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

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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.

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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, Opidium 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 Hypochytriomycetes, 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 subiformis 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),...
suggested 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 zoosporangia. It is not a strictly obligate pathogenicic 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 Iguazu National Park (25° 41′ S, 54° 26′45″ W) in Misiones Province, Argentina. The native vegetation is typical of the edge of the Iguazu 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 YpsSs 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′-TGTTCCGTGTTTCAAGCAG-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; antheridal branches branched, mainly diclinous, rarely monoclinal or androgynous; typically coiled around main hyphae and wrapping oogonial stalks forming a cluster of oogonia, (25–)30–45 µm diam. Oospores subecentric.

Type: Argentina: Misiones: Iguazu National Park, in Aechmea distichantha water tank growing at Iguazu 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, with sparse, short, or long branches; 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 or sympodial, (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
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). Oospores mostly not maturing inside the oogonia. Oospores hyaline or brownish, thin-walled, contents finely granular, subbeccentric, spherical or ellipsoidal, or irregular when immature, one per oogonium, filling the oogonium, (20–)25–35(–42) μm diam. Antheridal 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; very branched; persisting. Antheridal 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 Geolegnia helicoides (CBS 392.81), plus a probably misidentified environment isolate plus two strains from the Thraustotheca clavata (CBS 392.81), 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 as a new clade arising within Leptolegnia (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.
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...
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, *Geolegania* 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 *Geolegania* 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 *Geolegania* (*G. inflata, G. septisporangia,* and *G. intermedia*) have been isolated from soils (Chiu & 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. *Geolegania* 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 *Geolegania* 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 *Geolegania* species (Fuller & Jaworsky 1987, Johnson et al. 2002). This possibly explains why so few occurrences have been recorded, and suggests that *Geolegania* 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 *Leptolegania* is paraphyletic if *Geolegania* 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. septisorangia
   Antheridial branches diclinous, rarely monoclinous; typically coiled around main hyphae, forming a cluster of oogonia ......................................................................................... G. helicoides

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