitate–cupulate basidiomata. The basidiomata of all the remaining genera are comparatively much less organized mycelial mats that are resupinate on the host plant. Another distinctive feature of *Auriculoscypha* is a subcortical

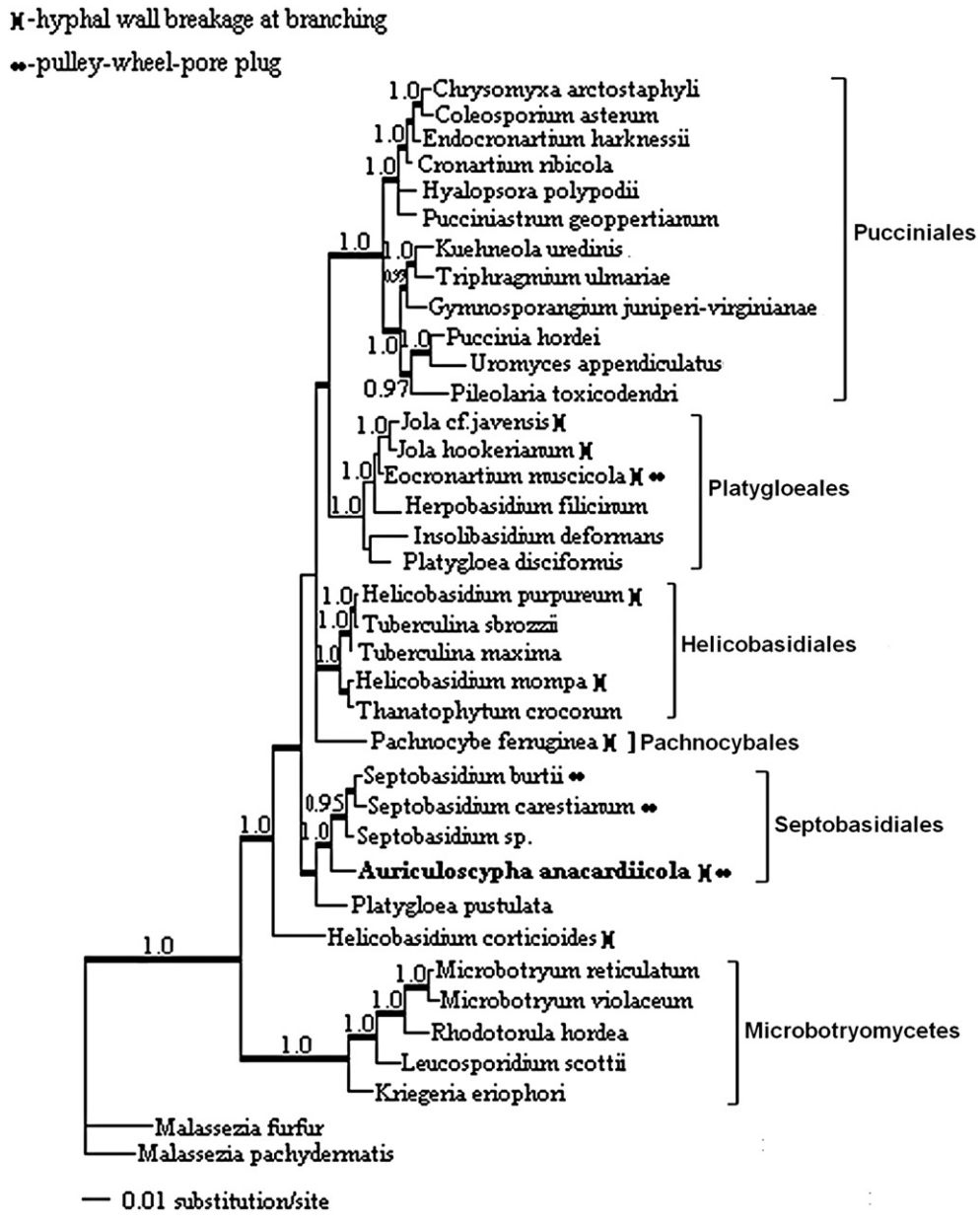

Fig 2 – The 50 % majority-rule consensus tree from 36K trees obtained from Bayesian MCMC analysis using nurDNA sequence data. Values above branches indicate the posterior probability of that clade. Only values equal or greater than 0.95 are shown. Branches receiving MP BS values above or equal to 50 are shown in double lines. *Auriculoscypha anacardiicola* is placed in bold font to highlight its phylogenetic position in the tree. The distribution of hyphal wall breakage on branching and of the pulley-wheel-shaped pore plugs in *Pucciniomycetes* is shown.

tubercle whose wall is composed of both fungal hyphae and plant tissues. Inside this tubercle, a single insect (usually) is trapped throughout its life. Insects thus parasitized by *Auriculoscypha* have no freedom of movement and remain immature and incapable of reproduction (Lalitha 1992). This kind of a relationship with the insect may be more primitive than that of *Septobasidium*, where multiple insects are associated with a single fungal thallus. Moreover, in the *Septobasidium*-scale insect association, many of the insects under each fungal colony are unparasitized and are free to move around and reproduce (Couch 1938) suggesting that the *Septobasidium*-scale insect association has evolved *via* kin selection on the part of the insect symbionts.

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