Ann Microbiol (2013) 63:253–258
DOI 10.1007/s13213-012-0468-x

ORIGINAL ARTICLE

A new species, *Pythium echinogynum*, causing severe damping-off of tomato seedlings, isolated from Tunisia, France, and India: morphology, pathology, and biological control

Afef Balghouthi · Rinita Jonathan · Sabine Gognies · Ahmed Mliki · Abdelkader Belarbi

Received: 23 February 2012 / Accepted: 12 April 2012 / Published online: 25 May 2012

Abstract *Pythium echinogynum*, sp. nov. was isolated from soil samples taken from the vineyards of Tunisia, France and also from a lawn sown with turf grass in India. The oomycete is characterised by the presence of both ornamented and smooth walled oogonia. The ornamented oogonia are provided with blunt spines that can be at times curved, 1–2 monoclinous antheridia that can at times wrap around the oogonia, and mostly aplerotic oospores. The oomycete also produces elongated oogonia measuring up to 65 μm long and 24 μm in breadth. The internal transcribed spacer (ITS) region of the rRNA of this new species is comprised of 975 bp and closely resembles (95.9 %) that o *P. spiculum* and forms a clade together with members of the ornamented or spiny oogonia like *P. mammilatum*, *P. spinosum* and *P. irregulare* but also with those producing smooth-walled oogonia like *P. paroecandrum*, *P. sylvaticum* and *P. cylindrosporum*. However, it has its own characteristics, which are quite distinct from all other species of this genus described so far. In pathogenicty tests, the oomycete was found to be a severe "damping off pathogen" to tomato and cucumber seedlings. However these symptoms were not produced when the new species was grown together with *P. lycopersicum*—a newly described mycoparasite. The taxonomic description of this new species, its comparison with related oomycetes, the sequence of the ITS region of its rRNA gene, phylogenetic tree with related species and some account of its pathology to tomato and cucumber seedlings are discussed in this article.

Keywords *Pythium echinogynum* · Tomato pathogen · Damping off · Mycoparasite

A. Balghouthi · A. Mliki
Laboratoire de Physiologie moléculaire des Plantes,
Centre de Biotechnologie de Bordj Cedria,
Hammam Lif, Tunisia

R. Jonathan (✉) · S. Gognies · A. Belarbi
Laboratoire de Microbiologie Générale et Moléculaire,
Université de Reims Champagne-Ardenne,
Moulin de la Housse, BP 1039, 51687 Reims, France
e-mail: rjonathan86@gmail.com

Introduction

Members of the genus *Pythium* are distributed throughout the world (Middleton 1943). Most of these are "amphibians" that are found in terrestrial as well as aquatic environments. The "oomycetes" are no longer classified as 'true fungi" and are now thought to be closer to 'algae' than to the fungal word. However, their behaviour, mode of nutrition, reproduction and their complete lack of chloroplasts are all reminiscent of their fungal nature. Quite a number of members of the genus *Pythium* are known for their ornamental oogonia (female gametangia), of which the most common is *Pythium echinulatum*. The spines found on the oogonia are of great taxonomic value. Plaats-Niterink (1981) recognised 21 species having ornamented oogonia. Since then, six more species with ornamented oogonia have been added to the group: *Pythium ornamentum*, *P. radiosum*, *P. ornacarpum*, *P. spiculum* and *P. apiculatum* (Paul 1987, 1992, 1999, 2006; Paul et al. 2006), and, *P. solare* (De cock et al. 2008).

The new species, *P. echinogynum* is being erected on the basis of its morphological characteristics, and the sequence of the ITS region of its rRNA. *P. echinogynum*, was isolated from soil samples taken from the vineyards of Tunisia, France and also from a lawn sown with turf grass in India. This new oomycete has been found to provoke serious damping off disease in tomato and cucumber.

Materials and methods

Oomycete isolates

Soil samples, together with plant root debris were collected in sterile capped bottles from vineyards in Nabeul, Tunisia (latitude 36.45, longitude 10.73), Marsannay, France (latitude 47.26, longitude 4.98) and from turf grass in Nagpur India (latitude 21.15, longitude 79.1). These were brought to the laboratory in France (Laboratoire de Microbiologie, Université de Reims, Champagne Ardenne). Oomycetes were isolated from these samples by usual baiting techniques as described elsewhere (Middleton 1943; Paul 1987), and purified by repeated culturing in sterile distilled water. Oomycetes were ultimately grown and maintained on a solid medium such as potato carrot agar (PCA) or potato dextrose agar (PDA). The temperature-growth relationship of the oomycetes was determined as grown on PCA incubated at 25°C. The type culture has been deposited at the CBS collection (Utrecht, the Netherlands).

Three isolates were identified as *Pythium echinogynum* with the help of keys provided by Middleton (1943), Waterhouse (1967), and Plaats-Niterink (1981), and also by its sequences using the BLAST search. The isolates taken from soil samples in Nabeul, Tunisia (Tun-11A), Marsannay, France (F-1234A), and from turf grass roots in Nagpur, India (SU 8A) showed 100 % similarity in the sequences of the ITS region of their rDNA. The type specimen is SU 8A as it readily forms the characteristic ornamented oogonia.

DNA extraction and PCR amplification

Mycelium of all isolates was taken from a 3-day-old culture on PCA. A small block of this medium together with the oomycete was introduced aseptically into a sterile bottle containing potato dextrose broth (PDB). This was then placed on an orbital shaker for 3 days at 25°C. The mycelium was then harvested and oomycete DNA extracted and purified using protocols described earlier (White et al. 1990; Paul et al. 2006; Moralejo et al. 2008; Belbahiri et al 2006). The ITS region of the oomycete rRNA was amplified by polymerase chain reaction (PCR) with universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC). The amplification program contained three steps: an initial denaturation step of 95°C for 2 min, followed by 30 cycles of denaturation at 95° C for 20 s, annealing for 25 s at 55°C and extension for 50 s at 72°C (Cooke et al. 2000). PCR products were separated on 1 % agarose gels in 1 % TAE, subjected to 100 V for 1 h and visualized under UV light.

The amplified PCR products were sequenced by Cogenics France (Beckman Coulter Genomics, Grenoble, France). The sequences obtained were compared with the ITS sequences of related species of *Pythium*: *P. intermedium* (AY598647), *P. attrantheridium* (AY 286014), *P. abappressorium* (DQ091294), *P. viniferum* (AY455694), *P. debaryanum* (AY598704), *P. sylvaticum* (AY598645), *P. kunmingense* (AY598700), *P. spinosum* (AF492017), *P. macrosporum* (AY598646), *P. cylindrosporum* (AY 083591), *P. regulare* (AF492018), *P. cryptoirregulare* (AY907896), *P. paroechandrum* (AY598644), *P. mamillatum* (AY 598703), *P. spiculum* (DQ 205094), *P. terrestris* (AY039714), *P. apiculatum* (DQ211530) and *P. violae* (AY598706). The sequence of the ITS region of the rRNA of the new species has been deposited with GenBank (accession FJ 660490).

Pathology and control

To check whether *P. echinogynum* was a plant pathogen, we conducted a series of experiments on cucumber and tomato seeds. We also used a recently described mycoparasite (*Pythium lycopersicum*) to biologically control any disease caused by this new species. Two sets of three plastic pots containing sterile compost were sown with cucumber and tomato seeds. (A = seeds alone, B = seeds + *P. echinogynum*, C= seeds + *P. echinogynum* + *P. lycopersicum*). This experiment was repeated three times. All the pots were incubated at room temperature (20°C).

After completion of the experiment, the tomato and cucumber seedlings were taken out of the pots, washed thoroughly and placed in Petri-dishes containing sterile distilled water and boiled hemp seed halves in order to re-isolate the oomyceteous organism from the diseased seeds.

Results

Pythium echinogynum sp. nov.

Pythium echinogynum sp. nov. is illustrated in Figs. 1, 2, 3, and 4.

Sporangio et zoosporis non observata, Corporibus hypharum globosa, cylindrosa, intercalaria, vel terminalia, 10–35 μm diam., zoosporae non observata. Oogonia intercalaria, catenaria raro terminalia, globosa, cylindrosa, 9–23 μm diam. ornata spiculis 3–7 μm longis. Antheridia, monoclinata, circa singulum oogonium formans nodum circum,complectentia oogonium; cellulae antheridiales inflatae Oosporas, pleroticas vel apleroticas, globosas vel cylindrosas, 8–26 μm diam, paries 0.5–1,5 μm crassus. Incrementum radiale quotiadianum 25 mm 25°C in agaro Solani tuberosi et Dauci carotae (PCA). Holotypus in herbario Universitatis Bourgogne conservatus (SU-8A).

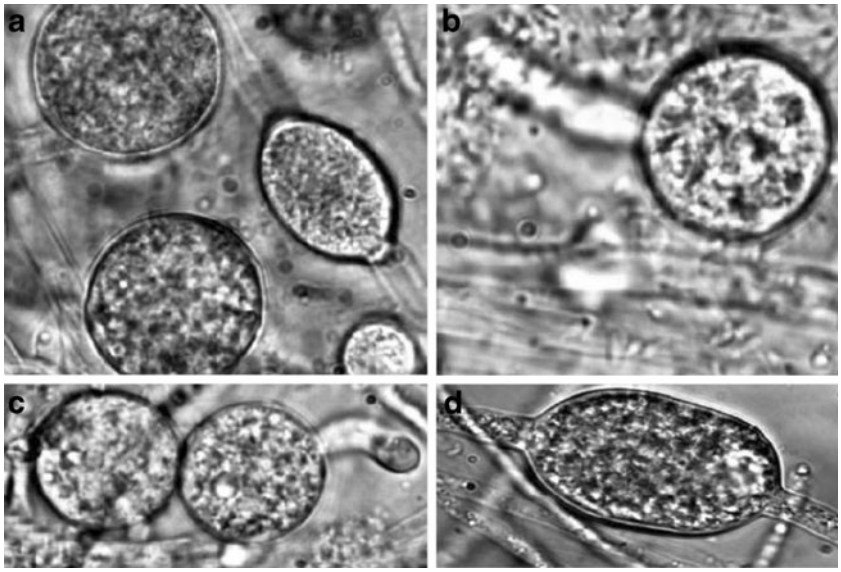

Fig. 1 Asexual and sexual reproductive bodies of *Pythium echinogynum*. **a** Spherical to elongated hyphal bodies. **b**, **c** Germinating hyphal bodies. **d** Elongated, intercalary hyphal body. *Bar* 30 μm

Morphological characteristics

Pythium echinogynum

The oomycete (SU 8A) grows well both on solid media as well as on hemp-seed halves in water. Its mycelium in water is hyaline-to-whitish. Colonies on PCA are submerged and show a broad Chrysanthemum pattern. Average radial growth of the fungus at 25°C on PCA is 25 mm day^{-1}.

Sporangia or hyphal bodies are produced in plenty (Fig. 1a–d). These are spherical (Fig. 1a), elongated or cylindrical (Fig. 1d) and mostly intercalary or catenulate. The spherical hyphal bodies measure from 10 to 35 μm in diameter (average 23 μm). The elongated hyphal bodies can measure up to 50 μm in length and 30 μm in breadth. Although produced plentifully, these structures fail to produce any zoospores. Repeated flooding of the cultures with sterile distilled water, tap, pond water, and also their incubation at different temperatures failed to sporulate the oomycete. However, the hyphal bodies devoid of zoospores were formed readily and these germinated through one to three germ tubes to form new colonies (Fig. 1b,c).

The female gametangia (oogonia) are mostly ornamented with spines but in young cultures these can be smooth walled. The oogonial spines measure between 1.5 and 7 μm in length and are mostly blunt and at times slightly curved (Fig. 3i). The smooth walled oogonia are mostly terminal (Fig. 2a–c) while the ornamented ones are usually intercalary or catenulate

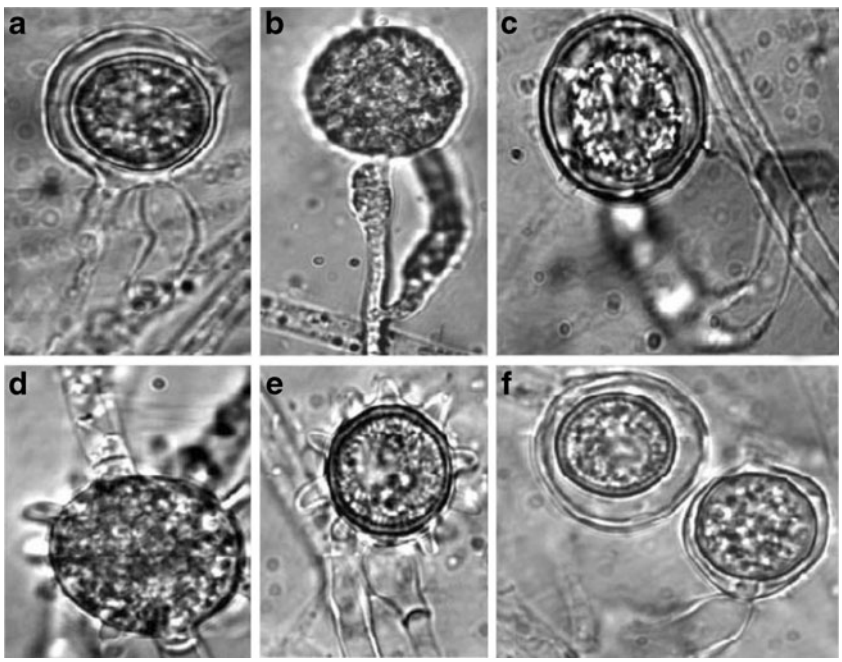

Fig. 2 Sexual reproduction of *P. echinogynum*. Oogonia, antheridia and oospore. **a**–**c** Smooth walled oogonia with monoclinous antheridia. **d**–**e** Ornamented oogonia with blunt spines. **f** Aplerotic oospores. *Bar* 25 μm

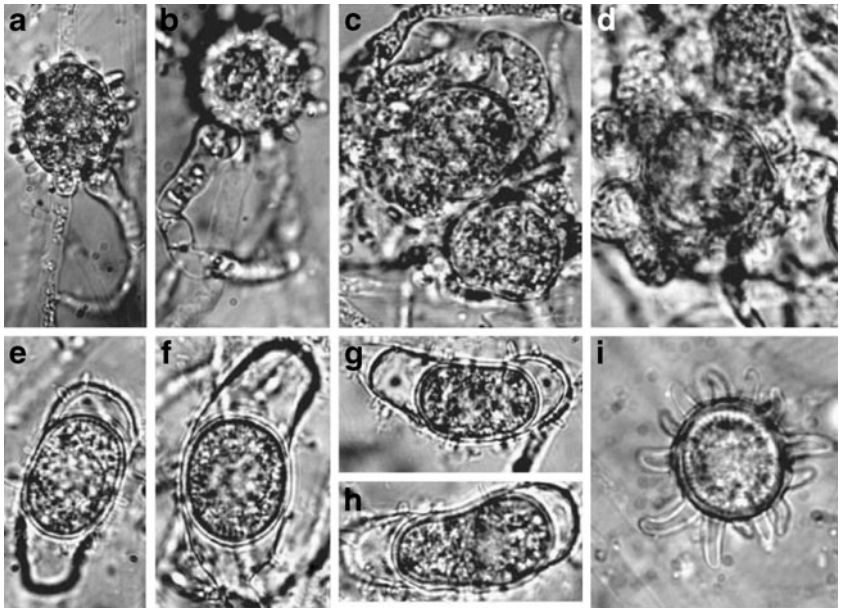

Fig. 3 Sexual reproduction of *P. echinogynum*. Ornamented and elongated oogonia. **a**–**b** Ornamented oogonia with monoclinous antheridia. **c**–**d** Inflated antheridial branches wrapping around the oogonia. **e**–**h** Elongated to cylindrical oogonai having oval to peanut shaped aplerotic oospores. **i** Ornamented oogonia with slightly curved spines and plerotic oospore. *Bar* 35 μm

(Fig. 2d–e). Oogonia (without spines) measure 9–23 μm in diameter (average18 μm). These are often spherical, but at times elongated. (Fig. 3e–h).The elongated oogonia can measure up to 65 μm long and 24 μm wide.

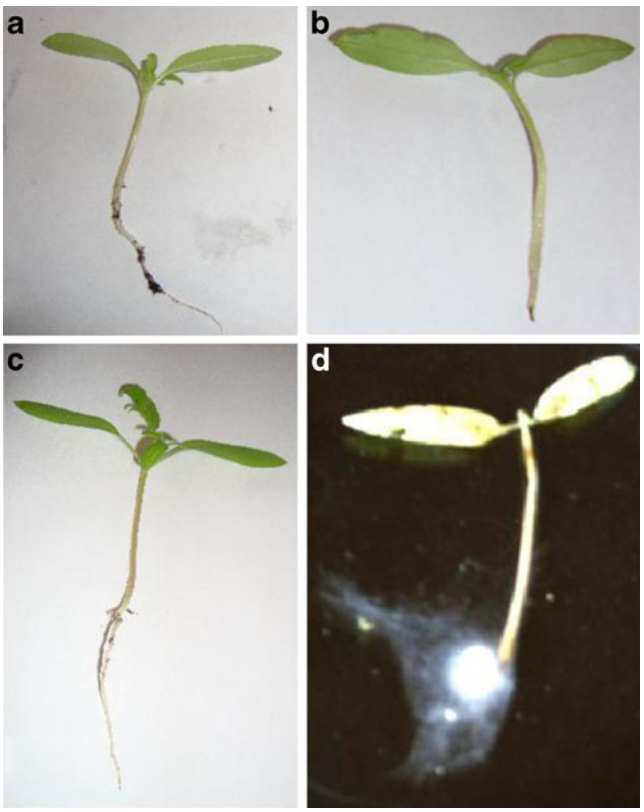

Fig. 4 Damping off of tomato seedlings. **a** Normal tomato seedling. **b** Damping off of tomato seedling caused by *P. echinogynum*. **c** Healthy growth of tomato seedling in the presence of *P. echinogynum* and *P. lycopersicum*. **d** fishing out of *P. echinogynum* from diseased tomato seedling on hemp seed

The male gametangia (antheridia) are mostly monoclinous and unique giving rise to one prominent antheridial cell having an apical contact with the oogonia (Figs. 2a–c, 3a–b) However, some of the oogonia are supplied with many-branched antheridia that completely wrap the female gametangia. The antheridial elements in this case are inflated to toruloid elements, which makes it difficult to observe the number and the mode of contact of the antheridia (Fig. 3c–d).

The zygotes (oospores) in the spherical oogonaia are mostly aplerotic (Fig. 2a,f) but at times these can be plerotic (Figs. 2c,e, 3i). However in the elongated oogonia these are invariably aplerotic (Fig. 3e–h). They are generally spherical, but in cylindrical oogonia these can be somewhat elongated or peanut-shaped. The spherical oospores measure between 8 and 26 μm in diameter, while elongated or cylindrical oospores can measure up to 45 μm in length (Fig. 3e–h).The oospore wall is relatively thin, measuring 0.5–1.5 μm.

In the presence of *P. echinogynum*, the tomato plant is wilted (Fig. 4b), showing characteristic ‘damping-off’ symptoms. In others pots, where the oomycete was introduced together with the mycoparasite *P. lycopersicum*, the seedlings were healthier (Fig. 4c) despite the presence of the pathogen. The diseased seedlings, when floated and baited with hempseed in a Petri dish containing sterile distilled water, produced luxuriant colonies on the hemp seeds within days. Hence we were able to fish out oomycetes corresponding to our new species, *P. echinogynum* from the diseased seedlings (Fig. 4d).

Discussion

Isolates of *Pythium echinogynum* were collected from soil samples taken from a vineyard in Nabeul, Tunisia (Tun-11A), Marsannay, France (F-1234A), and from turf grass

Table 1 Some morphological features and molecular characteristics of *Pythium spiculum* and *Pythium echinogynum*. *ITS* Internal transcribed spacer

Characters	*P. spiculum*	*P. echinogynum*
ITS region	945 bases	957 bases
Sporangia	Both terminal and intercalary, 15-33 μm zoospore not produced	Spherical, 10-35 μm, at times elongated up to 30×50 μm intercalary, catenulate, irregular, zoospores not produced
Oogonia	Spherical, cylindrical to peanut-shaped	Mostly spherical (9-23 μm in diameter) and ornamented with blunt spines, spherical but at times elongated and cylindrical measuring up to 24×65 μm, terminal or intercalary
Antheridia	Monoclinous and diclinous, one to three in number, never surrounding or encircling the oogonia	Monoclinous, usually one per oogonia. Sometimes many antheridial branches wraps around the oogonia
Oospores	Globose, cylindrical to peanut-shaped, plerotic and aplerotic, double oospores present, oospore wall 0.5-1 μm in diameter	Spherical to peanut shaped, plerotic, aplerotic in cylindrical oogonia, double oospores present, wall 0.5-1 μm in diameter

roots in Nagpur, India (SU 8A). All three isolates are morphologically similar and the ITS region of their rRNA has 100 % similarity. The type material SU 8A from India has been deposited with the CBS culture collection in Utrecht. The presence of this oomycete in three different countries indicates its adaptation to different climatic conditions. The sequence of the ITS region of its rRNA, which consists of 975 bases (ITS1, 5.8 S and ITS2), has been submitted to GenBank (accession no. FJ660490).

Pythium echinogynum lacks zoosporangia and zoospores and hence is perfectly adapted to its terrestrial habitat. It is provided with intercalary or catenulate hyphal bodies, ornamented oogonia, and thick-walled oospores. In old cultures, the oogonia and oospores are elongated and somewhat irregular in shape. The morphology and temperature–growth relationship of the oomycete brings it close to other species of the genus bearing ornamented oogonia, such as *P. mamillatum*, *P. spinosum*, *P. apiculatum*, *P. spiculum*, and also with non-ornamented species like *P. paroecandrum*, *P. cylindrosporum*, *P. macrosporum*, *P. intermedium*, *P. viniferum* and *P. regulare*. The closest relative is *P. spiculum* (GenBank accession, DQ205094.1)—a new species described from soil samples taken in France, Spain and Portugal (Paul et al. 2006) with a 95.9 % resemblance. However, some morphological features and molecular characteristics differ (Table 1).

Pathological experiments show clearly that *Pythium echinogynum* is a plant parasite as it can rapidly induce the "damping off" of tomato and cucumber. In our experiments the oomycete can induce both "pre-emergence" and "post emergence" damping off. The growth of the oomycete on the infected seedling when kept in water together with hemp seed is yet another proof that the oomycete is the causative agent of the disease. In bio-control experiments, when tomato and cucumber seeds were inoculated with *Pythium echinogynum* in the presence of a recently described mycoparasite, *Pythium lycopersicum* (Karaca et al. 2008), no

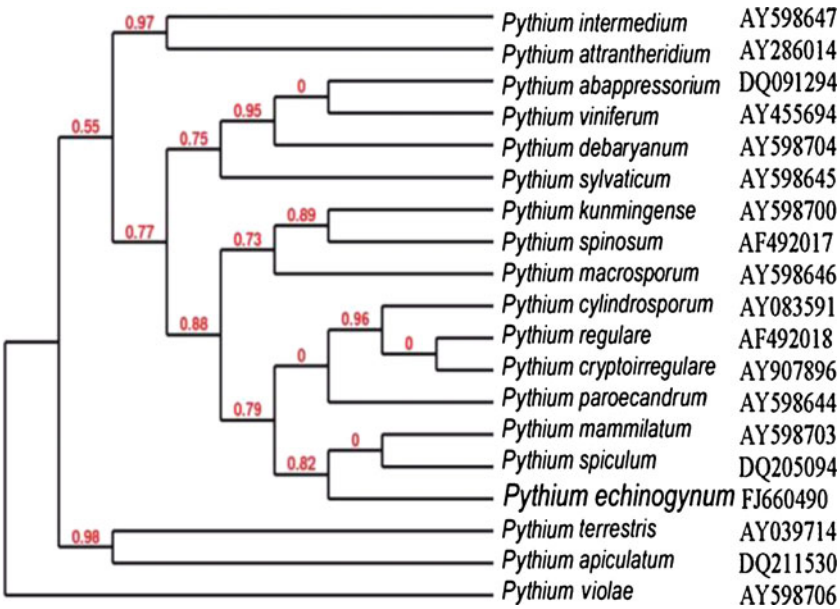

Fig. 5 Cladogram of some species related to *P. echinogynum* belonging to "Clade F"

"damping off" symptoms were produced and the plants were healthy. This shows that *Pythium lycopersicum* can protect both cucumber and tomato against the new plant parasite.

Pythium echinogynum fits well in "Clade F" as defined by LéVesque and De Cock (2004). This 'clade' includes 17 species, into which we can add some newly described species like *P. regulare*, *P. spiculum*, *P. apiculatum*, *P. attrantheridium* and now our new species *P. echinogynum*. The cladogram in Fig. 5 includes this new oomycete together with some related species belonging to "Clade F".

The morphological and molecular characteristics of strain SU8A enables us to place a new species, *Pythium echinogynum*, within the genus *Pythium*.

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