Multiple barcode assessment within the *Saprolegnia-Achlya* clade (*Saprolegniales, Oomycota, Straminipila*) brings order in a neglected group of pathogens

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Abstract: The *Saprolegnia-Achlya* clade comprises species of major environmental and economic importance due to their negative impact on aquaculture and aquatic ecosystems by threatening fishes, amphibians, and crustaceans. However, their taxonomy and phylogenetic relationships remain unresolved and suffer from many inconsistencies, which is a major obstacle to the widespread application of molecular barcoding to identify pathogenic strains with quarantine implications. We assessed phylogenetic relationships of major genera using three commonly used markers (ITS, SSU rRNA, and LSU rRNA). A consensus tree of the three genes provided support for nine clades encompassing eleven documented genera and a new clade (SAP1) that has not been described morphologically. In the course of this study, we isolated a new species, *Newbya dichotoma* sp. nov., which provided the only culture available for this genus. In parallel, we attempted to summarize the evolution of traits in the different genera, but their successive reversals rendered the inference of ancestral states impossible. This highlights even more the importance of a bar-coding strategy for saprolegniacean parasite detection and monitoring.

INTRODUCTION

Oomycetes (or water moulds) are protists with a mycelial growth that were traditionally classified within *Fungi*, and that have been relocated within *Heterokonta*, also known as *Straminipila*, based on their molecular phylogeny. They are ubiquitous in freshwaters, but also in soils and in marine environments, where they can be found either as parasites or free-living. *Saprolegnia-Achlya* is a monophyletic clade of mainly freshwater species that forms a sister clade to *Aphanomyces* and includes notorious animal pathogens. These organisms are abundant in the environment, where they behave as destructive pathogens in fish (Phillips *et al.* 2008), *Daphnia* (Wolinska *et al.* 2009), insects (Pelizza *et al*. 2011), amphibians (Fernández-Beneítez 2011), and crayfish (Krugner-Rigby *et al.* 2010). As such, they are responsible for millions of dollars losses to the worldwide aquaculture industry (van West 2006), with considerable damage to highly valued fish such as salmonids (Hussein & Hatai 1999). They can also threaten endangered wildlife, and cases of local extinctions of amphibian populations due to members of this group have been reported (Bragg 1958, 1962), as well as declines of wild fish stocks (van West 2006). It is therefore of crucial importance to detect these organisms and

identify them quickly and reliably, in order to apply quarantine measures (Lara & Belbahri 2011).

General strategies for detecting these parasites rely on isolation and cultivation of strains isolated from infected animals followed by morphological identification, a strategy that is time-consuming and requires highly trained specialists. As morphological features used for identification of species and genera require the presence of reproductive and disseminative structures which appear sometimes only after weeks (Steciow 2003), urgent measures cannot be taken early enough to prevent epidemics. For this reason, a molecular barcoding approach could bring a valuable tool for quickly and accurately monitoring disease outbreaks prior to taking appropriate measures. However, this approach is dependent on the existence of a reliable database that allows confident assignment of pathogen-derived or environmental sequences to known species. In particular, a well-documented molecular systematics study can provide a base for identifying known species and placing newly discovered strains. Unfortunately, existing databases suffer major drawbacks caused by: (1) taxonomic inconsistencies; and (2) misidentified strains sent to and preserved in public collections of fungus cultures. It is therefore urgent to clarify the taxonomy within this group

by providing a reliable phylogenetic framework based on sequence information and morphological features. In addition, it is useful to address inconsistencies that limit the use of culture collection resources and propagate errors in subsequent studies. Several species have been described but later assigned to either *Achlya* or *Saprolegnia*. These two genera are traditionally distinguished by the mode of discharge of the zoospores; in *Saprolegnia* flagellate stages encyst to give rise to a second flagellated form (diplanetism), whereas in *Achlya* the released zoospores are the only dispersive form (monoplanetism) (Daugherty *et al.* 1998, Johnson *et al.* 2002). However, these two gross types have several subtypes, and species identification can only be achieved using a combination of characters. In addition, these characters can only be assessed after the generation of sexual and asexual (dispersive) forms, which requires the testing of many different culture conditions and long incubation periods, a time-consuming process that can be precious in the case of a disease outbreak. Therefore, development of a robust barcoding strategy to identify possible pathogens or implement quarantine measures would be extremely useful.

In order to overcome these caveats, we recompiled the morphological features used to identify species of *Saprolegniaceae*. In parallel, we performed phylogenetic analyses of the group based on the three most frequently used markers, ITS, SSU and LSU rRNA, and compared their respective resolution powers. We also inferred a consensus tree and inferred possible scenarios for trait evolution. And, finally, we described a new species from a little-known genus, *Newbya dichotoma* sp. nov. isolated from floating organic matter associated with fishpond water.

MATERIALS AND METHODS

Isolation of *Newbya dichotoma*

Floating organic matter (twigs, leaves) were collected from the Chimehuin River (Neuquén Province, Argentina) and taken to the laboratory in separate sterile polyethylene bags. The methods for collection and isolation of mycelium-forming organisms with a flagellated stage described by Coker (1923), Johnson (1956), Sparrow (1960), and Seymour & Fuller (1987) were used. Baits were placed in water culture in sterilized Petri dishes containing several halves of hemp seeds (*Cannabis sativa*) and incubated at room temperature (15–20 °C). When mycelial growth was observed on the seeds, a single hypha was isolated and transferred to cornmeal-agar medium (CMA) to obtain an axenic culture. After 3–4 d, a block of agar from the edge of each colony was cut off and placed in sterilized Petri dishes containing water. Several preparations were made for each sample and zoosporic organisms were identified using the vegetative organs (shape and size of the hyphae), asexual structures (shape of zoosporangium and spores), and sexual organs (structure of the oogonium and antheridium). Observations and measurements were made with an Olympus BX 40 microscope (Olympus Optical, Tokyo) equipped with phase contrast optics. During our investigations this new species was repeatedly isolated from the same locality in May and June 2009, and May 2010.

DNA extraction, PCR, sequencing and phylogenetic analyses

Reference strains used in this study were obtained from CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) and VKM (All-Russian Collection of Microorganisms, Moscow, Russian Federation) and are listed in Table 1. 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 (White *et al.* 1990) for the ITS region, EK 42F and EK 1498R for SSU rRNA gene (López García *et al.* 2001), and ITS4 and 28S-564R as described in Steciow *et al.* (2013). 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 provided in Table 1. Sequences from representatives from all genera from the *Saprolegnia-Achlya* clade present in GenBank for the three marker genes have been surveyed and collected to complete the alignments that generated the trees presented here. Some sequences from the sister-clade *Aphanomyces* were added as outgroup. Although several diverging nomenclatures were used by different sequence submitters, we kept original names as they were. The nomenclature used in this study is that proposed by Dick (2001) and Spencer *et al.* (2002). Sequences were aligned manually using BioEdit software (Hall 1999). The phylogenetic trees were reconstructed using a Maximum Likelihood approach as implemented in MEGA v. 6 (Tamura *et al.* 2013) using the following parameters: general time reversible model with invariant sites and four categories among site variation, and 1000 bootstraps to evaluate node robustness. Alignments for the three genes comprised respectively 559 bp for ITS region, 1633 bp for SSU rRNA gene and 785 bp for the LSU rRNA gene. Trees were rooted with sequences derived from *Aphanomyces* species and relatives, as these organisms are the closest known relatives of the *Saprolegnia-Achlya* clade (Lara & Belbahri 2011). Because publicly available sequences from different gene markers did not derive from the same organisms, we could not concatenate the three markers and proceed to a joint phylogenetic analysis. Therefore, we inferred a consensus tree on the basis of keeping every node that was supported robustly by at least one marker and not contradicted by another.

RESULTS

### Gene trees and consensus tree

The tree based on the ITS region showed a robust support for the monophyly of all genera except *Achlya*, with bootstrap supports ranging between 0.89 and 1 (Fig. 1). The genus *Leptolegnia* appears paraphyletic if *Geolegnia* is accepted as a separate genus; likewise, *Thraustotheca* appears nested within *Achlya*, turning the latter paraphyletic too. Deeper branchings (i.e. relationships between genera) are generally not supported, except for SAP1 and *Leptolegnia/Geolegnia* (bootstrap value = 0.97) and *Aplanes/Aplanopsis/Newbya* (bootstrap value = 0.99). This tree placed *Newbya,* at the base of the genera *Aplanes* and *Aplanopsis*. This branching

Table 1. List of the cultures analyzed in this study, including the CBS accession number and GenBank accession numbers for each gene sequenced.

Species	VKM, LPSC and CBS accession numbers	GenBank accession numbers		
		ITS	SSU	LSU
Achlya debaryana	VKM F-1904	KP098352	KP098371	KP098355
Achlya sparrowii	VKM F-2217	KP098344	KP098380	KP098356
Aplanopsis spinosa	CBS 576.67	KP098347	KP098365	KP098359
Aplanes treleaseanus	VKM F-2129	AB219373	KP098363	KP098360
Aplanopsis spinosa	CBS 577.67	KP098346	KP098366	KP098358
Leptolegnia caudata	CBS 113431	KP098341	KP098369	ND[1]
Leptolegnia caudata	CBS 680.69	KP098340	KP098370	KP098357
Leptolegnia sp.	CBS 392.81	KP098342	KP098367	ND[1]
Protoachlya paradoxa	CBS 261.38	KP098351	KP098375	ND[1]
Isoachlya humphreyana	CBS 110057	KP098343	KP098376	ND[1]
Pythiopsis intermedia	CBS 304.35	KP098348	KP098377	ND[1]
Pythiopsis terrestris	CBS 110058	KP098350	KP098378	KP098362
Pythiopsis terrestris	CBS 110059	KP098349	KP098379	ND[1]
Thraustotheca clavata	CBS 343.33	KP098354	KP098372	HQ665213
Leptolegnia caudata	CBS 359.35	KP098338	KP098368	ND[1]
Thraustotheca clavata	CBS 557.67	KP098353	KP098373	HQ665268
Newbya dichotoma	LPSC 877	KP098345	KP098364	KP098361

[1]ND = Non determined.

Table 2. Morphological features of the Saprolegnia-Achlya clade genera including discharge mode, sporangial characteristics and zoospore motile flagellate phases.

| Genus	Discharge mode	Sporangia	Flagellated zoospores
Saprolegnia	saprolegnoid	New sporangia renewed by internal proliferation	dimorphic (primary+secondary)
Pythiopsis	saprolegnoid	New sporangia renewed sympodially ,in cymose or basipetalous fashion	monomorphic (primary)
Protoachlya	protoachloid	Some zoospores swim and some encyst and form a loose cluster	dimorphic (primary +secondary)
Isoachlya	saprolegnoid	New sporangia formed by cymose branching or internal proliferation	dimorphic (primary+ secondary)
Scoliolegnia	saprolegnoid	New sporangia renewed by internal proliferation, cymose branching or basipetal succession	dimorphic (primary +secondary)
Newbya	achlyoid	Sporangia fusiform or filiform, renewed by cymose branching	monomorphic (secondary)
Aplanes	aplanoid or achlyoid	Sporangia cylindrical or fusiform; spores germinate in situ, inside sporangium	0 lack flagellate zoospores
Calyptralegnia	thraustothecoid or calyptralegnoid	Sporangia fusiform, cylindrical or clavate; renewed sympodially, or in a basipetalous or cymose fashion	monomorphic (secondary)
Aplanopsis	achlyoid or without sporangia	Sporangia usually unknown	no planonts were seen
Achlya	achlyoid	New sporangia renewed sympodially, in cymose or basipetalous fashion	monomorphic (secondary)
Thraustotheca	thraustothecoid	Sporangia clavate, obpyriform, fusiform, renewed sympodially or in cymose manner	monomorphic (secondary)
Leptolegnia	leptolegnoid	Sporangia cylindrical, elongate, sometimes renewed by internal proliferation, spores produced in a single row	dimorphic (primary+ secondary)
Geolegnia	geolegnoid	Sporangia cylindrical, elongate or swollen at intervals, nonseptate, formed sympodially, spores produced in a single row	0 lack flagellate zoospores
Dictyuchus	dictyucoid	Sporangia cylindrical to clavate, renewed sympodially or in basipetalous fashion, spores produced in a 1 or more than one row	monomorphic (secondary)
Brevilegnia	achlyoid, dictyucoid or brevilegnoid	Sporangia cylindrical to clavate, renewed sympodially, in cymose or basipetalous fashion, spores produced in a 1 or more than one row	monomorphic or not swimming
Aphanomyces	achlyoid	Sporangia filamentous, spores produced in a single row	dimorphic (primary lack flagella)
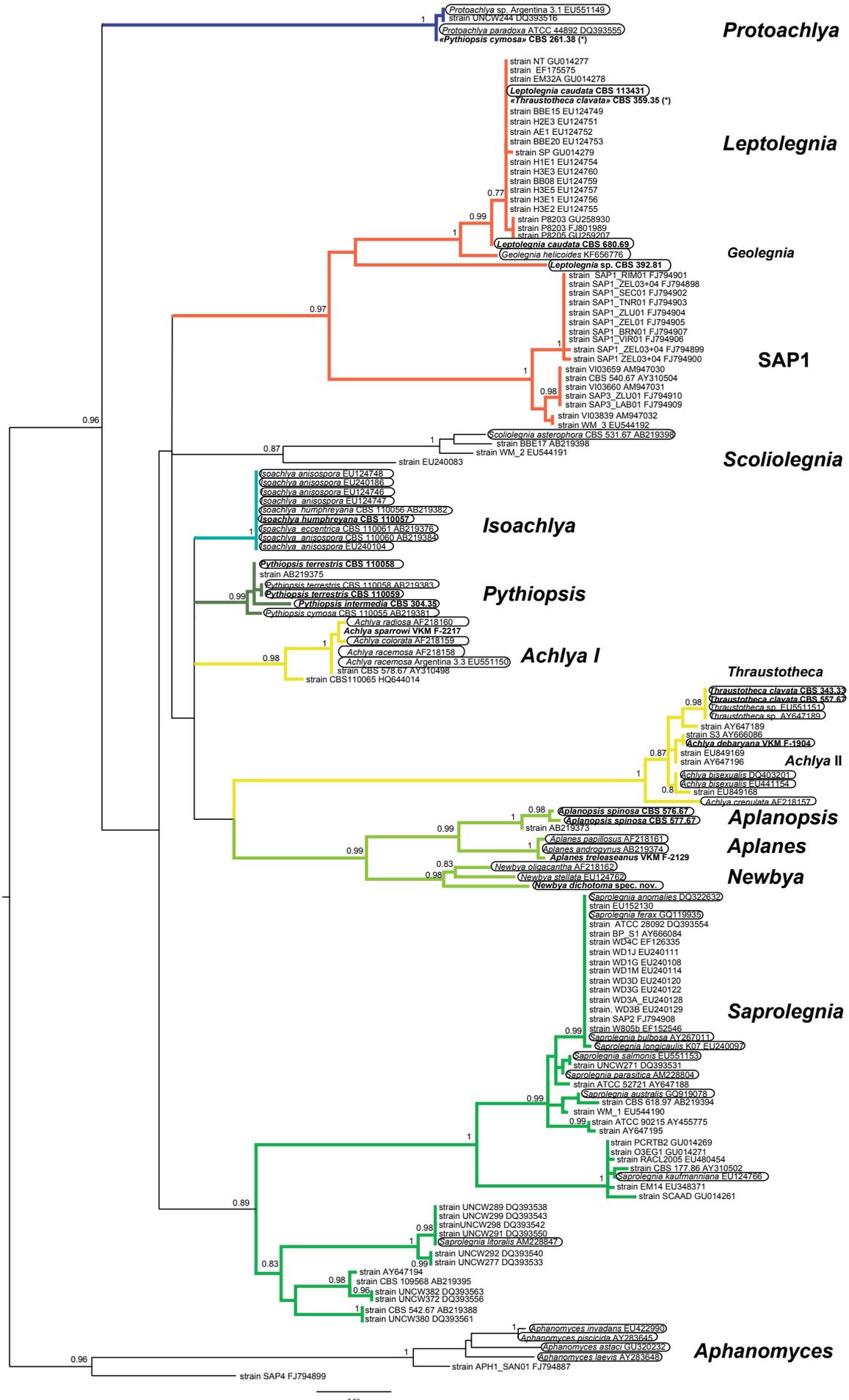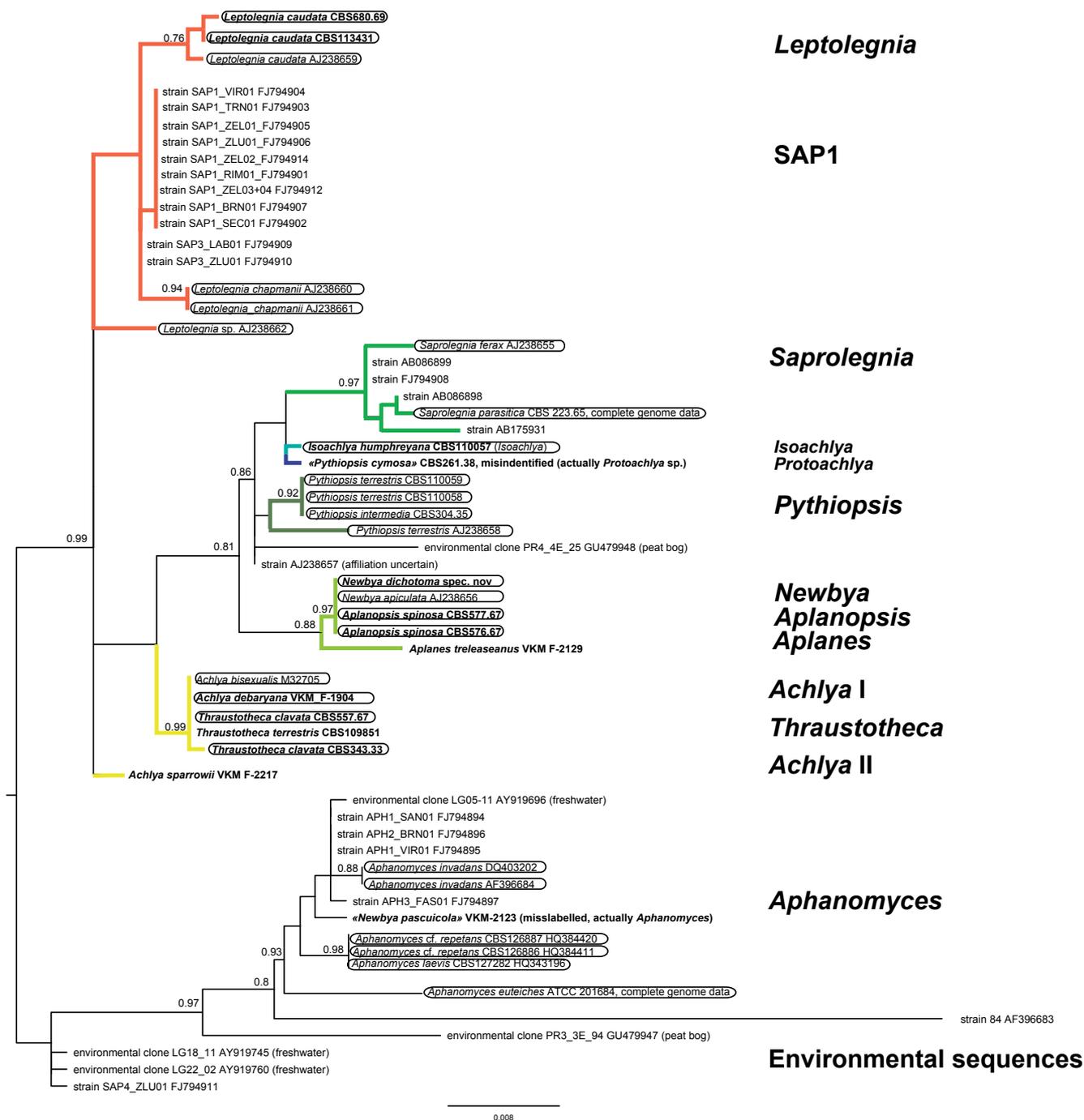
Fig. 2. Maximum Likelihood SSU rRNA gene phylogenetic tree showing the position of *genera* within *Saprolegniaceae*. The tree is rooted with sequences derived from *Aphanomyces* species and relatives. Species and isolates that have been studied morphologically have been enclosed. Sequences marked with an asterisk correspond to misidentified strains in culture collections.

pattern was contradicted by the SSU rRNA gene tree. The analysis based on the SSU rRNA gene (Fig. 2) respected the monophyly of all genera but did not give robust support to *Leptolegnia* and *Pythiopsis*. Genetic distances between sequences appeared visibly shorter on the tree, and distinct

species within genera or between closely related genera could not be discriminated; for example, *Aplanes treleaseanus* and *Newbya dichotoma* share the same sequence at the SSU rRNA level. Two supplementary deep branchings were supported by SSU rRNA, the first united *Saprolegnia*,

Fig. 1. (p. 442). Maximum Likelihood ITS phylogenetic tree showing the position of *genera* within *Saprolegniaceae*. The tree is rooted with sequences derived from *Aphanomyces* species and relatives. Species and isolates that have been studied morphologically have been enclosed. Sequences marked with an asterisk correspond to misidentified strains in culture collections.

Fig. 3. Maximum Likelihood LSU rRNA gene phylogenetic tree showing the position of *genera* within *Saprolegniaceae*. The tree is rooted with sequences derived from *Aphanomyces* species and relatives. Species and isolates that have been studied morphologically have been enclosed. Sequences marked with an asterisk correspond to misidentified strains in culture collections.
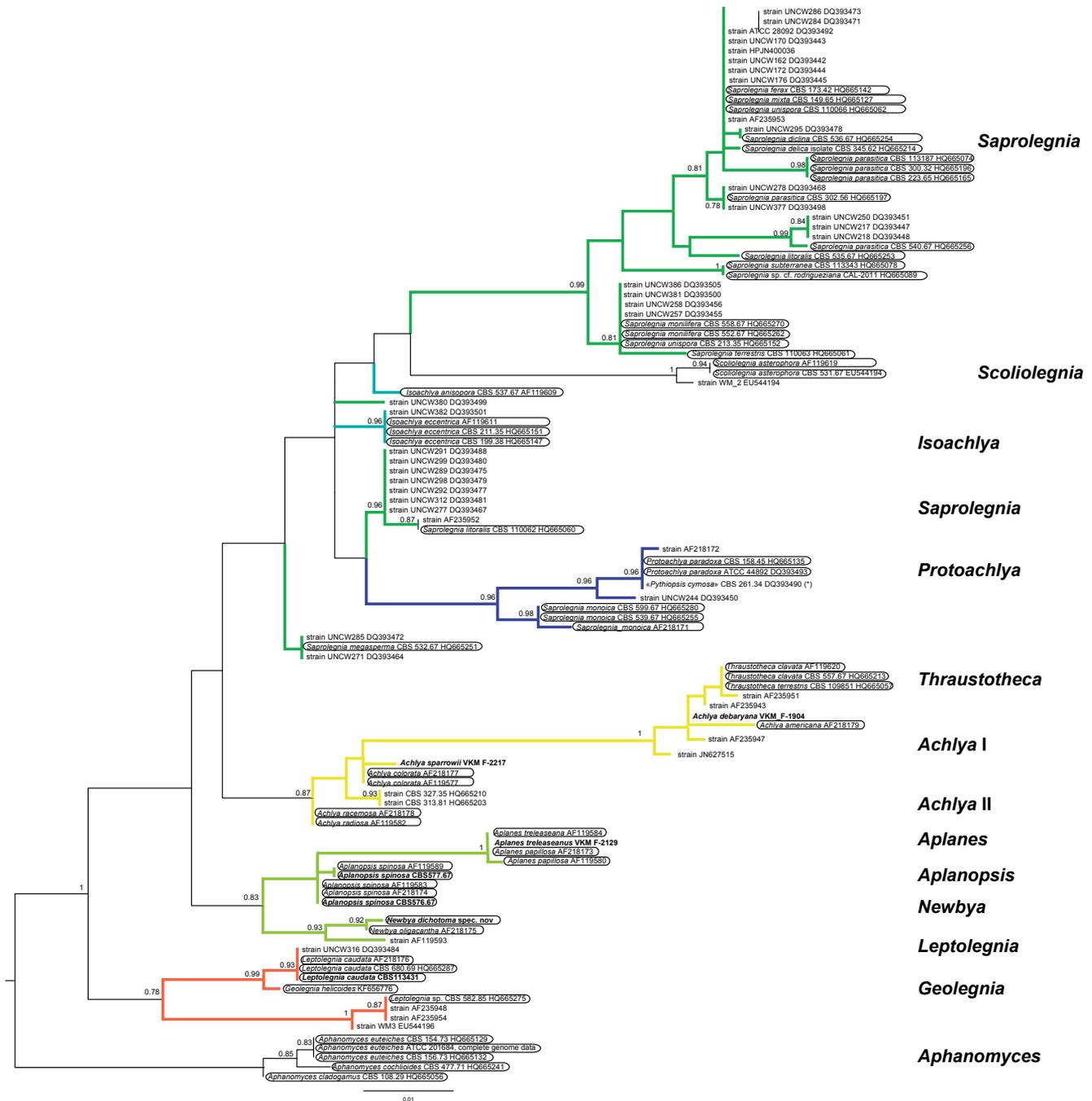
Isoachlya, *Protoachlya,* and *Pythiopsis* (bootstrap value = 0.86) and the second united this group to *Newbya* + *Aplanes* + *Aplanopsis* (bootstrap value = 0.81). In addition, the environmental sequence PR4_4E_25 GU479948 appears to belong probably to either a described genus that has not been surveyed with molecular tools, or perhaps even a totally new genus. Likewise, environmental clones LG18_11 (AY919745) and LG22_02 AY919760 seem to be related to both *Achlya-Saprolegnia* and the *Aphanomyces* group without any clear affinities for either of the two groups. In contrast, sequences PR3_3E_94 (GU479947) and LG05-11 (AY919696) branched within the *Aphanomyces* clade. The

phylogeny resulting from the LSU rRNA sequences (Fig. 3) showed a somewhat intermediate situation: some genera did not appear monophyletic (i.e. *Saprolegnia*, *Isoachlya*) or were not strongly supported (*Aplanopsis*). However, this analysis supported a robust (bootstrap = 0.87) branching between *Thraustotheca, Aplanes,* and *Aplanopsis*, a relationship that was not recovered in analyses based on the two other markers used (ITS and SSU rRNA).

The resulting consensus tree had the genera *Leptolegnia, Geolegnia*, and undescribed genus SAP 1 branching together, and also a group composed by *Aplanopsis, Achlya,* and *Thraustotheca* as the most basal known members of
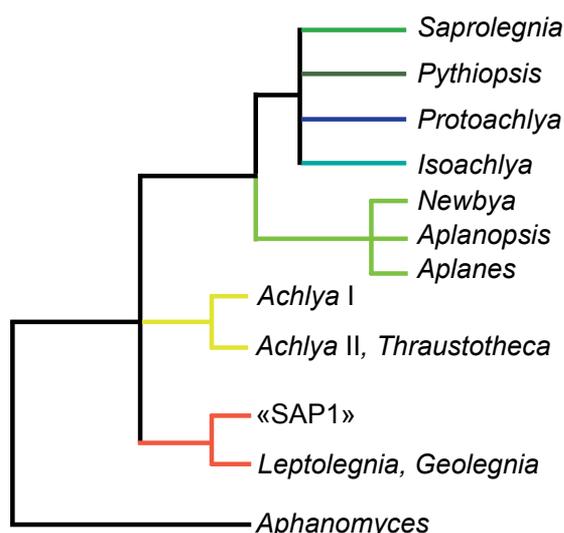
Fig. 4. Consensus tree of the Maximum Likelihood ITS, SSU and LSU rRNA phylogenetic trees showing the position of *genera* within *Saprolegniaceae*.

the group. *Newbya* and *Aplanes* occupied an intermediate position, while *Saprolegnia, Isoachlya*, *Protoachlya,* and *Pythiopsis* were resolved as the most derived (Fig. 4).

The genus *Achlya* divided into two relatively distinct groups, named here as *Achlya* I (containing *A. colorata* and *A. radiosa*), and *Achlya* II (for *A. bisexualis* and related strains).

Taxonomic reliability in GenBank

There were two types of errors in sequence assignment in this group of fungi in GenBank, taxonomic mistakes and incorrectly named material. The first type of mistake (referred to as type 1 hereafter) is caused by lumping different genera together by placing them into *Achlya, Saprolegnia* or *Pythiopsis*. This concerns, respectively, the genera *Scoliolegnia*, *Leptolegnia,* SAP1, part of *Isoachlya,* and *Protoachlya* all into genus *Saprolegnia*, and part of *Isoachlya* into *Pythiopsis* and *Aplanes,* and *Newbya* and *Aplanopsis* into *Achlya*. A second type (type 2) came from incorrect naming of strains, and occurs even when strains were apparently ordered from a public culture collection. Results of this investigation are presented in Table 2. We also faced the same problems with the strains we ordered: *Pythiopsis cymosa* (CBS 261.38) was actually a *Protoachlya*, *Thraustotheca clavata* (CBS 359.35) was *Leptolegnia caudata*, and *Newbya pascuicola* (the type species of *Newbya*), now lost in culture, possibly through contamination with an *Aphanomyces* (Fig. 2).

TAXONOMY

One strain isolated in the course of this study showed unique morphological features different from those of closely related species (Dick 2001) justifying its description as a new species. A detailed morphological description is provided below. Markers ITS and LSU rRNA successfully discriminated it from closely related species (Figs 1, 3), whereas the SSU rRNA gene was not variable enough to discriminate it from

"*Achlya*" *apiculata* (AJ238656; syn. *Newbya apiculata*), "*Achlya*" *spinosa* (CBS 576.67; syn. *Aplanopsis spinosa*), and *Aplanopsis spinosa* (CBS 577.67).

Newbya dichotoma Steciow, Lara & Belbahri, **sp. nov.**
MycoBank MB808617
(Figs 5–6)

Etymology: The name refers to the extremely well developed hyphae that are bifurcate (dichotomously branched), one or more times.

Diagnosis: Mycelium densum, cultura in seminibus *Cannabis sativa* ca. 1.5–5.0 cm diam. Hyphae ramosa, pleraque 30–90 µm late ad basim. Zoosporangia parcus in culturis juvenilibus filiformia vel fusiformia, 111–800(–1800) × (19–)24–72 µm, basipeta vel cymosa. Ejecto sporarum pro genus typica, cystae globosi 10–12 µm. Gemmae frequentis. Oogonia copiosa, apiculata, sphaerica, pyriformia vel doliiformia, vel paulo abnormia (27–)45–87(–146) µm. Paries oogoni foveatus, laevis; ramulus lateralibus vel terminalibus provenientia, 19–63(–437) µm. Oosporae subcentrici; (1–)2–5(–7) per oogonium, (19–)25–31(–41) µm. Ramulus antheridialis ramosus, monoclina, diclina, et androgina.

Type: **Argentina**: Neuquén Province, Chimehuín River, at 7 km from Junin de los Andes town, (39°54'59.64"S 71° 06'18.39"O), on floating organic matter, in water associated with aquaculture ponds where salmonids (*Onchorhynchus mykiss,* and *Salmo trutta*) and atherinopsids (Patagonian silverside: *Odonthestes hatcheri*) are raised, May 2010, *M. M. Steciow* (LPS 47444 – holotype; LPSC 877 – ex-type culture).

Description: Monoecious. Two-week-old pure cultures on hemp seeds 1.5–5.0 cm diam. *Mycelium* dense, extensive; principal hyphae slender or stout moderately branched, finishing in characteristic dichotomous branching near apices; 30–90 µm wide at the base. *Gemmae* sparse or abundant with the age of the water culture; cylindrical, fusiform, irregular, or branched; terminal or intercalary, single or catenulate, developed at the end of the hyphae functioning as zoosporangia. *Zoosporangia* sparse, slender; filiform, or fusiform; often tapering to the elongate apex; frequently straight, curved, bent or sinuous; (80–)150–800(–1300) × 20–30 µm; renewed sympodially or in cymose disposition, rarely in basipetalous succession. *Zoospores* monomorphic; discharge and behaviour achlyoid; primary spore cysts, 10–12.5 µm diam. *Oogonia* variable in abundance or abundant with the age of the culture; lateral or terminal, occasionally intercalary, single or catenulate; apiculate, often spherical, subglobose, pyriform, or very rarely oval or irregular, occasionally doliiform, very rarely filiform; oogonia very variable in size and number with the age of the culture, often 20–40 µm diam in average when small and developing as normal ones, and predominantly 45–90(–110) µm diam when bigger. *Oogonia* with oospores that can mature or not inside and develop into a lower number of oospores, often unique, mainly with a great size;
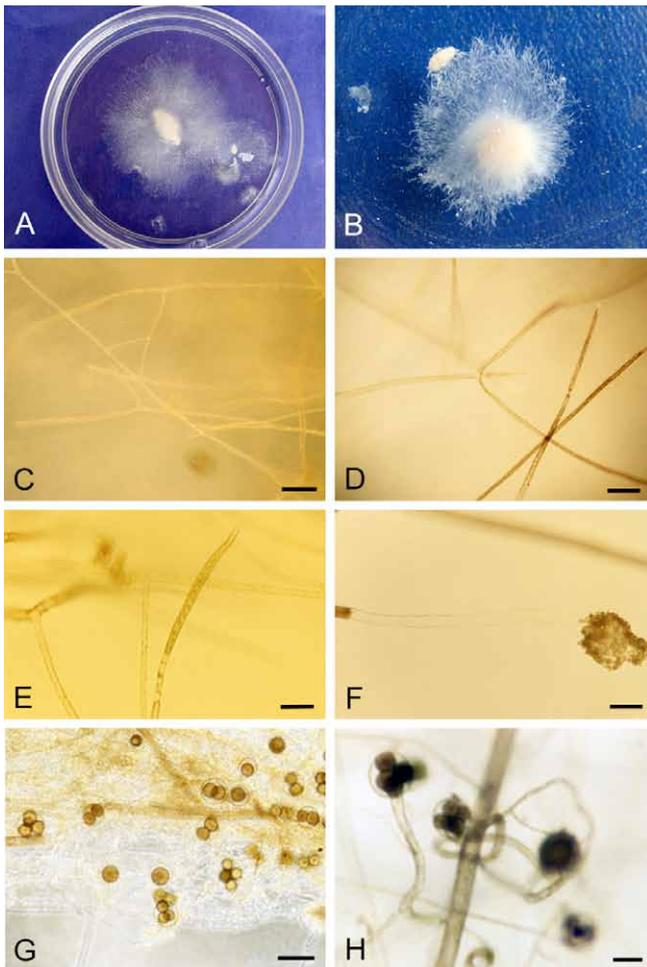
Fig. 5. Morphological features of *Newbya dichotoma* (LPS 47444) **A.** A young water culture with zoosporangia at the tips of the mycelium. **B.** A 2-wk-old hemp seed colony in water culture. **C–D.** Characteristic dichotomous branching of hyphae repeated in one or several times. **E.** Zoosporangium filiiform, tapering to the elongate apex. **F.** Zoosporangium showing typical achlyoid discharge. **G.** Ooogonia very variable in size, maturity and number of oospores inside, with the age of the culture. **H.** Oogonial stalks variable in length, curved, bent, coiled or straight, often branched. Bars: C = 200 μm, D–E, G = 100 μm; F, H = 25 μm.
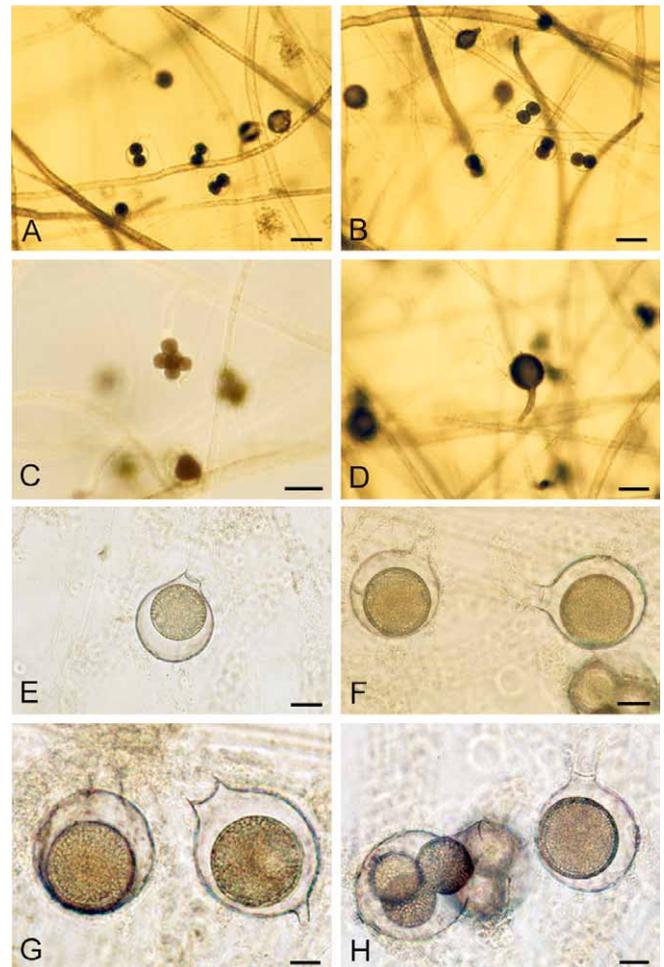

Fig. 6. Morphological features of *Newbya dichotoma* (LPS 47444) **A–B**. Mycelium with immature and some mature oogonia, often apiculate, on variable oogonial stalk, straight, bent or curved. **C.** Detail of a mature apiculate oogonium with a monoclinous antheridial branch. **D**. Immature oogonium with diclinous branches. **E–H.** Mature oogonia apiculate, spherical to subglobose, showing, in detail, the subcentric oospores type I, variable in number, size and maturity. Bars: A–D = 25 μm, E–H = 5 μm.

very frequent proliferation of immature and mature oogonia. *Oogonial wall* smooth, slender, (very rarely with a lateral papillate projection); unpitted, or pitted only under point of attachment of antheridial cells; inner surface occasionally irregular. *Oogonial stalks* variable in length, usually 0.5–4(– 9) times the diameter of the oogonium; slender and short or stout and longer; often curved, bent, twisted and often once-several times coiled, rarely straight; often branched. *Oospores* almost always maturing inside normal oogonia, or not maturing inside some of the greatest or abnormal ones; subcentric type I; spherical or ellipsoidal, or irregular when immature; (1–)2–5(–7) per oogonium, filling it or not filling the greatest or abnormal oogonia with often a greater normal-mature or abnormal not maturing oospore inside; (20–)25–35 μm diam inside smaller oogonia, and (35–) 50–70(–80) μm diam inside greater oogonia. *Antheridial branches* usually abundant; mainly monoclinous, often

diclinous, rarely androgynous; slender, irregular; abundantly branched; persisting. *Antheridial cells* simple, short; tubular to irregular; persisting; laterally appressed; fertilization tubes not observed.

Notes: The genus *Newbya s. str.* includes species previously named *Achlya* and now separated as: *N. apiculata, N. braziliensis, N. curvicollis, N. megasperma, N. oblongata, N. oblongata var. gigantica, N. oligacantha, N. pascuicola* (type species)*, N. polyandra, N. recurva, N. spinosa,* and *N. stellata* (Spencer *et al*. 2002).

Newbya dichotoma resembles *N. apiculata*. Both species have smooth oogonia that are spherical or apiculate, borne on slender or stout, straight, curved, bent, twisted or more characteristically coiled oogonial stalks, one to several times. The oogonial stalks of *N. apiculata* are branched, and the antheridial branches are predominantly monoclinous.

Newbya apiculata forms centric or preferentially subcentric oospores, reaching (20–)35–40(–48) µm diam.

In contrast, in *N. dichotoma*, the oogonia contain a more reduced number of only subcentric oospores (1–7 *vs*. 1–28 in *N. apiculata*), which are often variable in size and number within the same thallus. Oogonia containing only a few oospores predominate: one, two, or commonly up to four oospores, which can reach a relatively large diameter: 50–70(–80) µm diam. *Newbya dichotoma* also develops a considerable number of oogonia containing 3–5(–7) smaller oospores, that reach only 25–35 µm diam (Figs 5–6).

DISCUSSION

A major problem in the taxonomy of *Saprolegniales* that is encountered, especially in the *Saprolegnia-Achyla* clade, is the lack of clear and unique synapomorphies that can define genera. Rather, genera are defined by a combination of traits, which seem to revert frequently to ancestral states or even disappear along evolution (Dick *et al.* 1999, Dick 2001). A good example is the genus *Geolegnia*, represented here by *G. helicoides,* that has lost the entire flagellar apparatus in all life-stages seen in *Leptolegnia*-like ancestors (Steciow *et al*., 2013). An illustration of these changing characters is given in Table 2. It is, for instance, hazardous to infer the morphology of the ancestor of the whole clade. As in better known fungal groups such as the basidiome-forming *Agaricomycotina*, the combination of various traits, rather than single ones, is the only possibility to identify species morphologically (Zmitrovich & Wasser 2011). Some other traits that can possibly be discriminating for the different subgroups can be inferred by searching into the metabolism of these organisms and their enzymatic machinery. For instance, members of the genus *Leptolegnia* are known to degrade chitin (Hochwimmer *et al*. 2009), being important mosquito pathogens (Pelizza *et al*. 2011). The related *Geolegnia helicoides* has also been isolated from mosquitos (Steciow *et al*. 2013) and the equally related undescribed genus "SAP-1" (Wolinska *et al*. 2009) on cladocerans. It is therefore likely that the ability to use chitin is a common feature of this group (Fig. 4). Other biochemical characteristics, not necessarily directly related to pathogenesis, might be found in other clades, and a good barcoding strategy appears to be the most reasonable alternative way to identify these organisms.

For this purpose, we tested the resolution power of three common barcoding genes used for many protists and fungi (Pawlowski *et al*. 2012). ITS clearly had the best potential for species barcoding purposes, as it separated closely related species within genera most efficiently (Robideau *et al*. 2011). ITS has, however, been shown not to be reliable for other (presumably older) clades of oomycetes such as *Pythium s. lat.* (Levesque & DeCock 2004), but it is perfectly suited in the context of the *Saprolegnia-Achyla* clade. Deeper nodes appear most often unresolved by ITS, and there ribosomal genes are more useful and have been recommended for general oomycete phylogeny reconstruction (Riethmüller *et al*. 1999, Leclerc *et al*. 2000, Lara & Belbahri 2011). As the SSU rRNA gene is often used in environmental DNA surveys, it can be a potential source of discovery for new species

by revealing the phylogenetic position of organisms whose existence was unsuspected (Fig. 2) and which can potentially be emerging parasites, or possibly also important ecological actors, as the ecological role of free-living oomycetes remains a largely uncharted territory (Lara & Belbahri 2011).

Our study clarifies the phylogenetic relationships within the *Saprolegnia-Achyla* clade based on a combination of morphological features and a consensus tree derived from three phylogenetic markers. We demonstrate that the divisions between genera proposed by Spencer *et al.* (2002) were justified and further confirm that this clade is more genus-rich than initially proposed (Spencer *et al.* 2002, Hulvey *et al.* 2007). The formal description of *Newbya dichotoma* provided in this study illustrates the need for an effort towards culturing new strains and describing them morphologically prior to their genetic characterisation. Still, some genera probably associated with this clade have not been surveyed, such as *Brevilegnia*, *Calyptralegnia*, *Dictyuchus,* and *Scoliolegnia,* and they need to be targeted by these same markers in future studies. Here, we have, however, laid the basis of a taxonomic framework for the efficient recognition and description of pathogens within this clade of organisms of major economic and environmental concerns.

A good reference database is logically the most basic prerequisite for an effective barcoding strategy, the other being good marker genes that allow species discrimination (Hebert *et al.* 2003). It emerges from our survey that an uncritical use of GenBank as a reference database can be a source of mistakes that may only amplify the problems when taken as a basis for further studies. Type 1 mistakes (wrong taxonomy) might seem less problematic than type 2 (incorrect identifications); they are, however, a problem for non-specialists who want to use oomycete sequences as references in their phylogenies, thus encountering pervasively para/polyphyletic genera. These mistakes can be a problem in environmental DNA diversity surveys. Type 2 mistakes are of course of major concern when unknown strains have to be identified molecularly, and can lead to misinterpretations that can bear heavy consequences for wildlife management or economic issues. Therefore, we recommend that mistakes that have been identified are corrected, and further that cultures made available from culture collections are checked for such inconsistencies.

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