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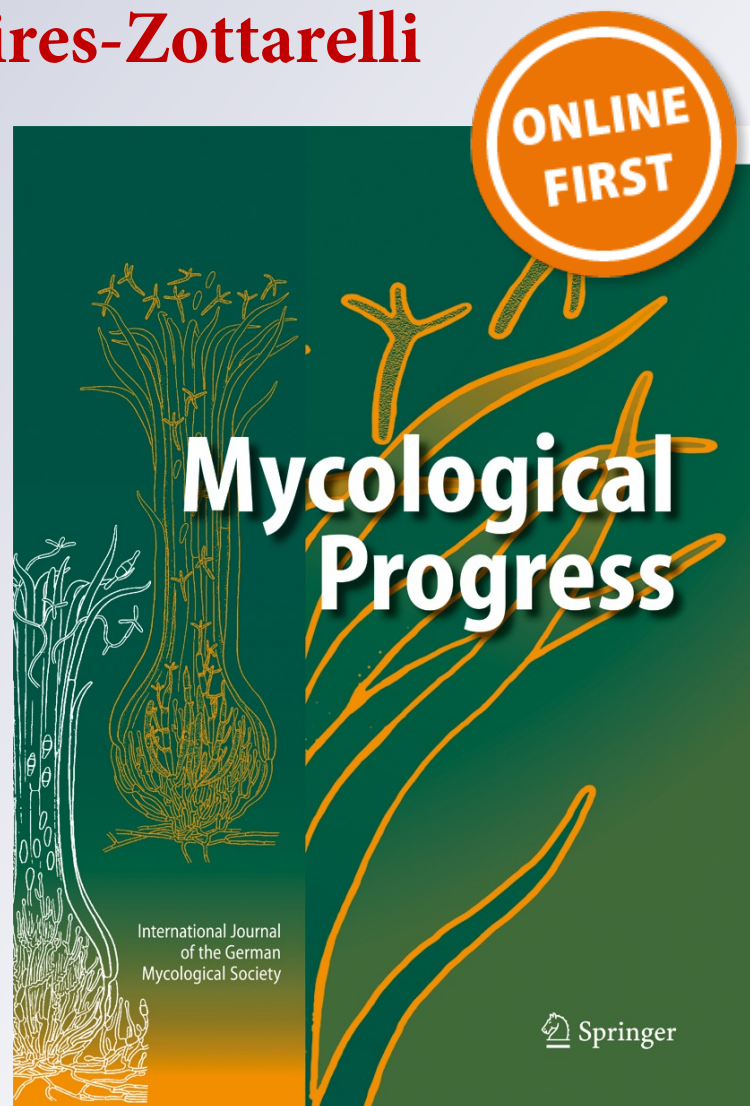
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Souza, Manuel E. Rueda-Páramo &  
Carmen L. A. Pires-Zottarelli**

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# New phylogenetic insights into *Saprolegniales* (Oomycota, Straminipila) based upon studies of specimens isolated from Brazil and Argentina

Sarah C. O. Rocha<sup>1</sup>  · Claudia C. Lopez-Lastra<sup>2</sup> · Agostina V. Marano<sup>1</sup> · José I. de Souza<sup>1</sup> · Manuel E. Rueda-Páramo<sup>2</sup> · Carmen L. A. Pires-Zottarelli<sup>1</sup>

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## Abstract

*Saprolegniales* is a complex and monophyletic order of oomycetes. Their members inhabit terrestrial, freshwater, and marine ecosystems and have a worldwide distribution. In these ecosystems, they are found as saprobes, parasites, or even pathogens of animals and plants of economic importance. In this study, a concatenate phylogeny of the partial LSU and complete ITS rDNA regions is presented, including isolates from Brazil and Argentina, which were sequenced after a detailed morphological analysis. Among the sequenced species, *Achlya orion*, *Leptolegnia eccentrica*, *Phragmosporangium uniseriatum*, and *Pythiopsis irregularis* are included for the first time in a phylogeny. Our results are in agreement with the recent informal proposals outlined in taxonomic overviews of the Oomycota of G.W. Beakes and collaborators, who placed the family *Verrucalvaceae* into the *Saprolegniales* and introduced the family *Achlyaceae* to group *Achlya s.s.*, *Brevilegnia*, *Dictyuchus*, and *Thraustotheca*. These results also support the transference of *Achlya androgyna* to *Newbya*. *Leptolegnia* appears as paraphyletic, with the separation of *L. eccentrica* from the other species of this genus. In addition, *Phragmosporangium*, which is herein sequenced for the first time, clustered as sister to some species of *Aphanomyces*, including the type species, *A. stellatus*.

**Keywords** *Achlyaceae* · Morphology · Phylogeny · *Saprolegniaceae* · *Verrucalvaceae*

## Introduction

*Saprolegniales* is a monophyletic order within the phylum *Oomycota* (*Straminipila*), which groups biflagellate heterotrophic organisms that have eucarpic, mycelial and coenocytic thalli of unlimited growth and produce asexual (sporangia) and sexual (gametangia) structures delimited by septa (Beakes and Sekimoto 2009). These organisms are usually found as saprobes in terrestrial and aquatic environments

(Beakes et al. 2014a). Some genera, such as *Achlya*, *Aphanomyces*, and *Saprolegnia* are important pathogens that infect a wide range of animal and plant hosts (Phillips et al. 2008, Diéguez-Uribeondo et al. 2009, Marano et al. 2017). As examples, *Aphanomyces euteiches* is a known pathogen of legumes (Gaulin et al. 2007, Madoui et al. 2009), and *Aphanomyces astaci* is responsible for the crayfish plague (Unestan 1965, Söderhäll and Cerenius 1999, Diéguez-Uribeondo et al. 2006, Kozubíková et al. 2009, Strand et al. 2012, Rezinciuc et al. 2016). *Saprolegnia* is one of the best-known pathogens of fish and their eggs (Noga 2010, Kiziewicz et al. 2013, Van Den Berg et al. 2013, Rezinciuc et al. 2014, Sandoval-Sierra et al. 2014a, Sarowar et al. 2014, Mastan 2015) and, together with *Achlya*, are reported as parasites in amphibians (Fernández-Benítez et al. 2007, Romansic et al. 2009, Ault et al. 2012, Gleason et al. 2014, Urban et al. 2015).

Although the number of studies reporting parasitic species of *Saprolegniales* is extensive, taxonomical studies of this group have traditionally been neglected (Riethmüller et al. 1999, Leclerc et al. 2000, Johnson Jr et al. 2002, 2005a, b,

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José Ivanildo de Souza is deceased.

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✉ Sarah C. O. Rocha  
sahtininha@gmail.com

<sup>1</sup> Instituto de Botânica, Núcleo de Pesquisa em Micologia, Av. Miguel Estéfano 3687, CEP, São Paulo, SP 04301-012, Brazil

<sup>2</sup> Centro de Estudios Parasitológicos y de Vectores, La Plata, Buenos Aires, Argentina

Hulvey et al. 2007, Steciow et al. 2014, Sandoval-Sierra and Diéguez-Uribeondo 2015, Rocha et al. 2016a, b), probably due the small number of specialists worldwide. Most studies hitherto have been based on morphological data, considering, e.g., the type of zoospore release from the zoosporangium and the characteristics of the oogonia and oospores (Johnson Jr et al. 2002, 2005a, b). Important reorganizations in the taxonomy of *Saprolegniales* have been proposed since the inclusion of molecular data from the SSU (small subunit) rDNA region (Dick et al. 1999, Spencer et al. 2002), which resulted in changes to the traditional classification. The most recently proposed genus in *Saprolegniaceae*, *Newbya*, was created by Spencer et al. (2002) as the result of a joint analysis of morphological (type of oospores: centric and/or subcentric) and molecular data from the SSU rDNA region of some species of *Achlya s.l.* The recent taxonomical revision by Beakes et al. (2014a) was based on the analysis of morphological and molecular data (LSU rDNA region), and resulted in the informal reorganization of the *Saprolegniales* into the families *Saprolegniaceae*, *Verrucalvaceae*, and a new family, *Achlyaceae*, for accommodating species of *Achlya* with eccentric oospores (*Achlya s.s.*), *Brevilegnia*, *Dictyuchus* and *Thraustotheca*. The genera *Aphanomyces* and *Plectospora* were transferred to *Verrucalvaceae*, a family that is currently composed of these two genera plus *Aquastella*, *Pachymetra* (this last genus still requires molecular analyses for confirmation), *Sommerstorffia* and *Verrucalvus* (Beakes and Thines 2017). All other related genera of this order were maintained in the *Saprolegniaceae*, including *Leptolegnia*, previously considered by Dick et al. (1999) as a member of the *Leptolegniaceae*. The placement of *Phragmosporangium* in one of these families is hitherto uncertain since it was described by Johnson Jr et al. (2005b) as a member of the *Saprolegniaceae* based solely on morphology, and Beakes et al. (2014a) and Beakes and Thines (2017) did not consider this genus in their overviews.

Studies carried out in Brazil and Argentina have resulted in an increased knowledge of the diversity of *Saprolegniales* (Steciow et al. 2012, Maia et al. 2015), although few studies have included isolates from these countries into a phylogeny (Spencer et al. 2002, Diéguez-Uribeondo et al. 2007, Steciow et al. 2013, 2014, Sandoval-Sierra et al. 2014b, Jesus et al. 2015, Rocha et al. 2016a, b, Gonçalves et al. 2017, Jerônimo et al. 2017). Thus, the aim of this study was to reconstruct a phylogeny for *Saprolegniales* based on the LSU and ITS rDNA regions, with the inclusion of Brazilian and Argentinean specimens.

## Material and methods

### Specimens

Thirty Brazilian and Argentinean specimens were analyzed in this study, which represented 22 species and 13 genera

(Table 1). All specimens have been reactivated by cultivating them onto MP<sub>5</sub> culture medium (Beneke and Rogers 1962). Thereafter, the specimens were placed into Petri dishes containing sterile deionized water and baited with *Sorghum* spp. seeds and/or snake skin. The morphology of the specimens was observed under the microscope, in order to confirm their previous taxonomical identities.

### DNA extraction, PCR amplification and sequencing

For DNA extraction, mycelium of each isolate obtained from selected spores grown onto solid medium, was cultivated into three Falcon® tubes containing 20 mL of MP<sub>5</sub> liquid medium with 0.5 g L<sup>-1</sup> of streptomycin sulphate and penicillin G. After incubation for 3–5 days at 21 °C, the mycelium was harvested by centrifugation at 13000 rpm for 15 min to obtain mycelial pellets. The mycelial pellets of the three replicates were aseptically combined in order to obtain enough biomass. The supernatants were discarded and 1 mL of sterile reverse-osmosis water was added to the tubes followed by vortexing at 2.500 rpm. The tubes were again centrifuged and the supernatant discarded. Pellets were treated following the protocol described in the PureLink Genomic DNA kit (Invitrogen®). The ITS1-5.8S-ITS2 and the LSU rDNA regions were amplified using the primers UN-up18S42 and UN-1o28S22 (Robideau et al. 2011) and LR0R/LR6-O (Riethmüller et al. 2002), respectively. DNA was amplified with the PCR SuperMix kit (Invitrogen®) for a final volume of 25 µL in a C1000 Touch™ Thermal Cycler Bio-Rad following the conditions described by Marano et al. (2014). Amplicons were purified with AxyPrep PCR Clean-Up Kit (Axygen®). Sequencing was performed in an ABI 3730 DNA Analyzer (Life Technologies™). Assembly of contigs and correction of ambiguous bases were manually performed in Sequencher™ (version 4.1.4).

### Phylogenetic analyses

Phylogenetic analyses were performed using a concatenated dataset of partial LSU and complete ITS rDNA sequences of the specimens. Both LSU and ITS sequences were compared with published sequences deposited in GenBank (Table 1), using *Apodachlya brachynema* (GenBank accession No. JX436350, HQ643125) and *Atkinsiella dubia* (GenBank accession No. AB285221, AB285522) as outgroups. The LSU and ITS sequences were aligned separately using MAFFT version 7 (Kazutaka and Daron 2013) and the ambiguously aligned characters edited with Geneious version 8.0.5. Both sequences were concatenated with Mesquite version 3.2 and the better substitution model (GTR + G + I) was selected based on the Akaike Information Criterion (AIC) and ran with jModelTest version 2.1.5 (Darriba et al. 2012). The Maximum Likelihood (ML) phylogenies were reconstructed with PhyML 3.1 (Guindon and Gascuel 2003) using the best model for nucleotide



**Table 1** Taxa of *Saprolegniales* considered in our phylogenetic analyses. CCIBt: “Coleção de Cultura de Algas, Cianobactérias e Fungos do Instituto de Botânica”; CEP: “Centro de Estudios Parasitológicos y de Vectores”; AVM: isolates of Dr. Agostina V. Marano; ATCC: American Type Culture Collection; CBS: CBS-KNAW Fungal Biodiversity Centre. (–) Data unknown \*Taxonomic changes proposed by Spencer et al. (2002)

Taxa	Voucher/ Collection	LSU	ITS	Origin	Country
<i>Oomycota Saprolegniales</i>					
<i>Achlya androgyna</i>	CCIBt 3993	KP006452	KP006459	Water	Brazil
<i>A. caroliniana</i>	CCIBt 3870	KP006451	KP006458	Water	Brazil
	AVM 15	KX395725	KX395728	Soil	Argentina
<i>A. flagellata</i>	CCIBt 3980	KT063221	KR063222	Soil	Brazil
	CBS 52867	AF218180	AF218143	–	UK
<i>A. orion</i>	CCIBt 3984	KT964700	KT213550	Soil	Brazil
	CCIBt 4100	KT935267	KT935272	Water	Brazil
<i>A. primoachlya</i>	CCIBt 3982	KM058757	KM058754	Water	Brazil
	AVM 7	KX395726	KX580959	Soil	Argentina
<i>Aphanomyces astaci</i>	CBS 121537	JX115216	EU477365	–	–
<i>A. cladogamus</i>	CBS 10829	HQ665056	HQ643113	<i>Lycopersicon esculentum</i>	USA
	CCIBt 3854	KT935289	KT935285	Water	Brazil
<i>A. cochlioides</i>	CBS 47771	HQ665241	HQ643115	<i>Beta vulgaris</i>	–
<i>A. euteiches</i>	CBS 15473	HQ665129	HQ643119	<i>Pisum sativum</i> root	Norway
	CBS 15673	HQ665132	HQ643117	<i>Pisum sativum</i> root	Norway
<i>A. laevis</i>	CCIBt 4070	KP006453	KP006463	Water	Brazil
	CBS 127282	HQ395650	HQ111469	–	–
<i>A. salsuginosus</i>	ATCC MYA-4775	JQ070132	JQ070107	Rostrum of <i>Salangichthys microdon</i>	Japan
<i>A. sinensis</i>	ATCC MYA-4825	JQ070142	JQ070116	Carapace of <i>Pelodiscus sinensis</i>	Japan
<i>A. stellatus</i>	CCIBt 3994	KP006454	KP006462	Water	Brazil
<i>Aplanopsis terrestris</i>	CCIBt 4037	KM058753	KM058756	Soil	Brazil
<i>Brevilegnia longicaulis</i>	CCIBt 3989	KM245939	KM245940	Soil	Brazil
<i>B. minutandra</i>	CCIBt 3351	KT935270	KT935274	Soil	Brazil
<i>B. variabilis</i>	CBS 110006	HQ665058	HQ643130	–	–
<i>Dictyuchus pseudodictyon</i>	CCIBt 4028	KT935271	KT935276	Water	Brazil
	CCIBt 4108	KT964702	KT213552	Water	Brazil
<i>Leptolegnia caudata</i>	CBS 68069	KP098357	KP098340	–	Canada
<i>L. chapmanii</i>	CEP 010	KT935280	KT935277	Parasitic in <i>Aedes albifasciatus</i>	Argentina
	CEP 433	KT935281	KT935278	Parasitic in <i>Aedes aegypti</i>	Argentina
<i>L. eccentrica</i>	CCIBt 3991	KT935282	KT213553	Soil	Brazil
	CCIBt 4125	KT935283	KT935279	Soil	Brazil
<i>Leptolegnia</i> sp.	CBS 58285	HQ665275	HQ643138	–	–
<i>Newbya dichotoma</i> *	MB 808617	KP098361	KP098345	–	Argentina
<i>N. oligacantha</i> *	CBS 10144	AF218175	AF218162	Pond water	Netherlands
<i>N. pascuicola</i> *	CBS 57667	KP098359	KP098347	–	–
	CBS 57767	KP098358	KP098346	–	–
<i>N. recurva</i> *	CBS 10152	AF119580	HQ643102	Soil from bog	USA
	CBS 10152	AF218173	AF218161	Soil from bog	USA
<i>Phragmosporangium uniseriatum</i>	CCIBt4107	KT935291	KT935286	Soil	Brazil
	CCIBt 3986	KT935290	KT213554	Soil	Brazil
<i>Plectospora myriandra</i>	CCIBt 3992	KR063219	KR063220	Water	Brazil
<i>Protoachlya hypogyna</i>	CCIBt 4079	KP165040	KP006461	Hydroponic nutrient solution	Brazil
<i>P. paradoxa</i>	CBS 15845	DQ393491	DQ393553	Water	Netherlands
	ATCC 44892	DQ393493	DQ393555	Water	Netherlands
<i>Pythiopsis irregularis</i>	CCIBt 3985	KT935284	KT213555	Soil	Brazil
<i>P. terrestris</i>	CBS 110058	KP098362	KP098350	Soil	Japan
<i>Saprolegnia aenigmatica</i>	CCIBt 3998	KT964704	KT336498	Water	Brazil
	CCIBt 4106	KT964703	KT964707	Water	Brazil
<i>S. ferax</i>	CCIBt 4099	KT964705	KT964708	Water	Brazil
	CBS 30537	HQ665199	DQ393565	–	–
<i>S. subterranea</i>	CBS 113343	HQ665078	HQ644009	Nematode, pond	Ukraine
	AVM 3	KX115431	KX084706	Soil	Argentina
<i>Thraustotheca clavata</i>	CBS 55767	HQ665268	HQ644017	–	UK
	CBS 34333	HQ665213	HQ644018	–	Netherlands
	AVM 2	KX395727	KX580960	Soil	Argentina

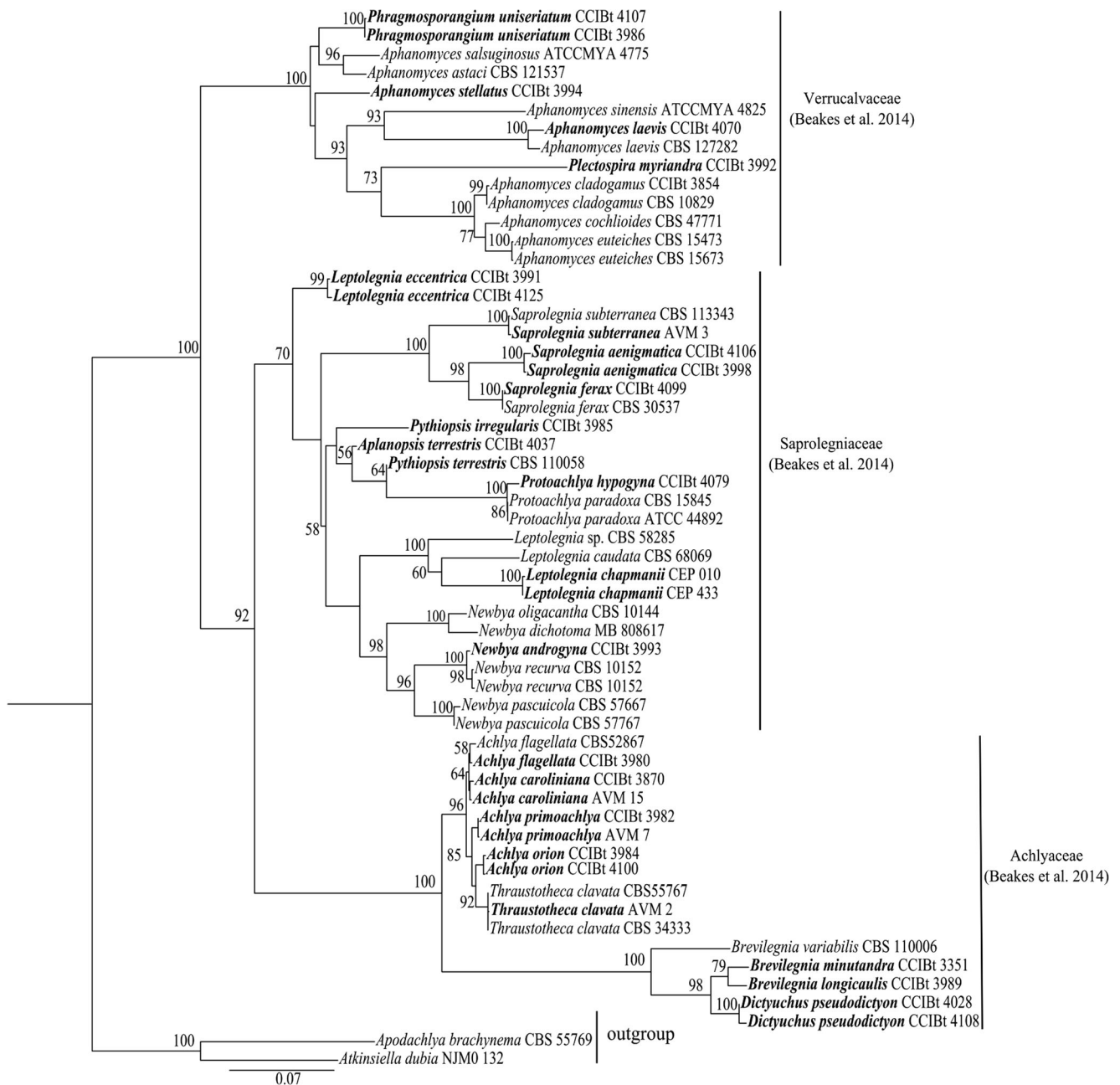
substitution, branch swapping by best of NNI and SPR, and support for nodes obtained by 1000 bootstrap pseudo-replicates.

## Results

### Phylogenetic placement of the species

Our phylogenetic analysis of the concatenated tree of members of *Saprolegniales* showed three well-supported major

clades, which correspond to the families *Saprolegniaceae*, *Achlyaceae* sensu Beakes et al. (2014a) and *Verrucalvaceae* (Fig. 1, Table 2). *Saprolegniaceae*, the largest family of this order, is herein represented by *Aplanopsis*, *Leptolegnia*, *Newbya*, *Protoachlya*, *Pythiopsis*, and *Saprolegnia*. Within the *Saprolegniaceae*, all species of *Saprolegnia* considered in this study form a well-defined clade (100% of bootstrap support). Species of *Protoachlya* form a subclade, closely related to species of *Pythiopsis* and *Aplanopsis*. Unfortunately, this subclade is not consistently supported (< 50% of



**Fig. 1** Concatenate maximum likelihood tree inferred from LSU and ITS rDNA sequences of *Saprolegniales*. Numbers next to branches indicate bootstrap support (%) and the bar shows the number of substitutions per

site. Only branches with > 50% of bootstrap support are shown. The species included in this study are indicated in bold

**Table 2** Key characteristics of the families in the order *Saprolegniales* and the genera included in each family according to molecular studies. Taxa included in this study are indicated in bold

Family	Genus
Achlyaceae: Thallus monoecious or dioecious. Hyphae stout. Asexual reproduction present; sporangia usually fusiform or filiform, terminal or intercalary; spores monomorphic or dimorphic with discharge type achlyoid, aplanoid, dictyucoid, brevilegnoid or thraustothecoid. Sexual reproduction normally present; oogonia terminal, lateral or intercalary, with one or more oospores per oogonium, eccentric. Antheridia present or absent, when present androgynous, monoclinal, declinal, hypogynous or exigynous.	<i>Achlya s.s.</i> <b><i>Brevilegnia</i></b> <b><i>Dictyuchus</i></b> <b><i>Thraustotheca</i></b>
Saprolegniaceae: Thallus monoecious. Hyphae stout or slender. Asexual reproduction present or absent, when present sporangia usually fusiform, filiform or irregular; terminal or intercalary; spores monomorphic or dimorphic with discharge type achlyoid, aplanoid, leptolegnoid or saprolegnoid or by aplanospores. Sexual reproduction present; oogonia terminal, lateral or intercalary, with one or more oospores per oogonium, normally subcentric, centric or subeccentric. Antheridia present or absent, when present androgynous, monoclinal, declinal, hypogynous or exigynous.	<i>Aplanes</i> * <b><i>Aplanopsis</i></b> <i>Calyptralegnia</i> * <i>Geolegnia</i> * <i>Isoachlya</i> * <b><i>Leptolegnia</i></b> <b><i>Newbya</i></b> <b><i>Protoachlya</i></b> <b><i>Pythiopsis</i></b> <b><i>Saprolegnia</i></b> <i>Scoliolegnia</i> * <b><i>Aphanomyces</i></b> <i>Aquastella</i> * <i>Pachymetra</i> * <b><i>Phragmosporangium</i></b> <b><i>Plectospira</i></b> <i>Sommerstorffia</i> <i>Verrucalvus</i> *
Verrucalvaceae: Thallus monoecious. Hyphae slender and delicate. Asexual reproduction present or absent, when present by undifferentiated or differentiated hypha-like sporangia (usually filamentous or lobulated); spores monomorphic or dimorphic usually with discharge type aphanomycoïd or dictyucoid; some sporangia showing internal proliferation. Sexual reproduction present; oogonia terminal, lateral or intercalary, usually with one oospore per oogonium, centric or subcentric. Antheridia present or absent, when present androgynous, monoclinal or declinal.	

\*Taxa assigned to these families according to Riethmüller et al. (1999), Steciow et al. (2013, 2014), Beakes et al. (2014a), Molloy et al. (2014) and Beakes and Thines (2017)

bootstrap support) and the relationship between these three genera needs further analysis and clarification.

*Achlya androgyna* clustered together with other centric and subcentric species of *Achlya* (98% of bootstrap support) that were previously transferred to *Newbya* (Spencer et al. 2002, Steciow et al. 2014). In addition, the morphological characteristics of this species (Fig. 2), which were carefully scrutinized in this study, also corroborate the need of transference of *A. androgyna* to *Newbya*. The species of *Leptolegnia* included in our phylogeny form a well-defined clade, except for the isolates of *L. eccentrica* (Fig. 3), which form a separate clade from the other species of this genus (Fig. 1).

The second major clade observed in our phylogeny corresponds to the family *Achlyaceae* sensu Beakes et al. (2014a), which groups all genera with eccentric oospores (Fig. 1). Our analysis shows that the sequences of *Achlya* and *Thraustotheca* have few divergences of nucleotides, although all isolates that belong to the same species clustered together and thus each species is phylogenetically well recognized. The genus *Thraustotheca* shows a close relationship with the eccentric species of

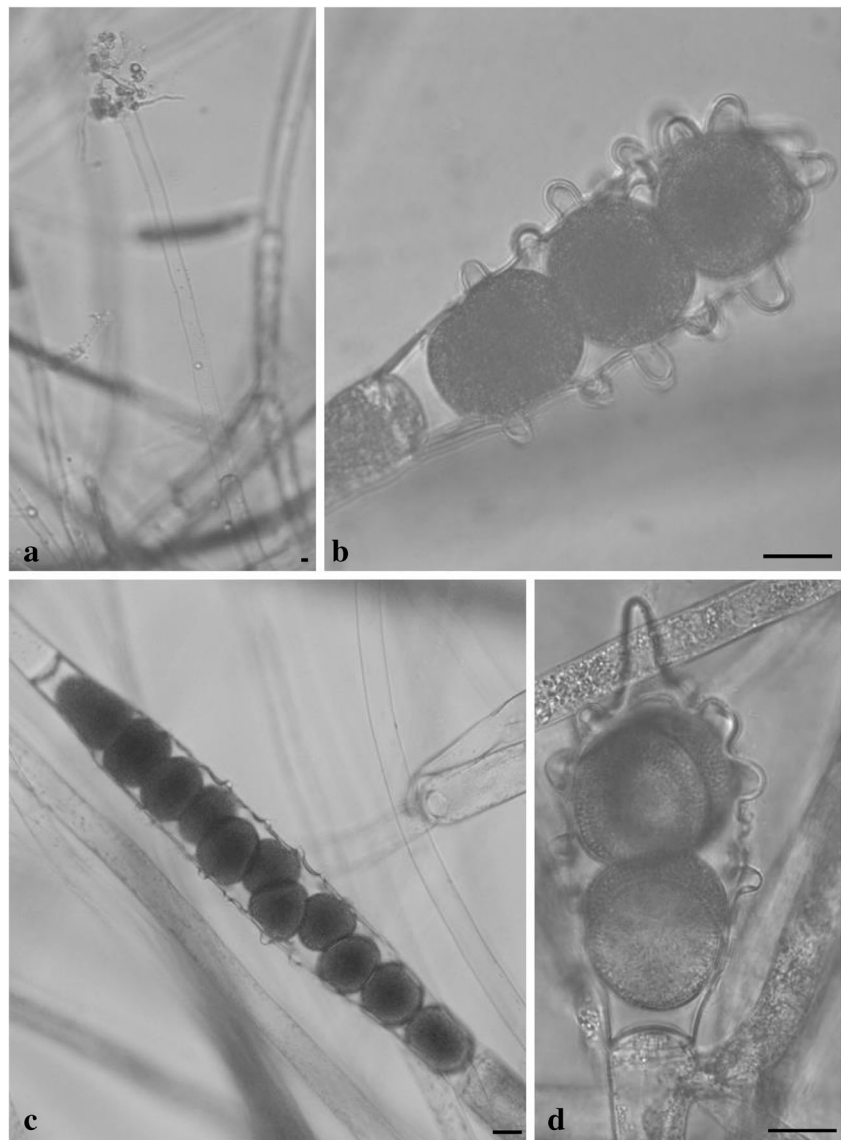
*Achlya s.s.*, as previously reported (Riethmüller et al. 1999).

The third major clade (100% of bootstrap support), corresponds to the family *Verrucalvaceae* and included the genera *Aphanomyces*, *Plectospira*, and *Phragmosporangium*. This last genus, whose characteristics were already commented and illustrated in detail in Pires-Zottarelli et al. (2007), was originally described as a member of the *Saprolegniaceae* based solely on morphology (Johnson Jr et al. 2005b). In addition, the results from our phylogeny confirm previous studies (Diéguez-Urbeondo et al. 2009) that indicate that *Aphanomyces* is not monophyletic and needs a further and detailed revision.

## Taxonomy

As the result of our concatenate phylogenetic analysis of LSU and ITS rDNA sequences and a careful analysis of the morphological characteristics of the specimen CCIBt 3993, we propose the transference of *Achlya androgyna* to *Newbya*.

**Fig. 2 a–d** *Newbya androgyna* (Archer) Pires-Zottar. & S.C.O. Rocha—**a** Sporangium with achlyoid zoospore release. **b** Terminal and ornamented oogonium with oospheres. **c** Intercalary and ornamented oogonium with oospheres. **d** Ornamented oogonium with subcentric oospores. Bars = 10  $\mu$ m



*Newbya androgyna* (Archer) Pires-Zottar. & S.C.O. Rocha, **comb. nov.** MB 823973, Fig. 2a–d.

≡ *Saprolegnia androgyna* Archer, Quart, J. Microscop. Soc. (N.S.) 7: 123, pl. 6. Fig. 1. 1867.

≡ *Aplanes androgynus* (Archer) Humphrey, Trans. Amer. Phil. Soc. (N.S.) 17: 134. 1893.

≡ *Achlya androgyna* (Archer) T.W. Johnson and R.L. Seym., Mycotaxon 92: 14. 2005.

=*Achlya braunii* Reinsch, Jahrb. Wiss. Bot. 11: 284, pl. 14, fig. 1–6. 1878.

≡ *Aplanes braunii* (Reinsch) de Bary, Bot. Zeitung (Berlin) 46: 650, pl. 9, fig. 2. 1888.

=*Saprolegnia treleaseana* Humphrey, Trans. Amer. Phil. Soc. (N.S.) 17: 111, pl. 17, figs 56–59. 1893.

≡ *Achlya treleaseana* (Humphrey) Kauffman, 8 Rep. Michigan Acad. Sci., Arts, Ltrs. 8: 27. 1906.

≡ *Aplanes treleaseanus* (Humphrey) Coker, J. Elisha Mitchell Sci. Soc. 42: 217, pls. 34, 35. 1927. [*non Aplanes treleaseanus* (Humphrey) Coker, Saprolegniaceae, p. 79, pl. 20. 1923.]

≡ *Aplanes treleaseanus* (Humphrey) Naumova, Bot. Mater. Qtd. Sporov. Rast. Bot. Inst. Akad. Nauk SSSR 10: 135, Fig. 3. 1955.

=*Achlya acadensis* Moore, Proc. and Trans. Nova Scotian Inst. Sci. 12: 229, figs. 16–19. 1908–09.

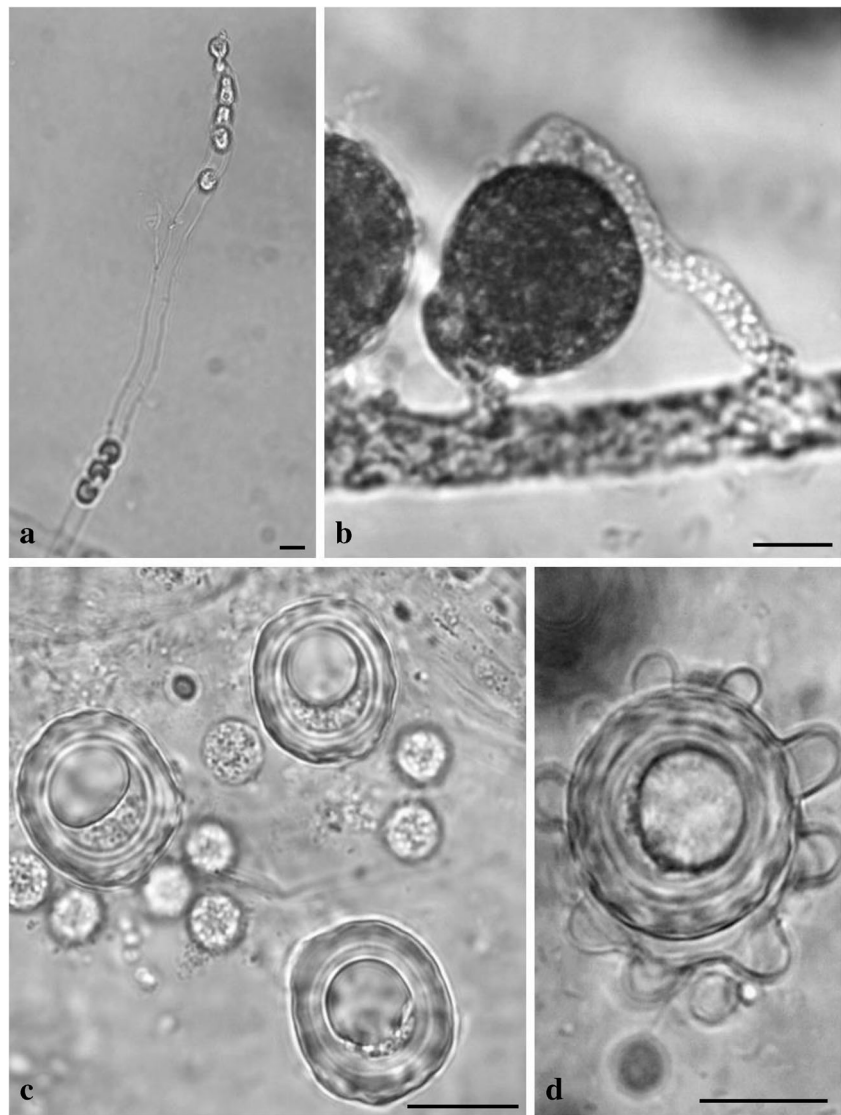
=*Aplanes braunii* var. *mindenii* Shkhorbatov, Bot. Mater. Inst. Sporov. Glavn. Bot. Sada RSFSR 2: 36. 1923.

≡ *Aplanes androgynus* var. *mindenii* (Shkhorbatov) Cejp, Oomycetes I, Flora CSR, Ser. B, Part 2, p. 271. 1959.

Monoecious. Zoosporangia scarce, terminal, fusiform; renovation not observed. Zoospore discharge and behavior



**Fig. 3 a–d** *Leptolegnia eccentrica*—**a** Spores discharge by the zoosporangium. **b** Oogonium in development and monoclinal antheridium. **c** Oogonia with an eccentric oospore. **d** Detail of an ornamented oogonium with an eccentric oospore. Bars = 10  $\mu\text{m}$



achlyoid. Zoospore cyst, 7.5–12.0  $\mu\text{m}$  diam. Gemmae present. Oogonia terminal and intercalary, sometimes lateral, filiform or naviculate, 60.0–430.0  $\times$  20.0–40.0  $\mu\text{m}$ , some of them oval, 50.0–170.0  $\times$  22.5–32.0  $\mu\text{m}$ , rarely spherical, 70.0–95.0  $\mu\text{m}$  diam.; smooth-walled, unpitted; sometimes sparsely provided with papillate ornamentations, 7.5–12.5  $\mu\text{m}$  long; oogonial stalks simple, 1–3 times diam. of the oogonium. Antheridia scarce, when present androgynous; antheridial branches simple; antheridial cells simple, laterally appressed; fertilization tubes not observed. Oospheres with late maturation. Oospores subcentric, spherical, 17.5–25.0  $\mu\text{m}$  diam., some of them oval, 17.5–60.0  $\times$  17.5–47.5  $\mu\text{m}$ , 2–8 (18) per oogonium.

Specimen examined: BRASIL, São Paulo, Cananéia city, “Parque Estadual da Ilha do Cardoso”, 25° 03' 05"-25° 18' 18" S; 47° 53' 48"-48° 05' 42" W, “Núcleo Perequê”, from freshwater samples, on *Sorghum* spp. seeds, 20 Aug. 2012, C.L.A. Pires-Zottarelli *s.n.* (CCIBt 3993).

Comments—The morphological characteristics of the specimen are according to Johnson Jr et al. (2005b), who were the last authors to propose a new combination for this species. Its key characteristics are the rare production of sporangia, and the presence of various sizes of filiform and/or naviculate oogonia, papillate ornamentations, subcentric oospores, and androgynous antheridia. Some of these characteristics, such as ornamentation and type of oospores, are common to all species of *Newbya* already described (Spencer et al. 2002).

## Discussion

Results from our phylogeny also suggest that the organization of lipid droplets inside the oospores can be considered a relevant taxonomic trait for the taxonomy of *Saprolegniales* at the genus level, as recently proposed by Beakes et al. (2014a). On the contrary, the type of zoospore discharge from the

sporangia, traditionally used to differentiate between genera of *Saprolegniales*, does not appear to be a key character, at least at the genus level. Our concatenate LSU and ITS rDNA phylogeny also supports the establishment of the new family *Achlyaceae*, as proposed by Beakes et al. (2014a), although no reliable sequences (from both gene regions) of *A. prolifera*, the type species of the genus *Achlya*, are currently available. Therefore, we consider that further studies are still needed before the formal designation of this family. The main features that characterize the *Achlyaceae* are the presence of three different types of zoospore discharge, namely achlyoid, dictyucoid, and thraustothecoid, and one to more oospores per oogonium that are predominantly eccentric.

*Achlya androgyna* clustered together with high support with other species of *Newbya* in our phylogeny. Based on this and on the morphological characteristics of this species, we consider that *A. androgyna* should be transferred to *Newbya*, and therefore, we propose the new combination *N. androgyna*. Unfortunately, there are no other sequences available at GenBank for both LSU and ITS regions of the rDNA of *Achlya androgyna* and its synonyms to include in our analysis.

The type of zoospore discharge has been traditionally considered as the main character to separate between genera of *Saprolegniaceae*. *Leptolegnia eccentrica*, which was sequenced for the first time in this study, is moderately supported within the *Saprolegniaceae*, forming a clade out of the core of the family. Although *L. eccentrica* has leptolegnoid type of zoospore discharge as all other species of *Leptolegnia* (including *L. chapmanii*, whose morphology was carefully analyzed in this study), it did not cluster together with the rest of the species considered in our phylogeny (Fig. 1). Steciow et al. (2013), based on the ITS and LSU rDNA analysis of some members of the *Saprolegniales*, considered the genus *Leptolegnia* as paraphyletic. These authors hypothesized that the loss of flagella in *Geolegnia*, and the emergence of the new species *G. helicoides*, were the result of a recent evolutionary event within *Leptolegnia*. Because of the small number of sequences of *Geolegnia* available at GenBank, and particularly due to the absence of sequences of the type species, we did not include this genus in our phylogeny. Johnson Jr et al. (2002) suggested certain similarity of *L. eccentrica* with species of *Leptolegniella* (*Leptomitales*, *Oomycota*) with regard to the characteristics of oospores (Fig. 3) but since there are no sequences of *Leptolegniella* available, it is not possible to test this potential relationship. Our results corroborate the results of Beakes et al. (2014a) regarding the position of *Leptolegnia* within the *Saprolegniaceae*. Further phylogenetic studies should be carried out to resolve the seeming absence of monophyly in *Leptolegnia*, before a decision about this genus can be taken.

*Phragmosporangium* is a monospecific genus created to accommodate *Phragmosporangium uniseriatum* (Johnson Jr et al. 2005b). This species was reported in Brazil as the second

world occurrence (Pires-Zottarelli et al. 2007) and is a very common saprotroph in soil samples of Brazilian biomes (Miranda and Pires-Zottarelli 2012, Nascimento and Pires-Zottarelli 2012, Pires-Zottarelli and Michelin 2012). Our phylogeny undoubtedly indicates that this genus is a member of the *Verrucalvaceae*. Although the type of zoospore discharge is considered to be dictyucoid (Johnson Jr et al. 2005b) and, therefore, resembles *Dictyuchus*, a member of the *Saprolegniales*, this genus also shares some characteristics with *Aphanomyces*, such as thickness of hyphae and presence of one single oospore per oogonium, which in turn can explain its phylogenetic placement within the *Verrucalvaceae*. The dictyucoid pattern of zoospore discharge is also exhibited by *Chlamydomyzium dictyuchoides*, an *incertae sedis* member of the early diverging clades of *Saprolegniomycetes*, and might be the result of convergent evolution (Beakes et al. 2014b, Beakes and Thines 2017).

The present study provides a new glance at the phylogeny of *Saprolegniales*, considering genera and species that were not previously included into a phylogeny and gives relevant information for clarifying the relationships between the main genera, confirming recently proposed taxonomical modifications.

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