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Oomycota from "Parque Estadual da Ilha do Cardoso" (PEIC): first records for São Paulo State and Brazil

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Abstract – (Oomycota from "Parque Estadual da Ilha do Cardoso" (PEIC): first records for São Paulo State and Brazil). We studied the oomycetes diversity from soil and freshwater samples collected quarterly from August 2012 to June 2013 at "Parque Estadual da Ilha do Cardoso" (PEIC), São Paulo State, Brazil. Among the species identified by morphological and/or molecular (complete ITS rDNA region) analysis, four are recorded for the first time for Brazil: *Araiospora streptandra* var. *streptandra* Kevorkian, *Achlya primoachlya* (Coker & Couch) TW Johnson and RL Seym., *Aplanopsis terrestris* Höhnk and *Saprolegnia aenigmatica* Sandoval-Sierra & Diéguez-Uribeondo and three for São Paulo State: *Achlya crenulata* Ziegler, *Brevilegnia longicaulis* TW Johnson and *Saprolegnia truncata* RL Seym. All species are described, commented and illustrated herein. The specimens were deposited in the culture collection of the "Instituto de Botânica (CCIBt)" and/or in the Herbarium SP. The ITS rDNA sequences obtained were deposited in the GenBank.

***Achlya* / *Aplanopsis* / *Araiospora* / *Brevilegnia* / ITS rDNA/ phyogeny / *Saprolegnia* / taxonomy**

INTRODUCTION

Oomycota is a group of heterotrophic zoosporic organisms, which are phylogenetically distant from the true fungi and currently classified into the Kingdom Straminipila. They are commonly found in terrestrial and aquatic ecosystems as saprobes on animal and vegetable debris and also as parasites of seaweeds, invertebrates, fishes, fungi, plants and even humans, as in the case of *Pythium insidiosum* de Cock and *P. aphanidermatum* (Edson) Fitzp. (Calvano *et al*. 2011; Beakes *et al*. 2014).

Nine hundred and fifty six species of Oomycota are known worldwide (Kirk *et al*. 2008), but only 196 have been recorded in Brazil up to date (Maia & Carvalho 2015). This number is low as compared to the great diversity of Brazilian biomes and the total surface of the country. In Brazil, the Atlantic Rainforest is the most studied biome with 47% of the total number of recorded species (Beneke & Rogers 1962; Rogers *et al*. 1970; Schoenlein-Crusius *et al*. 1990, 1992; Pires-

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Zottarelli *et al*. 1995, 1996, 2007; Rocha & Pires-Zottarelli 2002; Gomes & Pires-Zottarelli 2006, 2008; Pires-Zottarelli & Rocha 2007; Miranda & Pires-Zottarelli 2012; Jesus *et al*. 2013; Marano *et al*. 2014).

In the last few years, phylogenetic studies based on molecular data have contributed to a great extent in clarifying both the evolutionary and the taxonomic relationships within the Oomycota (Dick *et al*. 1999; Riethmüller *et al*. 1999; Petersen & Rosendahl 2000; Hudspeth *et al*. 2000; Leclerc *et al*. 2000; Spencer *et al*. 2002; Petrisko *et al*. 2008; Robideau *et al*. 2011; Lara & Belbahri 2011; Kageyama 2014; Sandoval-Sierra *et al*. 2014; Steciow *et al*. 2014; de Cock *et al*. 2015; Sandoval-Sierra & Diéguez-Uribeondo 2015). Unfortunately, most of these studies do not combine molecular and detailed morphological data for characterization of the species. The ITS rDNA region has become a universal marker for differentiation of closely related species and recognition of new taxa (Robideau *et al*. 2011), although for Oomycota, and especially Saprolegniales, there is still a very limited number of reliable sequences available in GenBank. The inaccurate sequence assignment in databases and misidentification of strains in public culture collections (Sandoval-Sierra *et al*. 2014; Steciow *et al*. 2014) make identification challenging. Consequently, molecular characterization complemented with detailed morphological analysis of the specimens is highly recommended.

In this paper, seven taxa of Oomycota that are new records for Brazil and/or São Paulo State are described, commented and illustrated. The majority of the species were cultured and sequenced, and their ITS rDNA sequences were deposited in GenBank.

MATERIAL AND METHODS

Study area

The “Parque Estadual da Ilha do Cardoso” (PEIC), 25°03’05’-25°18’18’’S; 47°53’48’’-48°05’42’’W, is an island located in the Atlantic Rainforest Domain (Ab’Saber 1977), that is situated in Cananéia municipality, in the southern part of São Paulo State. The weather according to Köppen´s climate system is mega-thermal and super humid, with no defined dry season or excess of rainfall in summer (Funari *et al*. 1987). These conditions and its complex geographical configuration lead to the establishment of a well-developed Atlantic Rainforest with different types of vegetation and water bodies like waterfalls, streams and rivers (Barros *et al*. 1991).

Sampling and laboratory analysis

Four samplings were carried out in August/November 2012 and February/June 2013 at “Núcleo Perequê”, which is in the northeastern part of the PEIC. We collected samples from 30 locations, 15 samples are from freshwater bodies (waterfalls, rivers, streams, temporary and permanent ponds) and 15 others from soil near to the water bodies. In the laboratory, aliquots of these samples (30 mL of the water samples and 15 g of the soil samples dissolved in 30 mL of autoclaved reverse-osmosis water) containing plant and animal debris were placed in Petri dishes and

baited with *Sorghum* spp seeds, onion skin, corn leaves and snakeskin (Milanez 1989). The plates were incubated at room temperature (20-22ºC) and the baits were examined after 5d of incubation in order to isolate the Oomycota. Isolation and purification were made onto solid MP5 media (4 g maltose, 1 g peptone, 15 g agar and 1000 mL reverse-osmosis water). Taxonomic placement of the species followed Beakes *et al*. (2014) and morphological identification was according to Sparrow (1960), Johnson *et al*. (2002, 2005a, b), Sandoval-Sierra *et al*. (2014) and original descriptions. Species were deposited at the CCIBt culture collection (“Coleção de Culturas de Algas, Cianobactérias e Fungos do Instituto de Botânica”) or at the Herbarium SP if the isolation in pure culture was not possible.

DNA extraction, PCR amplification and sequencing

Molecular analyses were performed for all the specimens able to grow in pure culture. The mycelia for DNA extraction were obtained by cultivating each isolate in three falcon tubes containing 20 mL of liquid MP5. After incubation for 3-5d at 21ºC, the mycelia were centrifuged at 13000 rpm for 15 min in order to obtain mycelial pellets. The mycelial pellets of the three replicates were aseptically combined in order to obtain enough biomass for DNA extraction. The supernatants were discarded and 1 mL of sterile reverse-osmosis water was added to the tubes followed by vortexing at 2500 rpm. The tubes were again centrifuged and the supernatant discarded. Pellets were treated according to the protocol described in the PureLink Genomic DNA kit (Invitrogen®). The ITS1-5.8S-ITS2 rDNA region was amplified using the primers ITS4/ITS6 (Cooke *et al*. 2000) or UN-up18S42 and UN-1o28S22 (Robideau *et al*. 2011). To aid identification of the isolate CCIBt 4037, the SSU rDNA region was amplified using the primers SR1-R and NS4 (White *et al*. 1990). DNA was amplified with the PCR Super Mix kit (Invitrogen®) for a final volume of 25 µL in a C1000 Touch™ Thermal Cycler Bio-Rad following the conditions described in Marano *et al*. (2014). Amplicons were purified with AxyPrep PCR Clean-up kit (Axygen®) and sequencing was performed in an ABI 3730 DNA Analyser (Life Technologies™). Assembly of contigs and correction of ambiguous bases were manually performed in Sequencher™ Version 4.1.4. The ITS and SSU rDNA sequences were compared with similar sequences published in GenBank using the BLASTn.

Phylogenetic analyses

For phylogenetic reconstruction, the ITS rDNA sequences of the isolates this study were compared with published sequences of isolates available in GenBank that presented high molecular similarity, using *Aphanomyces stellatus* de Bary as outgroup. Sequences were aligned using MAFFT version 7 (Kazutaka & Daron 2013), and the ambiguously aligned characters removed manually using the program Geneious version 8. The best fitting model of evolution was selected using the Akaike Information Criterion in jModeltest 0.1.1 (Posada 2008). The Maximum Likelihood (ML) phylogenies were reconstructed with PhyML 3.1 (Guindon & Gascuel 2003) using the best model for nucleotide substitution, branch swapping by best of NNI and SPR, and support for nodes obtained by 1,000 bootstrap pseudo-replicates. Bayesian inferences (BI) were generated using MrBayes v. 3.2.1 (Ronquist & Huelsenbeck 2003) with Markov Chain Monte Carlo (MCMC) methodology to calculate posterior probabilities of the phylogenetic trees. The program was run for

5 million generations with the best fitting model of evolution was then selected using the Akaike Information Criterion in jModelTest. The first 10% of the iterations were discarded as burn-in.

RESULTS AND DISCUSSION

One hundred and twenty samples were analyzed. Among a total of 24 identified species, four of them are cited for the first time for Brazil: *Araiospora streptandra* var. *streptandra* Kevorkian, *Achlya primoachlya* (Coker & Couch) TW Johnson & RL Seym., *Aplanopsis terrestris* Höhnk and *Saprolegnia aenigmatica* Sandoval-Sierra & Diéguez-Uribeondo and three for São Paulo State: *Achlya crenulata* Ziegler, *Brevilegnia longicaulis* TW Johnson and *Saprolegnia truncata* RL Seym. Six of the species were isolated in pure culture and their ITS rDNA region sequenced, with *Brevilegnia longicaulis* and *Saprolegnia truncata* referenced for the first time in GenBank. Isolation of *Araiospora streptandra* var. *streptandra* into pure culture was not possible because contamination of cultures occurred at all attempts. The BLASTn results of the ITS region for the species which have sequences available in GenBank have complemented the morphological characterization and helped identification. In the case of the recently described species *Saprolegnia aenigmatica* (Sandoval-Sierra & Diéguez Uribeondo 2015), the ITS BLASTn results were determinant for identifying this species. Similarly, the SSU BLASTn results for the sequence KT336499 have corroborated our identification of *Aplanopsis terrestris*.

RHIPIDIALES

Araiospora streptandra var. *streptandra* Kevorkian, *Mycologia* 26:151. 1934 Fig. 1

Basal cell cylindrical, branched. Sporangia pedicellate; wall either smooth or with spines; when smooth oval, pyriform or fusiform, 30.0-120.0 × 12.0-60.0 in size, and when spiny spherical, 37.0-66.0 µm diam.; spines 11.0-23.0 µm long., some apical spines up to 40.0 µm. Oogonia spherical, 43.0-50.0 µm diam. Antheridia borne singly, irregular, coiling around the oogonial stalk, one per oogonium. Oospore single, spherical, surrounded by a layer of hexagonal peripheral cells, 34.0-45.0 µm diam.; germination not observed.

Material examined: BRAZIL. São Paulo: Cananéia, Parque Estadual da Ilha do Cardoso (PEIC), Núcleo Perequê, from freshwater samples, on submerged twigs, 05/XI/2012, SCO Rocha & CLA Pires-Zottarelli s.n. (SP 445754; GenBank accession number: not avaliable).

Commentary: The characteristics of our specimen are in agreement with the original description (Kevorkian 1934). Sparrow (1960) described oval and pyriform sporangia with longer spines (15.0-30.0 µm) and smaller oogonia (52.0-68.0 µm).

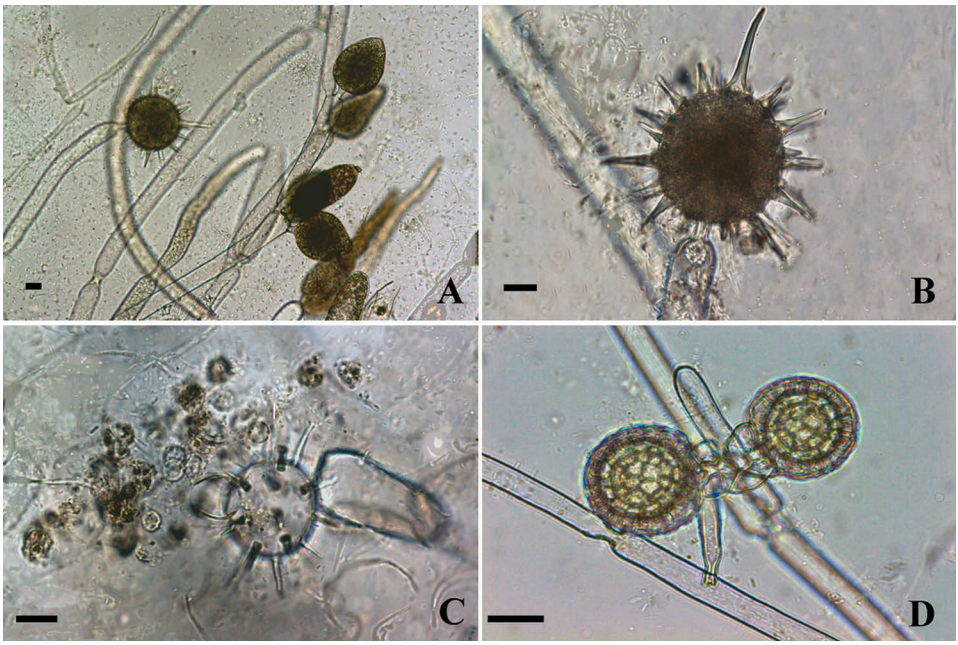

Fig. 1. *Araiospora streptandra* var. *streptandra*. **A.** General aspect of the colony and sporangia. **B.** Detail of the spines of different sizes in a spherical and terminal sporangium. **C.** Zoospore release. **D.** Detail of the oogonia with antheridia. Bar = 20 µm.

SAPROLEGNIALES

ACHLYACEAE

Achlya crenulata Ziegler, *Mycologia* 40:336. 1948 Fig. 2

Monoecious. Mycelium well-developed. Sporangia terminal, fusiform, sometimes naviculate, 100.0-170.0 × 20.0-30.0 µm; renewed sympodially. Discharge and behavior of the zoospores achlyoid; zoospore cysts 10.0-12.5 µm diam. Gemmae present. Oogonia lateral or terminal, spherical, 67.5-105.0 µm diam., including projections, if any, sometimes irregular; papillate and crenulate ornamentations; oogonial stalks 2-6 times the diam. of the oogonium. Oospheres usually not maturing. Antheridia diclinous; anteridial branches simple; antheridial cells simple, laterally appressed, sometimes in a digitate fashion; more than one per oogonium; fertilization tubes not observed. Oospores eccentric, 25.0-30.0 µm diam., 1-7 per oogonium.

Material examined: BRAZIL. São Paulo: Cananéia, “Parque Estadual da Ilha do Cardoso” (PEIC), “Núcleo Perequê”, from freshwater samples, on *Sorghum* spp. seeds, 28/XI/2012, SCO Rocha & CLA Pires-Zottarelli s.n. (CCIBt 3997; GenBank accession number: KP006457).

Commentary: The morphological characteristics of the examined specimen are in agreement with the original description (Ziegler 1948). This species was

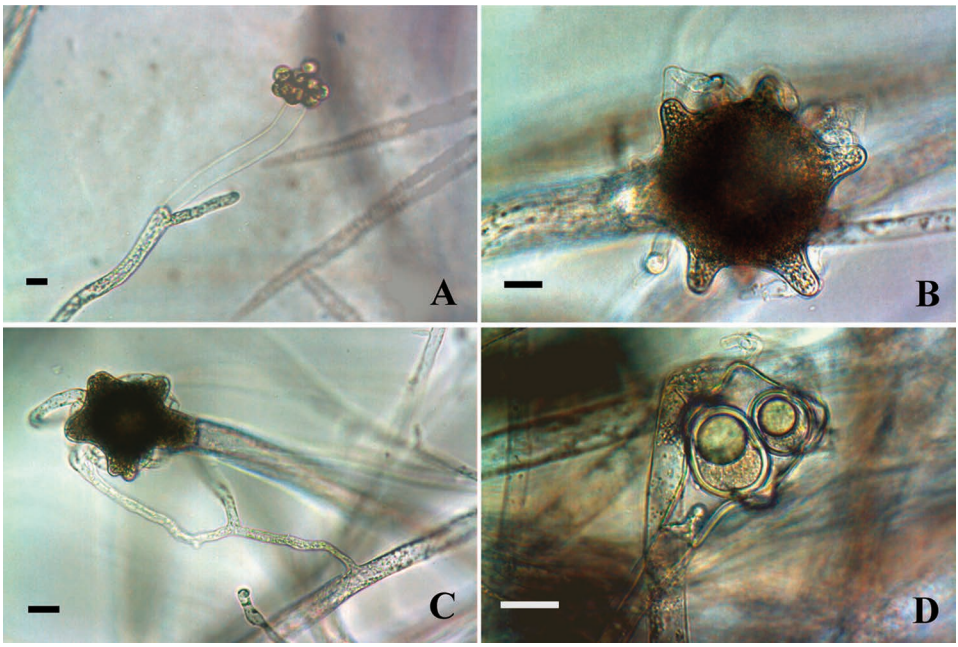

Fig. 2. *Achlya crenulata.* **A.** Achlyoid type of zoospore discharge from primary sporangia. **B.** Detail of the oogonia with papillate ornamentations. **C.** Oogonia with diclinous antheridia. **D.** Oogonia with eccentric oospores. Bar = 20 µm.

previously recovered in northeastern Brazil (Milanez *et al*. 2007). The sequence of our isolate (KP006457) clustered together with a single sequence of this species (AF218157) available in GenBank (Fig. 3).

Achlya primoachlya (Coker & Couch) TW Johnson & RL Seym., *Mycotaxon* 92:20. 2005
≡*Thraustotheca primoachlya* Coker & Couch, *Journal Elisha Mitchell Scientific Society* 40:198. 1924 Fig. 4

Monoecious. Mycelium well-developed. Sporangia terminal, fusiform, sometimes naviculate, 130.0-490.0 × 20.0-40.0 µm; renewed sympodially. Discharge and behavior of the zoospores from primary sporangia achlyoid; thraustothecoid and dictyuchoid from the secondary ones; zoospore cysts 10.0-12.0 µm diam. Gemmae absent. Oogonia lateral, spherical, 52.5-110.0 µm diam., including wall ornamentations; truncate, papillate and tuberculate ornamentations. Oogonial stalks ½-4 times the diam. of the oogonium, sometimes twisted. Oospheres generally maturing. Antheridia diclinous and monoclinous, not persisting, branched; antheridial cell simple, laterally and apically appressed; fertilization tubes not observed. Oospores eccentric, spherical, 15.0-22.5 µm diam., 1-13 (-20) per oogonium.

Material examined: BRAZIL. São Paulo: Cananéia, "Parque Estadual da Ilha do Cardoso" (PEIC), "Núcleo Perequê", from freshwater samples, on *Sorghum* spp seeds, 20/VIII/2012, SCO Rocha & CLA Pires-Zottarelli s.n. (CCIBt 3982; GenBank accession number: KM058754).

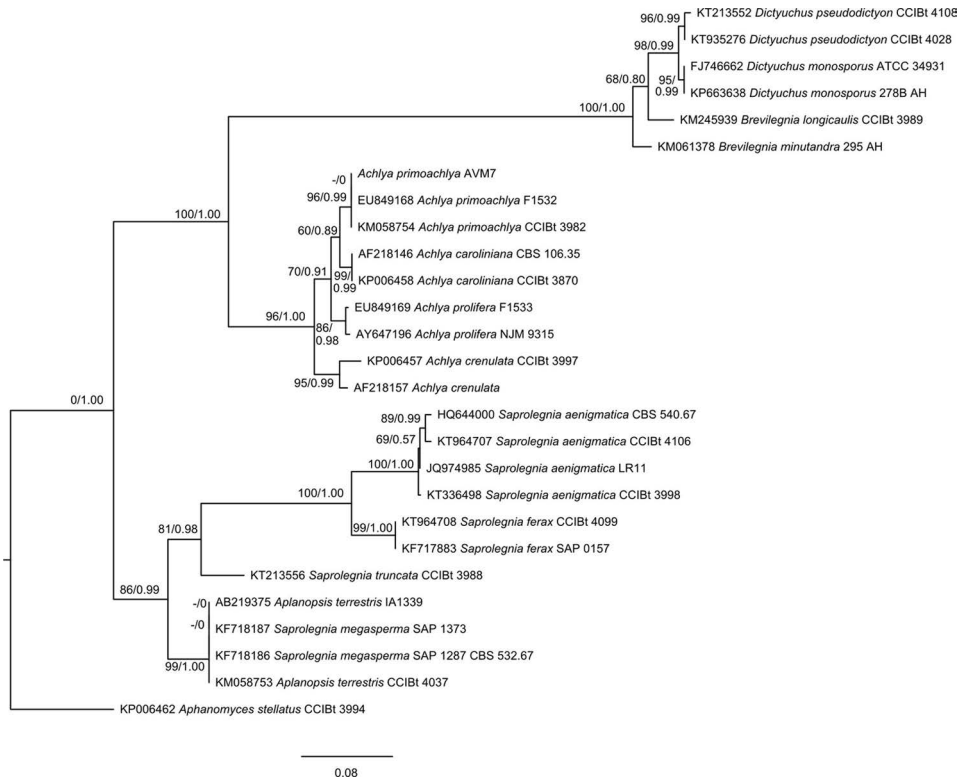

Fig. 3. Maximum likelihood tree of internal transcribed spacer (ITS) rDNA sequences of Saprolegniales. Maximum likelihood bootstrap support and posterior probability values large than 50% are indicated numerically, those under 50% are marked with (-). Those clades that were not present in the Bayesian trees are marked as (0). The scale bar represent the average number of substitutions per site.

Commentary: The sporangia are higher than those cited by Johnson *et al.* (2005b) which mentioned zoosporangia of 90.0-188.0 × 12.0-39.0 µm. The presence of irregular sporangia and cymose renewal were not observed in our specimen. The oogonia are also slightly larger than those cited by these authors (28.0-84.0 µm diam.). The dictyuchoid type of discharge from secondary sporangia and the presence of diclinous antheridia have not been previously referred for this species. It was first described by Coker & Couch (1924) as *Thraustotheca primoachlya* due to the presence of thraustothecoid type of zoospore discharge, however, Johnson *et al.* (2005b) observed that discharge from primary sporangia was achlyoid and transferred this species to *Achlya*. The result of BLASTn confirmed the morphological identification of this species. The ITS phylogeny (Fig. 3) showed that the sequence of our isolate (KM058754) clustered together with the single sequence of this species available in GenBank (EU849168) and one unpublished sequence from Argentina (AVM7).

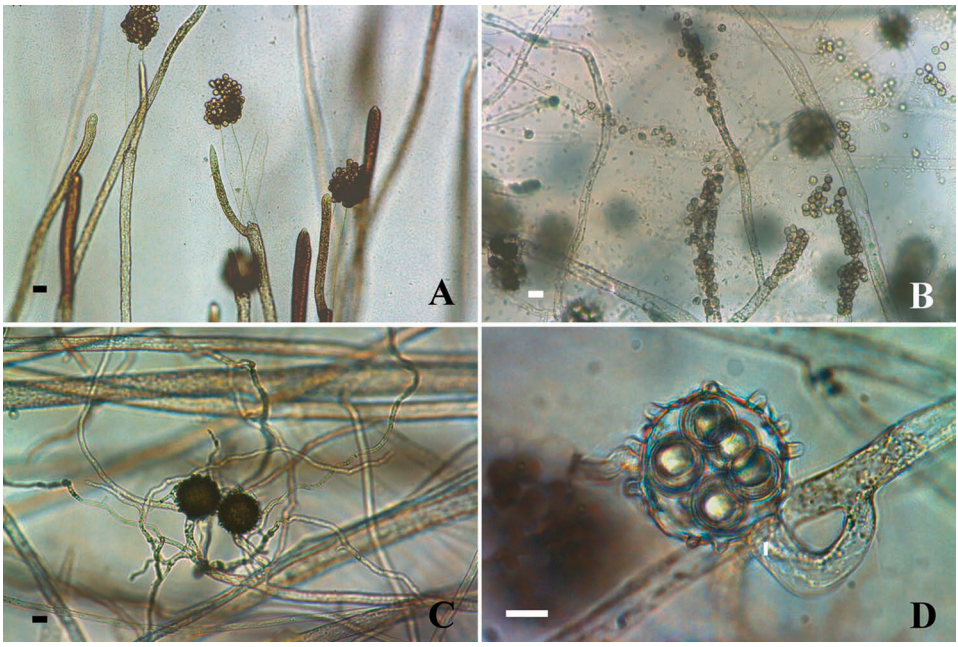

Fig. 4. *Achlya primoachlya.* **A.** Achlyoid type of zoospore discharge from primary sporangia. **B.** Thraustothecoid and dictyuchoid type of discharge from secondary sporangia. **C.** Oogonia with antheridial branches. **D.** Lateral oogonium with truncate and papillate ornamentations and eccentric oospores. Bar = 20 µm.

Brevilegnia longicaulis TW Johnson, *Mycologia* 42:244. 1950 Fig. 5

Monoecious. Mycelium well-developed. Sporangia terminal, cylindrical, 117.5-250.0 (-390.0) × 17.5-35.0 µm. Discharge and behavior achlyoid from primary sporangia, brevilegnoid from the secondary ones, rarely dictyucoid, occasionally germinating *in situ* (aplanoid); zoospore cysts 10.0-12.5 µm diam. Gemmae absent. Oogonia lateral, sometimes terminal, rarely intercalary, spherical, 27.5-35.0 µm diam., wall unpitted, smooth; oogonial stalks 1-6 times the diam. of the oogonium. Oospheres maturing. Antheridia diclinous, usually branched, long; frequently several antheridia attaching to the same oogonium; antheridial cells simple, laterally attached, rarely in a digitate fashion; fertilization tubes not observed. Oospore single, eccentric, spherical, 22.5-27.5 µm diam.

Material examined: BRAZIL. São Paulo: Cananéia, "Parque Estadual da Ilha do Cardoso" (PEIC), "Núcleo Perequê", from soil samples, on snakeskin, 20/VIII/2012, SCO Rocha & CLA Pires-Zottarelli s.n. (CCIBt 3989; GenBank accession number: KM245939).

Commentary: This species was recently recorded in northeastern Brazil (Rocha & Macêdo 2015). Most of the morphological characteristics of our specimen are in agreement with Johnson (1950), although we also observed achlyoid type of zoospore discharge. This is the first time that this species is sequenced. The ITS phylogeny (Fig. 3) placed our isolate (KM245939) together with sequences of other species of *Brevilegnia* and *Dictyuchus* obtained from GenBank.

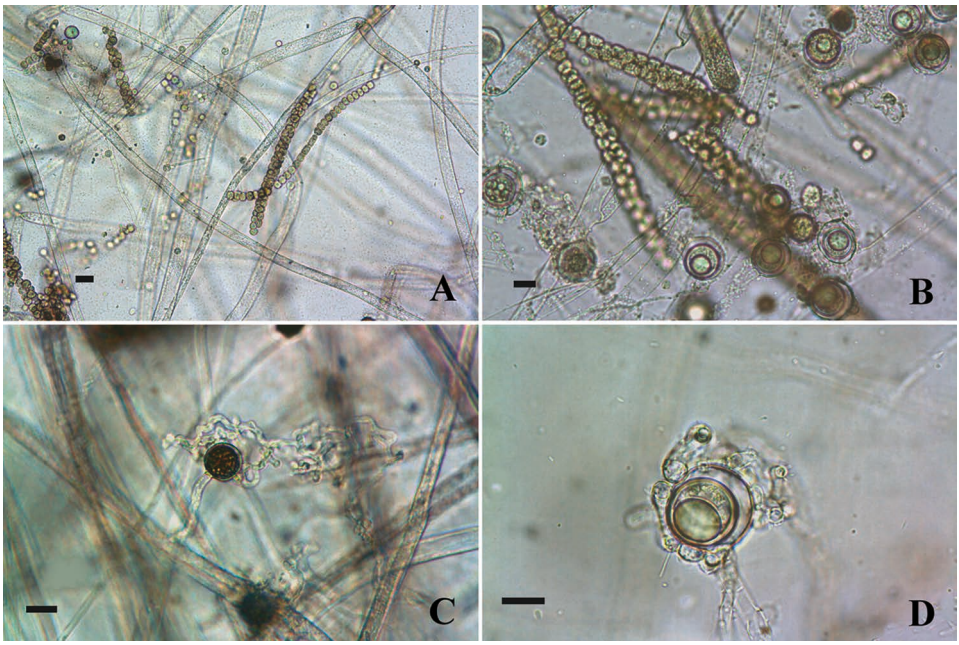

Fig. 5. *Brevilegnia longicaulis*. **A.** Types of zoospore discharge exhibited. **B.** General aspect of the colony with oogonia and sporangia showing brevilegnoid type of zoospore dicharge. **C.** Oogonia with diclinous antheridia. **D.** Oogonium with one eccentric oospore. Bar = 20 µm.

SAPROLEGNIACEAE

Aplanopsis terrestris Höhnk, *Veröff Inst Meeresforsch Bremerhav Sonderband* 1:127. 1952 Fig. 6

Monoecious. Mycelium well-developed. Hyphae 7.5-17.5 µm diam., thick. Sporangia not observed. Gemmae absent. Oogonia predominantly lateral, occasionally terminal, sometimes intercalary, spherical, 15.0-30.0 µm diam., some of them obovate or oval, 25.0-35.0 × 17.5-25.0 µm; wall generally smooth; sometimes with irregular ornamentations or with 1-2 long projections; oogonial stalks branched and irregular, 1-9 times the diam. of the oogonium. Oosphere generally maturing. Antheridia androgynous or rarely monoclinous, usually branched; antheridial cells simple, apically or laterally apressed; fertilization tubes not observed. Oospore single, maturing, centric and subcentric, spherical, 12.5-25.0 µm diam.

Material examined: BRAZIL. São Paulo: Cananéia, “Parque Estadual da Ilha do Cardoso” (PEIC), “Núcleo Perequê”, from soil samples, on *Sorghum* spp. seeds, 20/II/2012, SCO Rocha & CLA Pires-Zottarelli s.n. (CCIBt 4037; GenBank accession numbers: ITS = KM058753; SSU = KT336499).

Commentary: The morphological characteristics of the specimen examined are according to the original description (Höhnk 1952). ITS and SSU rDNA sequences confirmed the morphological identification according to BLASTn results. Our ITS phylogenetic analysis (Fig. 3) shows that our sequence (KM058753) have

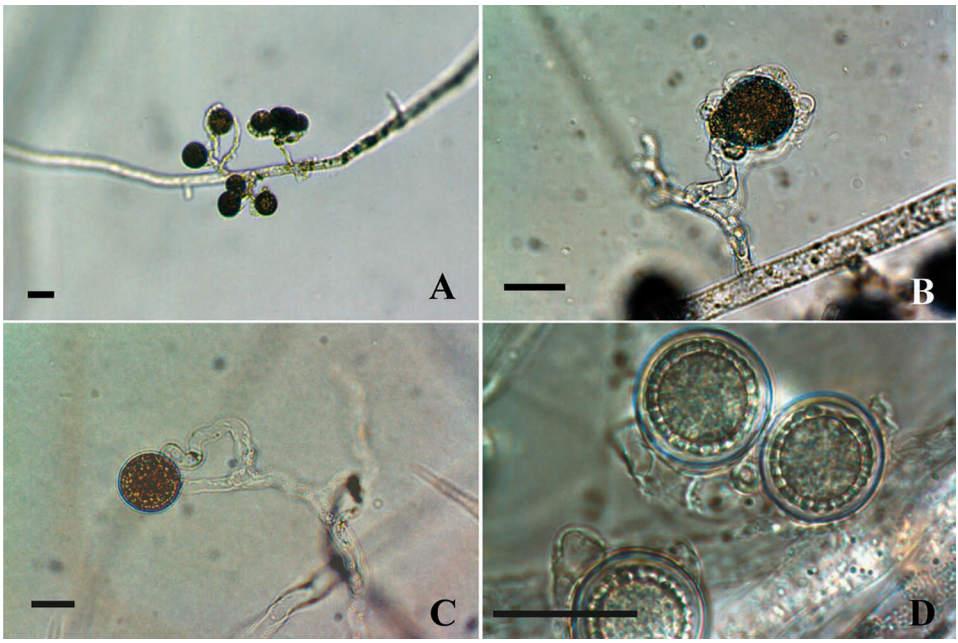

Fig. 6. *Aplanopsis terrestris.* **A.** Branched oogonial stalks. **B.** Lateral oogonium with antheridium. **C.** Androgynous antheridium. **D.** Oogonia with centric oospores. Bar = 20 µm.

formed a clade with both sequences of *Saprolegnia megasperma* (KF718187 and KF718186) and *Aplanopsis terrestris* (AB219375) from GenBank. Sandoval-Sierra *et al*. (2014) suggested that the ITS sequence deposited in GenBank as *A. terrestris* (AB219375) was misidentified and corresponds to *S. megasperma*. Our ITS phylogeny clearly showed that *S. megasperma* and *A. terrestris* sequences are placed in the same clade. Therefore, additional studies should be conducted in order to revise the voucher cultures of these species and to understand their relationship.

Saprolegnia aenigmatica Sandoval-Sierra & Diéguez-Uribeondo, *PLoS ONE* 10:7. 2015 Fig. 7

Monoecious. Mycelium well-developed. Sporangia terminal, clavate, sometimes fusiform, 80-250 × 17.5-37.5 µm; renewal by internal proliferation. Discharge and behavior saprolegnoid; zoospores encysted 10.0-12.5 µm diam.. Gemmae present. Oogonia lateral, sometimes terminal and intercalary, obpyriform, 50-125 × 45-80 µm, some of them spherical, 47.5-80.0 µm diam.; wall smooth, unpitted; oogonial stalks ½ -5 times the diam. of the oogonium. Oospheres maturing. Antheridia diclinous, branched, often coiling around the oogonia and stalks; antheridial cells simple, laterally apressed or in a digitate fashion; fertilization tubes not observed. Oospores subcentric, sometimes centric, 20.0-27.5 µm in diam., 5-20 per oogonium.

Material examined: BRAZIL. São Paulo: Cananéia, "Parque Estadual da Ilha do Cardoso" (PEIC), "Núcleo Perequê", from freshwater samples, on *Sorghum* spp. seeds, 20/VIII/2012 SCO Rocha & CLA Pires-Zottarelli s.n. (CCIBt 3998, CCIBt 4106; GenBank accession number: KT336498, KT964707).

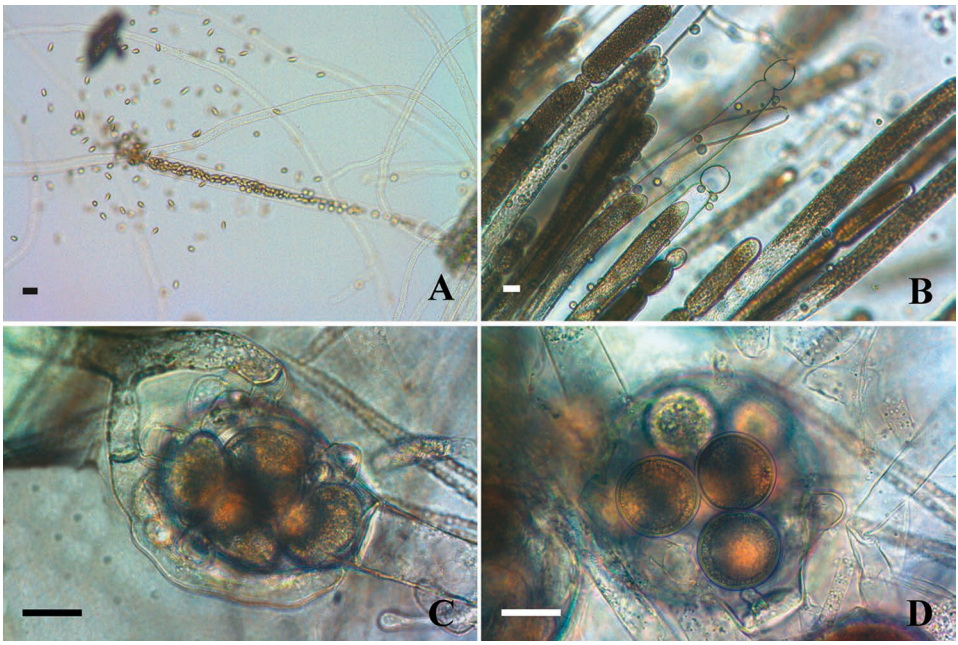

Fig. 7. *Saprolegnia aenigmatica*. **A.** Zoospore discharge saprolegnioid. **B.** Sporangia with renewal by internal proliferation. **C.** Diclinous antheridial branches. **D.** Oogonia with subcentric oospores. Bar = 20 µm.

Commentary: The ITS BLASTn showed that our sequences (KT336498 and KT964707) correspond to *Saprolegnia aenigmatica*, recently described by Sandoval-Sierra & Diéguez-Uribeondo (2015). Most of the characteristics are in agreement with the original description, except for the presence of subcentric oospores in our specimens. The identity of the two specimens obtained is certified by phylogenetic analysis of the ITS region, where the sequences are clustered together with those present in GenBank (Fig. 3).

Saprolegnia truncata RL Seym., *Mycotaxon* 92:9. 2005 Fig. 8

Monoecious. Mycelium well-developed. Sporangia terminal, clavate, 130.0-240.0 × 12.5-17.5 µm; renewal by internal proliferation. Discharge and behavior saprolegnoid; zoospores encysted 7.5-10.0 µm diam. Gemmae absent. Oogonia lateral, sometimes terminal, spherical, 37.5-70.0 µm diam., including papillae, if any; oogonial wall unpitted, with papillae and truncate projections, 7.5-12.5 × 10-22.5 µm; oogonial stalks ½-6 times the diam. of oogonium. Oospheres maturing. Antheridia androgynous; antheridial branches simple; antheridial cell simple, laterally and apically apressed; fertilization tube not observed. Oospores subcentric, 25.0-45.0 µm diam., 1-4 per oogonium.

Material examined: BRAZIL. São Paulo: Cananéia, Parque Estadual da Ilha do Cardoso (PEIC), Núcleo Perequê, from soil samples, on *Sorghum* spp. seeds, 20/VIII/2012, SCO Rocha & CLA Pires-Zottarelli s.n. (CCIBt 3988; GenBank accession number: KT213556).

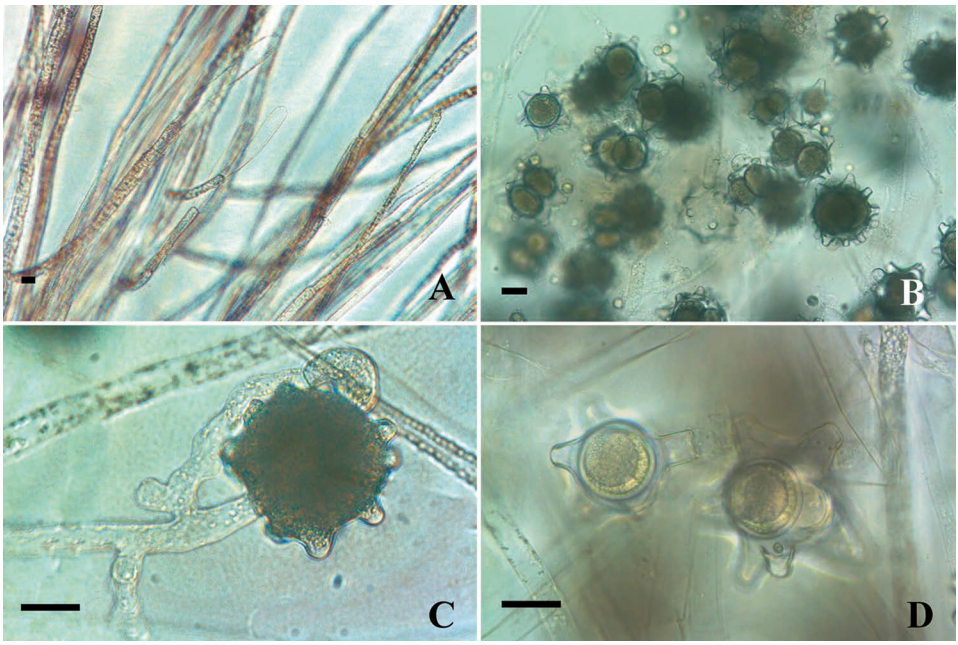

Fig. 8. *Saprolegnia truncata*. **A.** Sporangia terminal. **B.** General aspect of the mycelium with oogonia. **C.** Oogonium with papillae projections, and androgynous antheridium. **D.** Oogonia with truncate ornamentations and subcentric oospores. Bar = 20 µm.

Commentary: The morphological characteristics of the examined specimen conform to the original description (Johnson *et al*. 2005a). This species was originally isolated from roadside forest soil in Manaus municipality, Amazonas State, Brazil, in 1978. This is the second record of the species in the world. This species is included for the first time in a phylogenetic analysis (Fig. 3).

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