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Molecular Systematics of the Phyllachorales (Ascomycota, Fungi) Based on 18S Ribosomal DNA Sequences

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ABSTRACT

In order to evaluate the monophyly of the Phyllachorales from a molecular standpoint and elucidate its phylogenetic relationships with other orders, a segment of the 18S rRNA gene from several representatives of the Phyllachorales, including species of Glomerella, Phyllachora, Coccodiella (=Coccostroma), Sphaerodothis, Ophiodothella, *as well as* Magnaporthe *was sequenced. Maximum Parsimony analysis revealed that the Phyllachorales was a polyphyletic assemblage of taxa. None of the other members of the Phyllachorales, which produced either a clypeus or stroma, clustered with* Glomerella*. Of the taxa examined, was* Coccodiella *the closest relative of* Phyllachora.Magnaporthe *was closely related to the Diaporthales. Our 18S rDNA data highly supported* Glomerella *being accommodated in a separate family.*

Key words: DNA sequencing, 18S ribosomal DNA, PCR, Phyllachorales

INTRODUCTION

The Phyllachorales is a small order of mostly tropical perithecial ascomycetes (pyrenomycetes), and has generally been treated as comprising only one family, the Phyllachoraceae (=Polystigmataceae) (Eriksson and Hawksworth, 1993; Hawksworth et al., 1995). A major taxonomic problem with the order is the lack of reliable morphological characters that clearly delimit the entire group. Wehmeyer (1975) did not consider the characters used to define the family well established, and suggested that the Phyllachoraceae might include genera more closely related to other orders than to each other. Another factor that suggested that the family might be artificial was the emphasis that had been placed on only a few characters, such as ascospore shape, color, and septation, as well as on the extent of stromatic tissue (Cannon, 1991).

Despite the relatively high number of genera included in the family, only six have been commonly reported and cited in the literature: Coccodiella Hara (=Coccostroma Theiss. and Syd.), Glomerella Spauld. and H. Schrenk, Ophiodothella (Henn.) Höhn., Phyllachora Nitshke ex Fuckel, and Sphaerodothis (Sacc. and Syd.) Shear. The sixth genus, Magnaporthe R. A. Krause and R. K. Webster is considered by only a few investigators as a member of either the Phyllachorales (Barr, 1977) or Polystigmatales (Farr et al., 1989), whereas other authors have placed it in the Diaporthales (Krause and Webster, 1972; Yaegashi and Udagawa, 1978). The lack of distinct morphological characters and problems in determining homologous characters in

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fungi have contributed to the increasing interest in molecular systematics (Lutzoni and Vilgalys, 1994). One of the most effective methods to test hypotheses derived from morphology is the use of molecular data (Tehler, 1994), especially nucleotide sequencing, where several regions of the DNA molecule can be compared, according to the taxonomic level or group of fungi under study (Lutzoni and Vilgalys, 1994).

Sequences from the nuclear small-subunit ribosomal DNA (18S rDNA) are not available for taxa previously placed within the order Phyllachorales, except for Glomerella cingulata and Phyllachora graminis. The inability of some species to grow in culture is an obvious problem in working with molecular systematics of this order, and probably the cause of the lack of interest held by researchers in working with this important group.

The main purpose of this work was to investigate the relationships of the order Phyllachorales using partial 18S rRNA gene sequences. We further tested the feasibility of the two clades, "A" and "B", suggested by Spatafora and Blackwell (1994), after the inclusion of our isolates.

MATERIAL AND METHODS

The source of taxa included in the present work and the accession numbers for fungal strains and sequences deposited in GenBank are listed in Table 1.

DNA Purification: Mycelium was grown in 1.5 ml Eppendorf microcentrifuge tubes containing 1 ml of potato dextrose broth (PD broth), shaken for 5-7 days and washed twice with sterile distilled water. Total DNAs were isolated from macerated mycelium according to the CTAB modified method of Graham et al. (1994). The fungus tissue was macerated in CTAB (hexadecyltrimethyl-ammonium bromide) extraction buffer (2% CTAB; 100 mM Tris-HCl, pH 8; 1.4 M NaCl, and 20 mM EDTA) and the DNA was extracted with chloroform:isoamyl alcohol (24:1) and precipitated with ice-cold absolute ethanol.The DNA pellet was air dried overnight, dissolved in 50 µl of sterille distilled water, and stored at -20°C for later use.

For DNA extraction of Coccodiella and Sphaerodothis, both of which formed on their hosts superficial stromata composed only of fungus tissue, leaves were first placed in a moist chamber, and after approximately two hours stromata were removed from the host tissue, and placed in the tubes. Special care was taken with the genus Phyllachora once it produced a superficial pseudostroma composed of both plant and fungus tissue, the clypeus was cut off with a sharp blade, and only the centrum with fungus material was removed and placed in the microcentrifuge tubes. The DNA pellet was eventually treated with DNase free enzyme RNase (Boehringer Mannheim) (Ross, 1995).

PCR Amplification and DNA Sequencing: The 5' two-thirds of the 18S rDNA was selectively amplified using the primers NS1 and NS6, designed by White et al. (1990). PCR reactions for synthesis of double-stranded DNA were carried out in a Thermal Cycler (Perkin-Elmer Co., Branchburg, NJ), using the following program: 1 cycle of 2 min at 95°C, 55°C for 30 sec, and 72°C for 45 sec; an additional 38 cycles of 95°C/ 30 sec, 55°C/30 sec, and 72°C/45 sec; a final cycle was performed for 10 min at 72°C. Total reaction volume was 50 µl with the following components and final concentrations, according to the Repli-pack reagent set (Boehringer Mannheim Corporation, Indianapolis, IN): 1.5 mM of $MgCl_2$, 10x Reaction Buffer, 0.2 mM of a mix of the four dNTPs, 0.25 µM of each primer, 2.5 units of Taq DNA Polymerase, and 2 µl of templates. PCR products were visualized by electrophoresis in 1.0% agarose gel in the presence of ethidium bromide and purified using a Wizard™ Preps DNA purification system kit (Promega Corp., Madison, WI).

The purified double-stranded DNA was sent to the automated sequencing facilities (MGIF) of The University of Georgia. The primers NS1, NS2, NS3, and NS4 (White et al., 1990) were used to sequence both strands of DNA using an Applied Biosystems automated sequencer (model 373, version 1.2.1). Except for the NS3 region of Ophiodothella vaccinii, PCR products of all the other fungi have been sequenced in both directions. The sequence obtained with the primer NS4 in this fungus was not complementary to the sequence obtained with the primer NS3, perhaps due to the presence of an insertion at 3' end of NS3.

Sequence Analysis and Phylogenetics: The partial sequences of 18S rDNA were aligned using the "Pileup" progam (Genetics Computer Group, Madison, WI). The sequence alignment was further refined by eye to minimize gaps. Missing data (unreadable bases due to DNA polymorphisms, secondary structures or limitations of sequencing enzymes) (Seifert et al., 1995), were coded as

question marks (?), and gaps were coded as dots (.). Parsimony trees were obtained from the data by heuristic searches using the computer program PAUP 3.1.1 (Swofford, 1993). Bootstrap values, the statistical support for each node, were computed with 100 replications. Decay indices of various steps were performed within the computational confines of the computer. All sites, informative or not, were included in the analyses. Character polarity was determined by reference to designated outgroups.

Table 1 - List of taxa included in this study

TAXA AND ORDINAL RANK	ISOLATE OR SPECIMEN SOURCE	GENBANK ACCESSION
Phyllachora sp.	*Bauhinia* sp., Brazil	U78542
Glomerella cingulata	---	U48427
G. cingulata	Tomato, Brazil	U76338
G. septospora	*Stirax,* Taiwan	U78779
G. glycines	Soybean, USA	U63138
Colletotrichum gloeosporioides	Cashew, Brazil	U76339
C. gloeosporioides	---	M55640
Coccodiella toledoi	*Miconia* sp., Venezuela	U78544
C. melastomatum	*Miconia* sp.,Venezuela	U78543
Ophiodothella vaccinii	ATCC 36333[1]	U78777
Sphaerodothis acrocomiae	Coconut, Brazil	U76340
Magnaporthe salvinii	ATCC 44756[1]	U78546
Diaporthe phaseolorum	---	L36985
Phomopsis longicolla	PL526[2]	U78778
Cryphonectria parasitica	RTH1135[2]	U78541
Endothia gyrosa	RTH0139[2]	U78540
Leucostoma persoonii	---	M83259
Diatrype disciformis	---	U32403
Daldinia concentrica	---	U32402
Hypoxylon atroroseum	---	U32411
Xylaria hypoxylon	---	U20378
Pestalosphaeria sp.	RTH5147[2]	U78545
Nectria cinnabarina	---	U32412
Hypomyces chrysospermus	---	M89993
Hypocrea lutea	---	U32407
Epichloë festucae	---	U44113
Myriogenospora atramentosa	---	U44114
Microascus trigonosporus	---	L36987
Ceratocystis fimbriata	---	U32418
Halosphaeriopsis mediosetigera	---	U32420
Chaetomium globosum	---	U20379
Neurospora crassa	---	X04971
Pleospora rudis	---	U00975
Botryosphaeria dothidea	RTH0183[2]	U79482
B. rhodina	---	U42476
Saccharomyces cerevisiae	---	J01353
Taphrina deformans	---	U20376

[1] American Type Culture Collection.
[2] R.T. Hanlin's personal culture collection.

RESULTS

A total of 947 sites were included in the broad (37 taxa) analyses. A heuristic search produced 44 most parsimonious trees of 666 steps long, with consistency (CI) and retention (RI) indices of 0.578 and 0.701, respectively, for which the strict consensus is shown in Fig. 1. Due to computer constraints, for the bootstrap resampling, the number of taxa was reduced to 30 (Fig. 2). Of the 947 included bases, 180 sites (19%) were phylogenetically informative. Twenty-eight equally most parsimonious trees of 622 steps long were recovered. The phylogram compatible with the 50%

majority rule bootstrap consensus was 589 steps long, and had a CI , RI, and HI of 0.615, 0.677, and 0.385, respectively (Fig. 2). Decay analysis of the larger data set was performed only to one step longer than the minimum because of computer limitations (Fig. 1).

Two subclades were observed (Fig. 2). The subclade "A" was highly supported by bootstrap value of 98%. It was represented by the orders Hypocreales (including *Sphaerodothis*), Microascales and part of the order Phyllachorales (*Glomerella* spp. and its anamorph *Colletotrichum*). On the other hand, the subclade designated "B" had bootstrap of 62%. It encompassed the orders Sordariales, Xylariales (including *Ophiodothella*), Diaporthales, the genus *Magnaporthe*, and part of the order Phyllachorales (*Coccodiella* spp., and *Phyllachora*).

The non-stromatic genus *Glomerella* and its anamorph *Colletotrichum* did not group with the stromatic or pseudostromatic species (*Phyllachora, Coccodiella, Sphaerodothis,* and *Ophiodothella*).

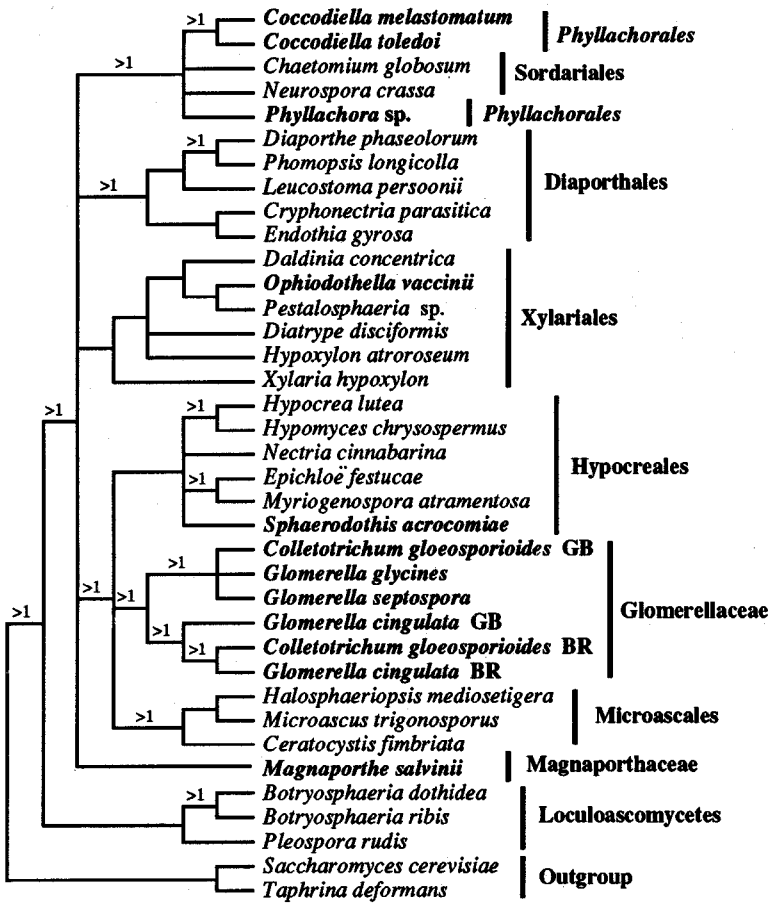

Figure 1 - Strict consensus of 44 most parsimonious cladograms based on 18S rDNA sequences from 37 taxa. Length=666 steps, consistency index=0.578, and retention index=0.701. Decay values >1 are indicated above internodes. Taxa in bold script represent species traditionally placed in the order Phyllachorales. The tree was rooted using *Saccharomyces* and *Taphrina* as outgroups. The current ordinal disposition of each taxon sampled is indicated on the right. GB and BR indicate GenBank and Brazil, respectively.

The three species of *Glomerella* (*G. cingulata, G. glycines, G. septospora*) and the anamorph *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. formed a monophyletic group supported by 100% of the bootstrap replicates in the parsimony analysis (Fig. 2). Additionally, *Glomerella* species

and *Colletotrichum gloeosporioides* were united by very short branches (Fig. 2), suggesting rapid radiation which in turn might have contributed to the unresolved branching order (polytomy) shown in the consensus cladogram (Fig. 1).

The clade formed by the two species of *Coccodiella, C. melastomatum* (Lév.) Hino and Katumoto and *C. toledoi* (Chardon) Hino and Katumoto had a bootstrap value of 100% (Fig. 1). They grouped with *Phyllachora* sp. in 58% of the parsimony trees (Fig. 2). They form a sister group to the Sordariales, and together seem to be a sister group to the Diaporthales. Despite the high bootstrap value connecting the orders Phyllachorales (*Phyllachora* and *Coccodiella*) and the Sordariales, they were morphologically very distinct and were accepted here as separate orders.

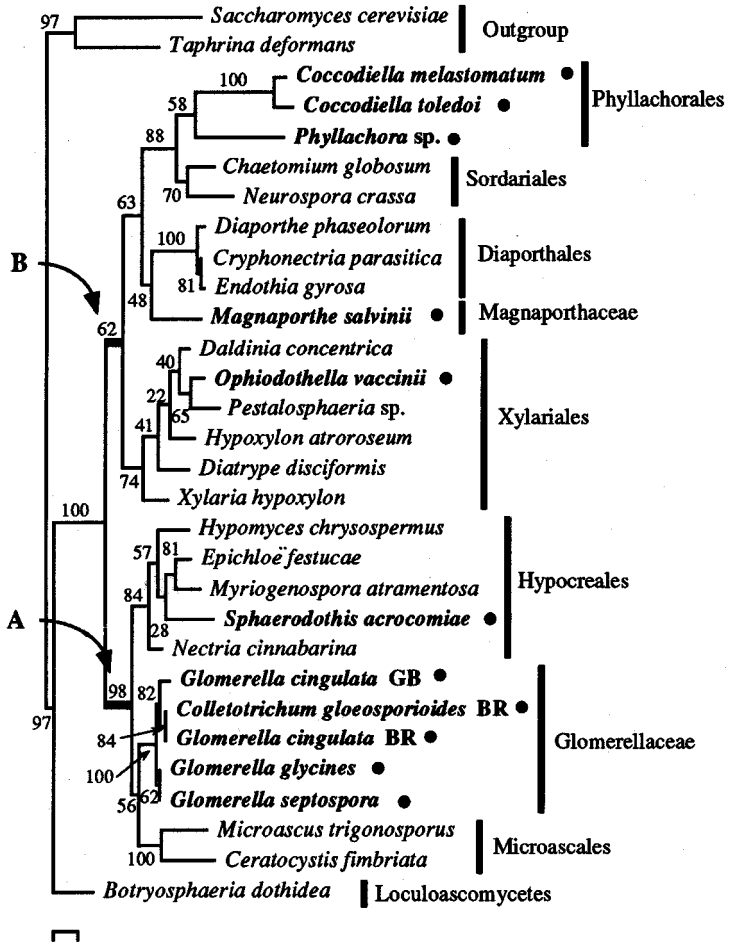

Figure 2 - Phylogram compatible with the bootstrap 50% majority-rule consensus tree from 18S rDNA sequences of a subset of 30 taxa. Values given at the branches are bootstrap values of 100 replicates. Branch lengths are proportional to the number of nucleotide changes. Scale bar corresponds to 10 nucleotide substitutions. The current ordinal disposition of each taxon sampled is indicated on the right. Taxa indicated in bold script followed by dots represent species traditionally placed in the order Phyllachorales. The tree was rooted using *Saccharomyces* and *Taphrina* as outgroups. "A" and "B" correspond to subclades according to Spatafora and Blackwell (1994).

Ophiodothella vaccinii was placed within the order Xylariales as a sister species to *Pestalosphaeria* in all trees. These two genera grouped in 65% of the bootstrap replicates (Fig. 2).Their placement within Xylariales was supported by 74% of the bootstrap replicates (Fig. 2). *Sphaerodothis acrocomiae* did not group with the genus *Coccodiella* despite their superficial resemblance, but was placed in the order Hypocreales (Fig. 2), with 84% bootstrap support for the group. However, its position is not resolved.

The taxon sampled for the family Magnaporthaceae (Cannon, 1994), *Magnaporthe salvinii*, seemed to be a sister group to the Diaporthales, rather than being placed with the Phyllachorales as suggested by Barr (1977). The bootstrap value supporting the Diaporthales-Magnaporthaceae clade was only 48% (Fig. 2). The genera *Cryphonectria* (Sacc.) Sacc. and D. Sacc. and *Endothia* Fr. were treated by Barr (1978) in two different families of the order Diaporthales. However, in the broad analysis, these genera nested together in 81% of the parsimony tree (Fig. 2), and were probably part of the same family.

DISCUSSION

Our results derived from the molecular data supported the hypothesis that the Phyllachorales was a polyphyletic group, with some of the members having connections to three different orders: the Xylariales, the Hypocreales, and the Sordariales. The topology of our gene tree derived from the parsimony analyses, supported Spatafora and Blackwell's (1994) delimitations of the two subclades, "A" and "B". The position of *Glomerella* as a sister genus to the Microascales agreed with the results of Berbee and Taylor (1992), and Spatafora and Blackwell (1994). However, our results do not support the use of *Glomerella* to represent the order Phyllachorales, because this genus is very distantly related to *Phyllachora* and *Coccodiella*.

Glomerella species formed a highly monophyletic group in the parsimony tree. Morphologically, *Glomerella* is very distinct from the other Phyllachorales, and it is restricted to species that share a *Colletotrichum* anamorph (which are exclusively associated with species of *Glomerella*) and produce ascomata that lack stromatic tissue, unusual for members of the Phyllachorales. A clear distinction between *Glomerella* and the other Phyllachorales rests in its nutritional nature. *Glomerella* species are saprophytes or necrotrophs whereas the other members in the order are biotrophs and weak parasites that extract nutrients from the host without detrimental effect (Cannon, 1988; 1991). In addition, the concept of the genus *Glomerella* should be formally broadened to include not only fungi forming ascospores with one cell, but also ascospores with three to four cells, characteristic of the newly described species *Glomerella septospora* (Sivanesan and Hsieh, 1993) whose correct generic identity is confirmed by our data and analyses.

Due to the high support for the monophyly of species of *Glomerella* and its anamorph *Colletotrichum*, apart from the other taxa sampled, we suggest that the order Glomerellales, created by Chadefaud (1960) and further validated by Locquin (1984), and its monotypic family (Glomerellaceae), invalidly published by Locquin (1984), be reinstated to accommodate species of *Glomerella* and its anamorph, *Colletotrichum.*

The genus *Phyllachora* grouped with species of *Coccodiella,* had paraphyses, spermatial anamorphs, ascus and ascospore features and black stromatic tissue which was superficial and pseudostromatic (clypeus) in *Phyllachora* and erumpent and stromatic in *Coccodiella.* They were both biotrophic leaf parasites and were apparently the only true Phyllachorales sampled for this study. The moderately low statistical support for the *Phyllachora*/*Coccodiella* clade could be a consequence of their long branch lengths. Long branches may indicate an accelerated rate of evolution (Spatafora and Blackwell, 1994) or rapid divergence (Alexopoulos et al., 1996). It is also possible that the species of *Coccodiella* and *Phyllachora* sampled represent the most divergent taxa within the order.

Our 18S rDNA data supported the transfer of the genus *Ophiodothella* to the order Xylariales, probably within the family Amphisphaeriaceae, characterized by the formation of a blackened clypeus, ascus apical ring mostly amyloid, and anamorph coelomycetous (Barr, 1990). The traditional placement of the genus *Ophiodothella* in the Phyllachorales was probably due to the production of a superficial clypeus on leaves in the same manner as *Phyllachora*. However, *Ophiodothella* forms long filiform ascospores, which are atypical for the Phyllachorales, and shares with the other xylariaceous fungi the "*Xylaria*" type of centrum and an ascus tip that stains blue in iodine (J+), both of which are characteristic of the order Xylariales.

Sphaerodothis acrocomiae did not group within the Phyllachorales, and was placed in the order Hypocreales. The current taxonomic placement of *Sphaerodothis* in the Phyllachorales is based on the stromatic resemblance with other typical members of the order such as *Coccodiella*. Otherwise, *Sphaerodothis* and *Coccodiella* are distinguished by the color of the ascospores: they are brown before discharge in the former versus hyaline in the latter (in very old material they can turn brown but only after discharge). The ascus tip is undifferentiated in

the species *Sphaerodothis acrocomiae* and differentiated in an apical ring in *Coccodiella melastomatum* and *C. toledoi*. It is likely that stromata in these two genera have arisen convergently. It has been hypothesized that certain morphological characters in unrelated groups can evolve several times due to similar selection pressures (Hawksworth, 1986). Therefore, classifications which emphasize only a few taxonomic characters can artificially group polyphyletic taxa that morphologically resemble each other (Hausner et al., 1993; Spatafora and Blackwell, 1994).

It is possible that the order Hypocreales is broader than currently delimited (Glenn et al., 1996), and the relatively long branch presented by *S. acrocomiae* may be a consequence of missing intermediate taxa which could be undiscovered, extinct, or unsampled (Alexopoulos et al., 1996). Alternatively, the grouping of *Sphaerodothis* within the Hypocreales could also result from a faster rate of sequence change on *Sphaerodothis.* Only when additional sequences from other species of *Sphaerodothis* or other stromatic genera (especially from the tropics) are available, the phylogenetic position and the taxonomic importance of apical and lateral paraphyses, currently used to delimit the Hypocreales, can be more rigorously evaluated. For the time being the genus show be kept in the Phyllachorales. The taxonomic affinities of the genus *Magnaporthe* are equivocal and it has been placed in several different order. Despite Cannon's (1994) disagreement about a close link between *Magnaporthe* and members of the order Diaporthales, our molecular data suggest that they are probably closely related and may comprise a single order. Additional species of *Magnaporthe* as well as other genera accepted in the family Magnaporthaceae proposed by Cannon (1994) need to be sequenced in order to elucidate the taxonomic position of this group. Perhaps, the 28S rDNA, a less conserved molecule, would be more suitable to resolve the relationship between the Magnaporthaceae and the Diaporthales.

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RESUMO

A ordem Phyllachorales foi avaliada do ponto de vista molecular visando esclarecer a sua relação filogenética com outras ordens. Um fragmento do gene 18S rRNA de diversos representantes dos Phyllachorales, incluindo espécies de *Glomerella, Phyllachora, Coccodiella (=Coccostroma), Sphaerodothis, Ophiodothella,* como também *Magnaporthe* foi sequenciado. Análise de parsimônia máxima revelou que a ordem Phyllacholares é polifilética. Nenhum dos outros representantes dos Phyllachorales, que produzem um clipeu ou estroma, se agruparam com *Glomerella*. Dos taxa estudados*, Coccodiella* é o mais próximo de *Phyllachora*. Esses dois gêneros formam um grupo irmão dos Sordariales, que juntos são um grupo irmão dos Diaporthales. *Sphaerodothis* e *Ophiodothella* se agruparam dentro dos Hypocreales/Clavicipitales e Xylariales, respectivamente. *Magnaporthe* é o mais próximo de Diaporthales. Nossos dados de 18S rDNA fortemente suportam *Glomerella* ser acomodado em uma família distinta.

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