Incipient loss of flagella in the genus *Geolegnia*: the emergence of a new clade within *Leptolegnia*?

Mónica M. Steciow[1*], Enrique Lara[2*], Amandine Pillonel[2], Sebastián A. Pelizza[1,4], Eduardo A. Lestani[3], Gustavo C. Rossi[4], and Lassaad Belbahri[2]

[1]Instituto de Botánica Spegazzini, 53 N° 477, (1900) La Plata, Buenos Aires, Argentina; corresponding author e-mail: msteciow@fcnym.unlp.edu.ar
[2]Laboratory of Soil Biology, Department of Biology, University of Neuchâtel, 11 Rue Emile Argand, CH-2000, Neuchâtel, Switzerland
[3]Centro de Investigaciones Ecológicas Subtropicales, Parque Nacional Iguazú /Asoc. Civil Centro de Investigaciones del Bosque Atlántico, Pto. Iguazú, Misiones, Argentina
[4]CEPAVE-CCT –CONICET, La Plata, Argentina
*Both authors contributed equally to the work.

Abstract: The genus *Geolegnia* represents a poorly documented group of saprolegnialean oomycetes isolated from soils as free-living organisms. Although it is morphologically similar to the facultative parasitic genus *Leptolegnia, Geolegnia* presents the uncommon property of having lost a flagellate stage in its lifecycle. Based on ITS and large subunit (LSU) rRNA sequence data, we show *Geolegnia* to be basal to *Leptolegnia,* and also introduce *Geolegnia helicoides* sp. nov. Using sequence data of *Leptolegnia* available in GenBank, supplemented by data derived from culture collections, we show that *Geolegnia* is nested within *Leptolegnia*, a genus characterised by its "conventional" biflagellate life cycle. The emergence of *Geolegnia* is therefore seen as a recent event, and we suggest here an evolutionary context where this loss might have been advantageous. Based on this study, *Leptolegnia* remains paraphyletic, awaiting the redefinition of genera in this complex.

Key words:
Oomycetes
Fast evolution
Phylogeny
Saprolegniales
Internal transcribed spacer (ITS)
Large subunit (LSU) rRNA

Article info: Submitted: 19 December 2012; Accepted: 28 September 2013; Published: 25 October 2013.

INTRODUCTION

Oomycetes are a group of fungal-like heterokonts that are often associated with freshwater and terrestrial habitats, such as marginal sites around lakes, lagoons, streams, or isolated from seasonally or intermittently waterlogged soils, and also marine environments (Lara & Belbahri 2011). Although there are many free-living species, others are plant pathogens (e.g. *Phytophthora* species), while others are animal parasites, including one species (*Pythium insidiosum*) infecting humans, and another (*Saprolegnia parasitica*) parasitising fish in fish farms. The specificity of these parasites is considered to vary between groups, being maximal in the case of obligate parasitic species such as *Peronospora*, where particular strains may have a very limited host range (Goker *et al.* 2007). Amongst facultative oomycetes, there can also be specializations for broader groups; in particular, the genus *Leptolegnia* parasitizes invertebrates and has been studied for potential as an agent for mosquito biocontrol (Pelizza *et al*. 2011). Other members of the genus can be associated with cladocerans, fish, and amphibian eggs and larvae (Petrisco *et al*. 2008, Wolinska *et al.* 2009). The genus *Geolegnia*, with which it shares large thick-walled sporangiospores that are encysted in a single row, is not known to be associated with any organism. That genus is characterised by the absence of flagella in its mobile life-stage, an unusual trait in *Oomycetes*.

Flagellar loss has evidently occurred several times during the evolution of eukaryotes. In true fungi (*Fungi*), it occurred only once, at the divergence between *Chytridiomycetes* and *Blastocladiomycetes* and other *Fungi*, *Olpidium* standing as an exception (James *et al*. 2006, Sekimoto *et al*. 2011). A more recent flagellar loss has also been reported in *Blastocladiomycetes*, but this case remains exceptional (James *et al*. 2011). To our knowledge other osmotrophic filamentous groups, such as *Hyphochytriomycetes,* only have flagellated members. Flagella are used as dispersal means in aqueous habitats (Liu *et al*. 2006). Within *Oomycetes*, these losses have occurred several times; within basal genera, in *Haptoglossa*, one clade out of three comprises only organisms that produce non-flagellated (aplanosporic) zoospores (Hakariya *et al.* 2009), and *Chlamydiosporum aplanosporum* has also been reported to lack a flagellate stage (Glockling & Beakes 2000). In *Peronosporales*, *Myzocytiopsis subliformis* also has aplanosporic zoospores (Glockling & Beakes 2000), as well as other obligately pathogenic clades such as *Hyaloperonospora*, *Bremia,* and most *Peronospora* species (Beakes *et al.* 2012). All these taxa include only obligate parasites (Lara & Belbahri 2011),

suggesting a loss of structural complexity when adopting a parasitic life-style (Judelson *et al.* 2012).

Geolegnia is currently the only saprolegnialean fungal genus that has aplanosporic zoospores. It is not a strictly obligate pathogenic genus, since it grows easily in culture, and has been isolated from soil (Fuller & Jaworski 1987, Johnson *et al*. 2002). In this paper, we investigated the phylogenetic position of the genus based on a new species isolated from mosquito larvae living in the water-holding tanks supporting the plant *Aechmea distichantha* in a subtropical forest area of northern Argentina (Misiones Province).

MATERIALS AND METHODS

Isolation

Living larvae of *Culex* (*Microculex*) *imitator* were collected from *Aechmea distichantha* (*Bromeliaceae*) in Iguazú National Park (25 ° 41'4 "S, 54 º 26'45" W) in Misiones Province, Argentina. The native vegetation is typical of the edge of the Iguazú River, with small to medium sized trees, with bamboos and ferns in the understory.

Collected larvae were placed individually within sterile glass containers and taken immediately to the laboratory for further observation. After 48 h, all larvae of *C. imitator* died, and subsequent microscopic analyses showed the presence of dense hyphae. Dead mosquitoes were placed in sterile distilled water containing several sterile hemp seeds (*Cannabis sativa*) for baiting, and incubated at room temperature (15–20 °C). After seed colonization, a single hypha was isolated and transferred to YpSs Emerson medium (Yeast extract soluble starch agar, Fuller & Jaworski 1987) to obtain axenic cultures. Measurements and observations were made using an Olympus BX 40 microscope (Olympus Optical, Tokyo) equipped with phase contrast optics. Based on morphology, the fungus was found to be an undescribed species of *Geolegnia*, described here as *G. helicoides.* All other species included in this study were obtained from the CBS-KNAW Fungal Biodiversity Centre (CBS, Utrecht, The Netherlands), and are listed in Fig. 3.

DNA extraction, PCR and sequencing

DNA was extracted with a guanidine thiocyanate buffer protocol as in Lara & Belbahri (2011). PCR was performed using the wide-spectrum primers ITS4 and ITS6 for the ITS region and ITS4 and 28S-564R (5'-TGGTCCGTGTTTCRAGACG-3') for the LSU region (White *et al.* 1990). The PCR products were sequenced with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Geneva) using a BigDye™ Terminator Cycle Sequencing Kit (PE Biosystems). Sequences have been deposited in GenBank with accession numbers: KF656775 (ITS region) and KF656776 (LSU region). They were aligned manually using BioEdit software (Hall 1999). The phylogenetic tree was reconstructed using Maximum Likelihood with a RAxML algorithm (Stamatakis *et al*. 2008). The computations were performed at the Vital-IT (http://www.vital-it.ch) Centre for high-performance computing of the SIB Swiss Institute of Bioinformatics (Lausanne). As *Leptolegnia* sequences appeared to be the closest relatives of *Geolegnia helicoides,* we retrieved all available related sequences from GenBank. The genera *Saprolegnia* and *Achlya* were used as outgroups.

RESULTS

Morphology

Observations were made from axenic 2-wk-old cultures (4–9 cm diam), grown on hemp seeds at 20° C. The species possessed characters typical of the genus *Geolegnia*, i.e. aplanosporic propagules. It could be differentiated from congeneric species by a number of criteria that are detailed in Table 1.

Geolegnia helicoides M. M. Steciow, E. Lara, L. Belbahri, A. Pillonel, & S. A. Pelizza, **sp. nov.**
MycoBank MB805937
(Figs 1–2)

Etymology. Referring to the development of diclinous antheridal branches arranged in helicoidally around the main hyphae (Fig. 1D–E).

Diagnosis: On *Culex imitator* larvae, in *Aechmea distichantha* water tank. Sporangia cylindrical to broadly filiform or fusiform, (150–)190–600(–800) × 10–20 µm; antheridial branches branched, mainly diclinous, rarely monoclinous or androgynous; typically coiled around main hyphae and wrapping oogonial stalks forming a cluster of oogonia, (25–)30–45 µm diam. Oospores subeccentric.

Type: **Argentina**: *Misiones*: Iguazú National Park, in *Aechmea distichantha* water tank growing at Iguazú River margin, on *Culex imitator* larvae, 2 Aug. 2010, *Eduardo Lestani* (LPSC 1165 – holotype; culture ex-holotype LPS48465).

Description: *Monoecious*, mycelium dense, extensive; principal hyphae slender, moderately branched, straight or sinuous, bent or slightly curved at the tips; 10–25 µm wide at the base (Fig. 1B–C). *Gemmae* rare or sparse in water culture or in solid medium; cylindrical, fusiform, irregular or branched; terminal or intercalary, single or catenulate, developing at hyphal ends; functioning as zoosporangia (Figs 1D–E). *Sporangia* cylindrical to broadly filiform or fusiform, often tapering towards the apex, straight, usually curved, bent or somewhat sinuous, sparse, slender, renewed in a basipetalous fashion or sympodially, (150–)190–600(–800) × 10–20 µm (Fig. 2A). *Spores* non-motile, predominantly cylindrical, fusiform, or broadly ellipsoid, infrequently to rarely oval; released upon deliquescence of sporangium wall; always formed in a single row; directly producing hyphae at germination, 25–30 × 10–18 µm (Fig. 2B). *Oogonia* variable in abundance or becoming abundant with the age of the culture, lateral or terminal, often formed in dense clusters, brownish, single or catenulate when immature, spherical or subglobose, sometimes obpyriform, pyriform, or very rarely oval or irregular, (25–)30–45 µm diam, with a frequent proliferation of immature and mature oogonia. *Oogonial wall* smooth, thin, rarely with a lateral papillate projection, unpitted. *Oogonial stalks* variable in length, usually 0.5–4 times the diameter

Table 1. Comparison of the morphological features of the different *Geolegnia* species.

	G. helicoides	***G. inflata***	***G. intermedia***	***G. septisporangia***
Zoosporangial shape	Cylindrical to broadly filiform, fusiform; tapering to the apex; straight, usually curved, bent or somewhat sinuous	Cylindrical to filiform at first, becoming swollen at intervals (spores inside expanded portions), in its typical catenulate aspect	Cylindrical to filiform, becoming swollen at intervals (spores inside expanded portions)	Cylindrical to filiform, curved to somewhat sinuous
Zoosporangial size (µm)	(150–)190–600(–800) × 10–20	70–380(–655) × 8–29	As in *G. inflata*	38–187 × 10–24
Spores shape	Non-motile. Cylindrical, fusiform, or broadly ellipsoidal, infrequently to rarely oval	Non-motile. Spherical-oval, ovate; seldom elongate; formed in a single row	Non-motile. Spherical-oval, ovate; seldom elongate	Non-motile. Cylindircal, fusiform, or broadly ellipsoidal; rarely oval
Oogonial wall	Smooth, thin; unpitted (very rarely with a lateral papillate projection)	Smooth, thin; unpitted	Smooth, thin; unpitted	Smooth, thin; unpitted
Antheridial branches	Androgynous, mainly diclinous, often monoclinous, forming helicoidal spirals about the oogonial stalk, and extensively wrapping about themselves and around adjacent hyphae; slender, irregular; very branched; persisting	Mainly androgynous, infrequent. monoclinous, (diclinous); slender, irregular; unbranched or once-branched; persisting	As in *G. inflata*	Mainly androgynous, adjacent to oogonia; rarely monoclinous; slender, twisted; unbranched or once-branched; persisting
Oogonial shape	Spherical - subglobose, sometimes obpyriform, pyriform, or very rarely oval or irregular; often immature and proliferating	Spherical-subspherical, obpyriform	As in *G. inflata*	Spherical to obpyriform
Oogonial diam (µm)	(25–)30–45	(14–)18–22(–33)	As in *G. inflata* or larger	(15–)24–30(–38)
Oospore type	Subeccentric; hyaline or brownish, thin-walled, contents finely granular. Oospheres almost always not maturing inside normal oogonia	Eccentric; Oospheres almost always maturing inside normal oogonia	Eccentric; Oospheres almost always maturing inside normal oogonia	Eccentric; Oospheres almost always maturing inside normal oogonia
Oospore diam (µm)	(20–)25–35(–42)	(13–)15–20(–28)	16–19	(13–)22–29(–36)
Oospores per oogonium	Single; spherical or ellipsoidal, or irregular when immature; filling the oogonium	Single; spherical; filling the oogonium	Single; spherical; filling the oogonium	Single; spherical; filling the oogonium
Oogonial stalk	Slender and short or stout and longer; often straight, bent, twisted or curved; branched and somewhat irregular	Slender; curved, bent, twisted and irregular, unbranched or once-branched	Slender; curved, bent, twisted and irregular, unbranched or once-branched	Slender, straight, curved, or somewhat irregular; unbranched
Gemmae	Very rare; cylindrical, fusiform, irregular, or branched; terminal or intercalary, single or catenulate	Lacking	Lacking	Very rare; clavate-obpyriform; single, terminal

of the oogonium, rarely up to nine times; slender and short or stout and longer; often straight, bent, twisted or curved, often branched and somewhat irregular (Fig. 2C). *Oospheres* mostly not maturing inside the oogonia. *Oospores* hyaline or brownish, thin-walled, contents finely granular, subeccentric, spherical or ellipsoidal, or irregular when immature, one per oogonium, filling the oogonium, (20–)25–35(–42) µm diam. *Antheridial branches* usually abundant; androgynous, mainly diclinous, often monoclinous, forming helicoidal spirals about the oogonial stalk, and extensively wrapping about themselves and around adjacent hyphae; slender, irregular; abundantly branched; persisting. *Antheridial cells* simple, broadly clavate or tubular, usually bent, persistant, apically appressed, the portion nearest the oogonial wall sometimes constricted into a neck-like extension, occasionally laterally attached; fertilisation tubes not observed (Fig. 2D).

Molecular analysis

Both ITS and LSU markers place *Geolegnia helicoides* basal to the genus *Leptolegnia*, with a clade that comprises environmental isolates plus two *L. caudata* strains from the CBS (CBS 113431 and 680.69), a still undescribed species of *Leptolegnia* (CBS 392.81), plus a probably misidentified culture (CBS 359.35, as "*Thraustotheca clavata*"). Its basal placement to this group was robustly supported (bootstrap value 96 % and 99 % respectively for ITS and LSU). Another basal clade in the ITS trees represented a group of sequences wrongly identified as *Saprolegnia* (Fig. 3).

DISCUSSION

The presence and absence of flagella has been considered a trait of major taxonomical relevance. In this context, the genus *Geolegnia* has been considered as the most derived member

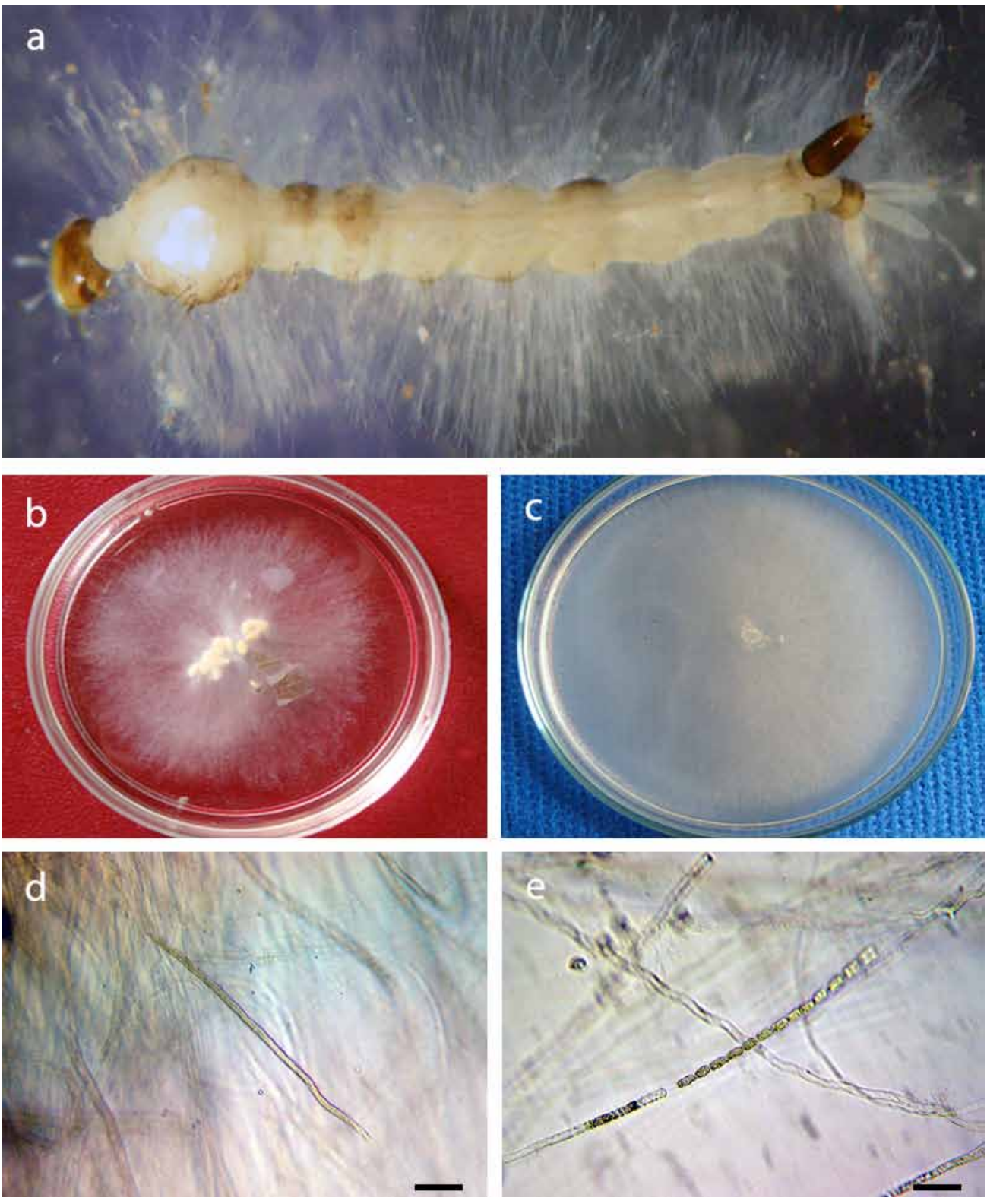

Fig. 1. *Geolegnia helicoides* (LPS 48465). **A.** Larvae, 48 h post infection of *Culex imitator* with mycelium. **B.** A 2-wk-old hemp seed colony in water culture. **C.** Colony growing on YPSs medium. **D.** Aspect of mycelium with zoosporangia in water culture. **E.** Zoosporangium showing typical geolegnoid spore release; spores formed in a single row. **B**ars: D–E = 30 µm.

of the *Saprolegniales* "galaxy" (Fuller & Jaworsky 1987). However, members of this genus possess all characteristics of *Leptolegnia,* with the exception of the presence of flagella. As *Geolegnia helicoides* is nested within *Leptolegnia*, it can be deduced that it emerged from a *Leptolegnia*-like ancestor, and therefore the loss of its flagella is certainly a recent evolutionary event.

As most *Geolegnia* species have been found in soils, where the dispersal potential of flagellated propagules is not as high as in freshwater, it can be deduced that there

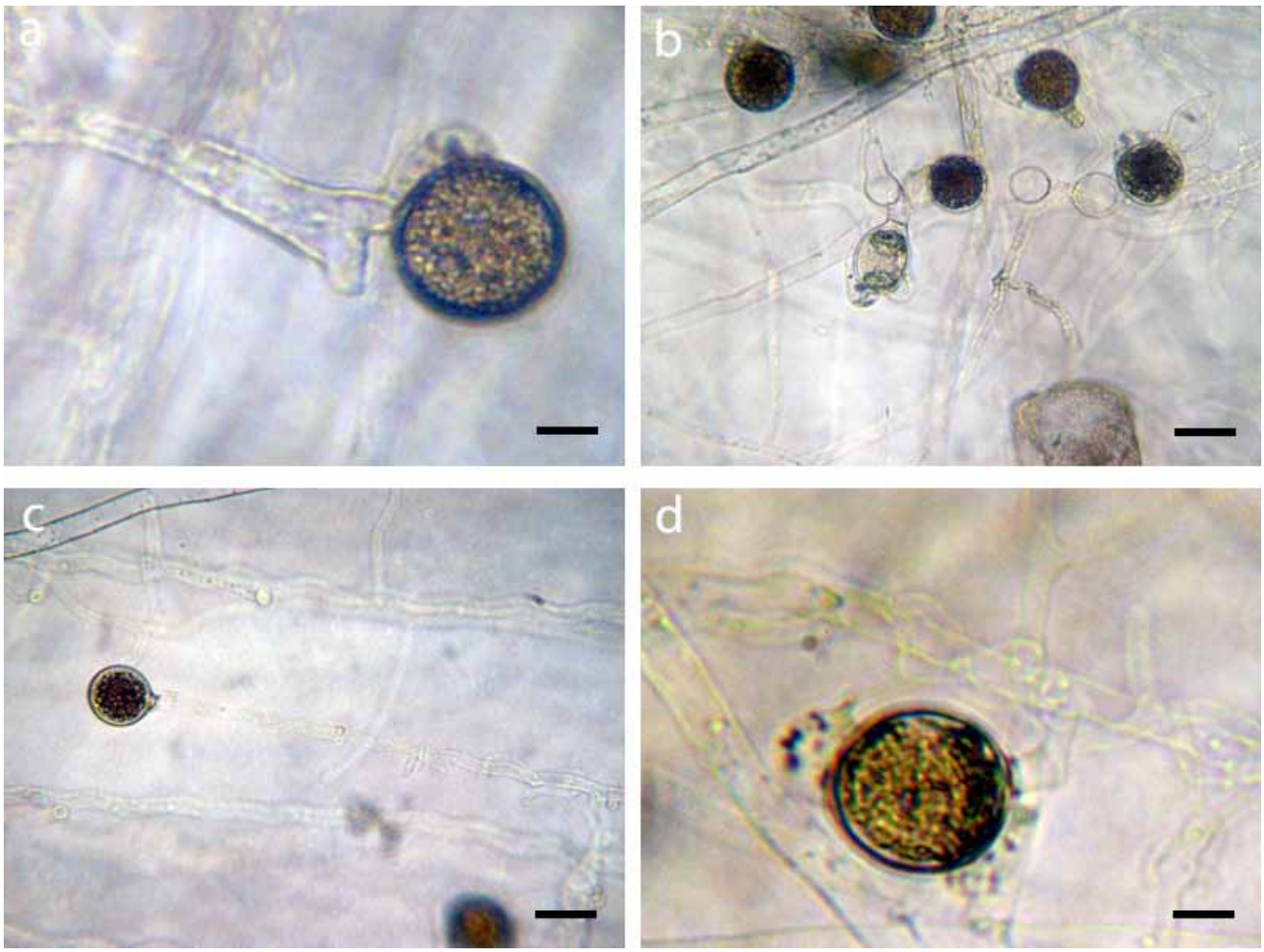

Fig. 2. *Geolegnia helicoides*. **A.** Androgynous antheridial branch, adjacent to the oogonium. **B.** Aspect of mycelium with immature and mature oogonia in clusters, and antheridial branches in water culture. **C, D.** Detail of characteristic diclinous and monoclinous coiling antheridial branches, around the oogonial stalks, oogonia and neighbouring vegetative hyphae. Bars: A and D = 10 µm; B–C = 30 µm.

should be at least no evolutionary disadvantage not to possess them. To the contrary, there is a genetic cost for maintaining useless flagellar machinery: no less than 257 proteins are associated with the flagella in *Phytophthora infestans* (Judelson *et al*. 2012). To date, *Geolegnia* is the first example of an oomycete with aplanosporic zoospores that is not an obligate parasite. We hypothesize that this trait has been conserved because *Geolegnia* never occurs in environments where it is disadvantageous in its competition with other *Saprolegniales*; soils are an environment where flagellated propagules cannot travel far. Indeed, most members of *Geolegnia* (*G. inflata, G. septisporangia,* and *G. intermedia*) have been isolated from soils (Chiou & Chang 1976), and members of the *Saprolegniales* are seldom isolated from edaphic systems (Johnson *et al*. 2002). In the case of *G. helicoides*, the distribution potential of propagules within a tank of bromeliad plants is relatively low. In addition, organic matter essential to its growth (i.e. dead insects and other detritus) will be concentrated at the bottom of the tank. *Geolegnia* propagules will also fall to the bottom of the tank; in such a situation also, flagella do not represent an advantage.

Members of the genus *Geolegnia* are thought to be rare, but since colonies not do not release motile or flagellated propagules, the species are not recovered in gross cultures unless the baits or available substratum are placed in direct contact with the environmental samples. Even then, the more rapidly developing species of *Oomycetes* tend to outcompete the slower-growing *Geolegnia* species (Fuller & Jaworsky 1987, Johnson *et al*. 2002). This possibly explains why so few occurrences have been recorded, and suggests that *Geolegnia* species may be more common than previously thought.

Taxonomic implication of the study

The taxonomy of saprolegnian oomycetes is only partly resolved as suggested by several studies (Dick 2001, 2002). In this study, we provide phylogenetic data to show that *Leptolegnia* is paraphyletic if *Geolegnia* is maintained as a separate genus. We consider this option as the most appropriate, since flagellar loss is a rare event. In addition, our study reveals the existence of a monophyletic group of sequences collectively mislabelled as *Saprolegnia*. Further investigations will be necessary to define synapomorphies with this clade and to further define these genera.

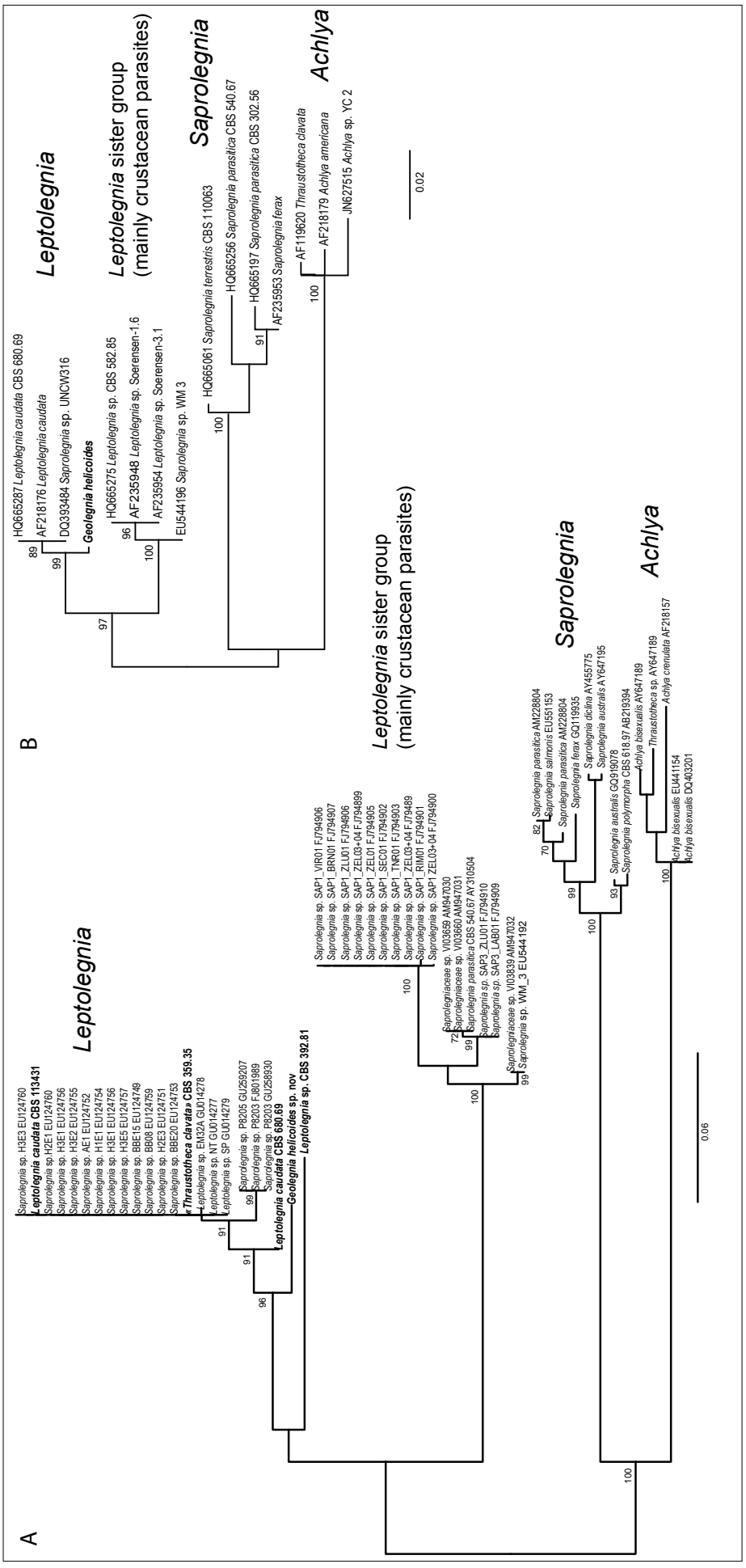

Fig. 3. Maximum likelihood phylogenetic trees showing the position of *Geolegnia helicoides* with respect to *Leptolegnia*, and sequences from closely related organisms. Tree is rooted with genera *Saprolegnia*, *Thraustotheca* and *Achlya*. **A.** ITS tree. **B.** LSU tree.

Key to *Geolegnia* species

Based on Johnson *et al*. (2002).

1 Spores generally spherical or ovoid; sporangium wall swollen at intervals; the swellings generally containing only one spore 2
Spores generally elongated, fusiform, broadly ellipsoidal or cylindrical; sporangium wall not swollen at intervals 3

2 (1) Oospores 13–15 µm diam ..... **G. inflata**
Oospores 16–19 µm diam ..... **G. intermedia**

3 (1) Antheridial branches androgynous, rarely monoclinous; never coiled around main hyphae and clusters of oogonia ..... **G. septisporangia**
Antheridial branches diclinous, rarely monoclinous; typically coiled around main hyphae, forming a cluster of oogonia ..... **G. helicoides**

ACKNOWLEDGEMENTS

This work was supported by the Swiss State Secretariat for Education and Research (grant reference: SER No. C09.0139) and the European Union for the projects ISEFOR “Increasing Sustainability of European Forests: Modelling for Security against Invasive Pests and Pathogens under Climate Change" (FP7-KBBE-2009-3 call, Proposal number: 245268) and the COST action FP0801 “Established and Emerging *Phytophthora*: Increasing Threats to Woodland and Forest Ecosystems in Europe”. We are indebted to Christophe Paul for technical assistance. Mónica Steciow wishes to thank CONICET (PIP 1422) and UNLP (Project N11/ 566) for financial support.

REFERENCES

Beakes GW, Glockling SL, Sekimoto S (2012) The evolutionary phylogeny of the oomycete “fungi” *Protoplasma* **249**: 3–19.

Chiou TS, Chang HS (1976) Aquatic phycomycetes of Taiwan. II. *Botanical Bulletin of Academia Sinica* **17**: 37–53.

Dick MW (2001) *Straminipilous Fungi: systematics of the Peronosporomycetes including accounts of the marine straminipilous protists, the plasmodiophorids and similar organisms*. Dordrecht: Kluwer Academic.

Dick MW (2002) Towards an understanding of the evolution of the downy mildews, in: *Advances in Downy Mildew Research* (Spencer-Phillips PTN, Gisi U, Lebeda A, eds): 1–57. Dordrecht: Kluwer Academic.

Fuller M, Jaworski A (1987) *Zoosporic Fungi in Teaching and Research*. Athens, GA: Southeastern Publishing,

Glockling SL, Beakes GW (2000) A review of the taxonomy, biology and infection strategies of “biflagellate holocarpic” parasites of nematodes. *Fungal Diversity* **4**: 1–20.

Goker M, Voglmayr H, Riethmuller A, Oberwinkler F (2007) How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. *Fungal Genetics and Biology* **44**: 105–122.

Hakariya M, Hirose D, Tokumasu S (2009) Molecular phylogeny of terrestrial holocarpic endoparasitic peronosporomycetes, *Haptoglossa* spp., inferred from 18S rDNA. *Mycoscience* **50**: 130–136.

Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

James TY, Hoffman Y, Zarka A, Boussiba S (2011) *Paraphysoderma sedebokerense*, gen. et sp nov., an aplanosporic relative of *Physoderma* (*Blastocladiomycota*). *Mycotaxon* **118**: 177–180.

James TY, Letcher PM, Longcore JE, Mozley-Standridge SE, Porter D, Powell MJ, Griffith GW, Vilgalys R (2006) A molecular phylogeny of the flagellated fungi (*Chytridiomycota*) and description of a new phylum (*Blastocladiomycota*). *Mycologia* **98**: 860–871.

Johnson TWJR, Seymour RL, Padgett DE (2002) *Biology and Systematics of the Saprolegniaceae*. http://wwwilumina-dliborg

Judelson HS, Shrivastava J, Manson J (2012) Decay of genes encoding the oomycete flagellar proteome in the downy mildew *Hyaloperonospora arabidopsidis*. *PloS One* **7**: e47624.

Lara E, Belbahri L (2011) SSU rRNA reveals major trends in oomycete evolution. *Fungal Diversity* **49**: 93–100.

Liu YJJ, Hodson MC, Hall BD (2006) Loss of the flagellum happened only once in the fungal lineage: phylogenetic structure of kingdom *Fungi* inferred from RNA polymerase II subunit genes. *BMC Evolutionary Biology* 6: 74.

Pelizza SA, Cabello MN, Tranchida MC, Scorsetti AC, Bisaro V (2011) Screening for a culture medium yielding optimal colony growth, zoospore yield and infectivity of different isolates of *Leptolegnia chapmanii* (*Stramenopila*: *Peronosporomycetes*). *Annals of Microbiology* **61**: 991–997.

Petrisko JE, Pearl CA, Pilliod DS, Sheridan PP, Williams CF, Peterson CR, Bury RB (2008) *Saprolegniaceae* identified on amphibian eggs throughout the Pacific Northwest, USA, by internal transcribed spacer sequences and phylogenetic analysis. *Mycologia* **100**:171–180.

Sekimoto S, Rochon D, Long JE, Dee JM, Berbee ML (2011) A multigene phylogeny of *Olpidium* and its implications for early fungal evolution. *BMC Evolutionary Biology* **11**: 331.

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML Web servers. *Systems Biology* **57**: 758–771.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds): 315–322. San Diego: Academic Press.

Wolinska J, Giessler S, Koerner H (2009) Molecular identification and hidden diversity of novel *Daphnia* parasites from European lakes. *Applied and Environmental Microbiology* **75**: 7051–7059.