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Morphological and phylogenetic analyses of three *Phytopythium* species (Peronosporales, Oomycota) from Brazil

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Abstract – We analyzed the morphology and phylogenetic placement of six isolates of *Phytopythium* belonging to *P. helicoides*, *P. palingenes* and *P. vexans* that were isolated from water bodies and substrates used for hydroponically grown crops. The molecular data are from the partial large subunit and the complete internal transcribed regions of the ribosomal DNA. These three species are characterized by the presence of ovoid to globose zoosporangia with papillae, internal proliferation as in *Phytophthora* and mode of zoospore discharge as in *Pythium*. All isolates showed high morphological and phylogenetic similarity with members of the Clades II and III of *Phytopythium*. In this paper, *Phytopythium palingenes* is included for the first time in phylogenetic analyses and our ITS and LSU phylogenies indicated that *Aquaperonospora taiwanensis* is a synonym of *Phytopythium helicoides.*

***Aquaperonospora* / ITS / LSU / phylogeny / taxonomy**

INTRODUCTION

The genus *Phytopythium* (Peronosporales *sensu lato*, Beakes *et al.* 2014) was described by Bala *et al.* (2010) with *P. sindhum* A.M. Lodhi, Shahzad & Lévesque as the type species. Members of this genus are inhabitants of terrestrial, fresh and estuarine environments where they play key roles as saprophytes or plant pathogens, causing disease in a large number of agricultural crops (Baten *et al.* 2015). Although many phylogenetic analyses (e.g. Lévesque & de Cock 2004; Villa *et al.* 2006; Marano *et al.* 2014a) evidenced that members of the ex *Pythium* Pringsh. clade K needed to be transferred to *Phytopythium*, it was only recently that de Cock *et al.* (2015) made the formal new combinations. So far, 17 species are included in *Phytopythium*. These species are organized in three monophyletic clades: Clade I, the largest, composed by 12 known species; Clade II with *P. chamaehyphon* (Sideris) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque and *P. helicoides* (Drechsler) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque and one potentially new species, and Clade III with *P. curcubitacearum* S. Takim and *P. vexans* (de Bary) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque (Baten *et al.* 2014, 2015), although, *P. cucurbitacearum* was considered to be invalid due to the absence of Latin diagnosis when originally described (de Cock *et al.* 2015).

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Phytopythium is characterized by the presence of ovoid to globose sporangia with papillae (except for *P. vexans*), internal proliferation which resembles *Phytophthora* and the type of zoospore discharge as in *Pythium*: a vesicle is formed outside the sporangia to where the undifferentiated protoplasm moves through a tube (Bala *et al.* 2010, Baten *et al.* 2015). Once in there, the zoospores are delimited and start moving outside once the vesicle wall disappears (van der Plaats-Niterink 1981). In the case of *Phytopythium kandeliae* (H.H. Ho, H.S. Chang & S.Y. Hsieh) Thines, the zoospores are developed partly inside the sporangium and partly inside the vesicle (Marano *et al.* 2014b).

During two different studies in São Paulo State, Brazil, we identified three *Phytopythium* species (*P. helicoides*, *P. palingenes* (Drechsler) Abad, de Cock, Bala, Robideau, Lohdi & Lévesque and *P. vexans*) based on morphology and phylogenetic analyses. In addition, *Phytopythium palingenes* is preserved in culture, sequenced and included for the first time in phylogenetic analyses.

MATERIALS AND METHODS

Isolates

The six isolates analyzed in this study (Table 1), were deposited in the culture collection ("Coleção de Culturas de Algas, Cianobactérias e Fungos do Instituto de Botânica – CCIBt", São Paulo, SP, Brazil). CCIBt 3981, CCIBt 4069 and CCIBt 4101 were originally isolated from fresh and brackish water collected at "Parque Estadual da Ilha do Cardoso" (PEIC), Cananéia city, southern coast of São Paulo state, in August and November 2012. CCIBt 4103, CCIBt 4104 and CCIBt 4097 were isolated from substrates used in hydroponically grown crops in Embu-Guaçu and Itapecerica da Serra, São Paulo state, in September 2014.

Laboratory analysis

In the laboratory, aliquots (30 mL) of water samples collected were plated and baited with *Sorghum* sp. seeds and pieces of onion (*Allium cepa* L.) skins (Sparrow 1960, Milanez 1989). In the case of substrate samples, 5 g were placed in

Table 1. Origin, CCIBt culture collection number and GenBank accession number of the newly sequenced *Phytopythium* isolates of this study. Substrate: growing substrates used in hydroponics. NA: not available. PEIC: Parque Estadual da Ilha do Cardoso

Taxa	*Isolate Origin*			*GenBank Accession Number*	
	N° CCIBt	*Samples*	*Collection area*	*LSU*	*ITS*
Phytopythium palingenes	3981	Water	PEIC	KR092143	KR092139
Phytopythium vexans	4069	Water	PEIC	NA	KR092140
Phytopythium vexans	4101	Water	PEIC	KR092144	KR092141
Phytopythium vexans	4097	Substrate	Itapecerica da Serra	NA	KR092142
Phytopythium helicoides	4104	Substrate	Embu-Guaçu	NA	KR092137
Phytopythium helicoides	4103	Substrate	Embu-Guaçu	NA	KR092138

a Petri dish together with 30 ml of autoclaved reverse-osmosis water and baited with the same baits. Gross cultures were incubated in an acclimatized room (21°C). After 4-7 days of incubation, baits were observed under the microscope and the specimens of *Phytopythium* isolated. The isolates were purified on CMA (corn meal agar) medium (Fuller & Jaworski 1987).

DNA extraction, PCR and sequence amplification

For DNA extraction, the isolates were cultivated initially onto solid medium with 0.20 gL^{-1} of each streptomycin sulphate and penicillin G as antibiotics. A small piece of agar with mycelium from the pure culture was transferred to Erlenmeyers containing 50 mL of MP_5 liquid medium (maltose-peptone) prepared with autoclaved reverse-osmosis water. After incubation for 5-10 d at 21°C, the mycelium was transferred to 2.0 mL microfuge tubes in order to obtain mycelial pellets with enough biomass for DNA extraction. DNA genomic extraction followed the protocol described in the "PureLink Genomic DNA Kit" (Invitrogen ™). Electrophoresis was performed using 1% (p/v) agarose gel.

The partial LSU and complete ITS1-5.8S-ITS2 (rDNA) region, were amplified using the primers LR0R/LR6-O (Riethmüller *et al.* 2002) and UN-up 18S42/UN-up 28S22 (Robideau *et al.* 2011) respectively. DNA was amplified with the PCR SuperMix kit (Invitrogen®) for a final volume of 25 µl in a C1000 Touch™ Thermal Cycler Bio-Rad. The PCR amplification technique was performed following the conditions described by Marano *et al.* (2014b). Amplicons were purified with AxyPrep PCR Clean-up kit (Axygen®). PCR products were analyzed by electrophoresis on a 1% agarose gel, and stored frozen at – 20°C. Sequencing was performed in an ABI 3730 DNA Analyser (Life Technologies™). Assembly of contigs and correction of ambiguous bases were manually edited using the program Sequencher™ version 4.1.4.

Phylogenetic analyses

LSU and ITS rDNA sequences were compared against BLASTn. Oomycete sequences from this study were deposited at GenBank (http://www.ncbi.nlm.nih.gov) and are shown in the Table 1. For phylogenetic reconstruction, the LSU and ITS rDNA sequences of the isolates were compared with published sequences of other *Phytopythium* species deposited in GenBank, using *Pythium takayamanum* as outgroup. The new species *Aquaperonospora taiwanensis* described by Ko *et al.* (2010) was included in this analyses due to morphological similarities with *Phytopythium* species. Sequences were aligned using MAFFT version 7 (Kazutaka & Daron 2013), and the ambiguously aligned characters removed manually. The Maximum Likelihood (ML) phylogenies were reconstructed with MEGA version 6 (Tamura *et al.* 2013) using the best model for nucleotide substitution and branch support based on 1,000 bootstrap pseudo-replicates.

Morphology

Sexual and asexual structures of the isolates were characterized and measured once the pure cultures were obtained. Identification was according to van der Plaats-Niterink (1981) and the original descriptions of the species.

Salinity tests

Since one of the isolates (CCIBt 4069) was collected in water with 0.05% of salinity, tests were performed in order to characterize the growth of the isolates at different salinities. The growth (colony diameter) of the isolates were measured on solid CMA culture medium prepared using different dilutions of reverse-osmosis water and filtered and autoclaved seawater to obtain the salinities: 0.0 (without seawater), 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (only seawater). Dishes were incubated at 23°C for 48 h and the diameters of the colonies measured with a millimeter rule.

RESULTS

Phylogenetic analyses

We used a total of 27 sequences from 20 species, including the outgroup *Pythium takayamanum* (CBS 121.492 and NBRC 104223) for the LSU rDNA analysis. In the case of the ITS rDNA, 43 sequences from 21 species, including the outgroup (CBS 121.492 and 2D5S071) were used. Maximum Likelihood trees (Figs 1 and 2) showed three major clades (Clades I-III), which are all moderately to well-supported in both LSU and ITS phylogenies (Clade I: 99% and 69% of bootstrap support in LSU and ITS, respectively; Clade II: 100% in both LSU and ITS; and Clade III: 99% and 100% in LSU and ITS, respectively). Our *Phytopythium* isolates are placed in Clades II and III. *Phytopythium palingenes* is included for the first time in phylogenetic analyses and grouped in Clade II together with *P. helicoides* and *P. chamaehyphon*. The three *P. vexans* isolates of this study, collected under different conditions (growing substrates for hydroponics, fresh and brackish water) were placed within Clade III, clustering together with other *P. vexans* sequences available in GenBank.

Species descriptions and salinity tests

Phytopythium helicoides (Drechsler) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque, Persoonia 34: 37. 2015. **Fig. 3 A-F**

≡ *Pythium helicoides* Drechsler, J. Washington Acad. Sci. 20: 413. 1930.

≡ *Ovatisporangium helicoides* (Drechsler) Uzuhashi, Tojo & Kakish., Mycoscience 51: 360. 2010.

= *Phytophthora fagopyri* S. Takim ex S. Ito & Tokun. Trans. Sapporo Nat. Hist. Soc. 14: 15. 1935.

= *Aquaperonospora taiwanensis* Ko, Bot. Stud. 51: 343-350. 2010.

Description: Mycelium well-developed. Zoosporangia terminal, subglobose, ovoid, 25.0-42.5 × 10.0-37.5 (av. 35.6 × 24.0) µm, proliferating internally or forming secondary sporangia on branches originated just below the septum of the primary ones. Encysted zoospores, spherical, 7.5-12.5 µm diam. (av. 10.5 µm), discharge tubes apical, differentiated into an evanescent vesicle. Sexual structures absent.

Material examined: BRAZIL. São Paulo State: Embu-Guaçu. From growing substrates used in hydroponics, 23/IX/14, on *Sorghum* sp. seeds. Leg. & det. D. R. Gonçalves & C. L. A. Pires-Zottarelli (CCIBt 4103, 4104).

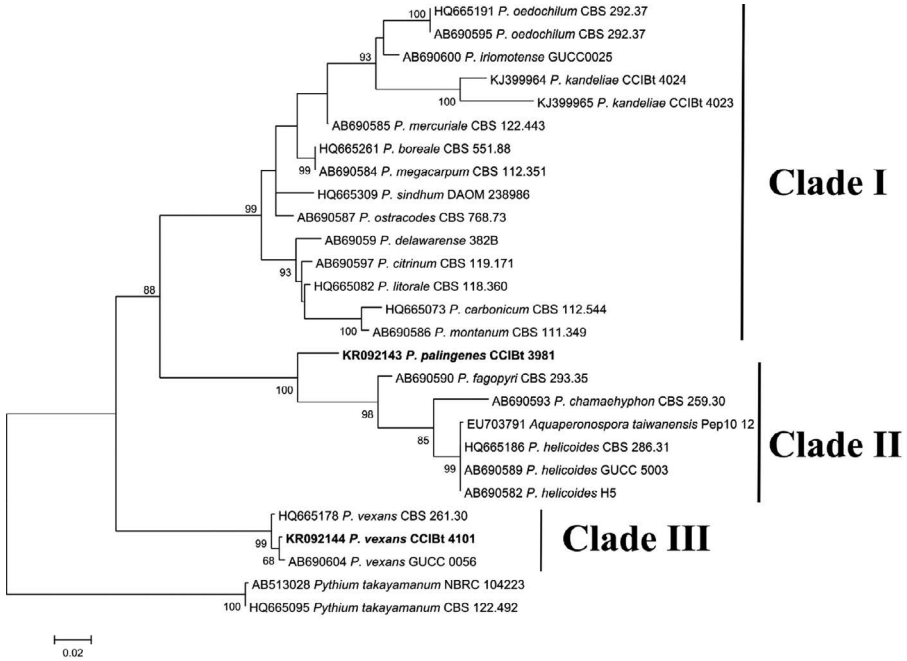

Fig. 1. Maximum likelihood tree inferred from partial LSU rDNA sequences of *Phytopythium* isolates. Numbers next to branches indicate bootstrap support (%) and the bar shows the number of substitutions per site. Only branches with > 70% of bootstrap support are shown.

Culture growth: colony pattern radiate, without aerial mycelium. Growth of the isolates after 48 h at 23°C: *(i)* CCIBt 4103: on CMA without salt: 8.5 cm; on CMA with 0.5% of salt: 8.5 cm; on CMA with 1.0% of salt: 6.6 cm; on CMA with 1.5% of salt: 5.9 cm; on CMA with 2.0% of salt: 5.6 cm; on CMA with 2.5% of salt: 4.3 cm; on CMA with 3.0% of salt: 2.0 cm. *(ii)* CCIBt 4104: on CMA without salt: 3.2 cm; on CMA with 0.5% of salt: 3.0 cm; on CMA with 1.0% of salt: 2.7 cm; on CMA with 1.5% of salt: 2.1 cm; on CMA with 2.0% of salt: 2.2 cm; on CMA with 2.5% of salt: 2.0 cm; on CMA with 3.0% of salt: 0.7 cm.

Remark: The characteristics of the asexual reproduction are in agreement with the original description of Drechsler (1930, 1941) and van der Plaats-Niterink (1981). Both isolates CCIBt 4103 and 4104 did not produce sexual structures and the identification was made based on its phylogenetic placement in the ITS phylogeny (Fig. 2). According to the morphological characteristics and phylogenetic placement, *Aquaperonospora taiwanensis* (Ko *et al.* 2010) is a synonym of *P. helicoides* (Figs 1 and 2).

Phytopythium palingenes (Drechsler) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque, Persoonia 34: 37. 2015. **Fig. 4 A-F**

≡ *Pythium palingenes* Drechsler, J. Washington Acad. Sci. 20: 416. 1930.

Description: Mycelium well-developed. Zoosporangia terminal, subglobose, ovoid, 32.5-37.5 × 30.0-35.0 (av. 35.0 × 33.0) µm, proliferating internally or forming

secondary sporangia on branches originating just below the septum of the primary ones. Encysted zoospores spherical 10.0-12.5 (av. 11.0) µm diam., discharge tubes apical, differentiated into an evanescent vesicle. Oogonia terminal with short or

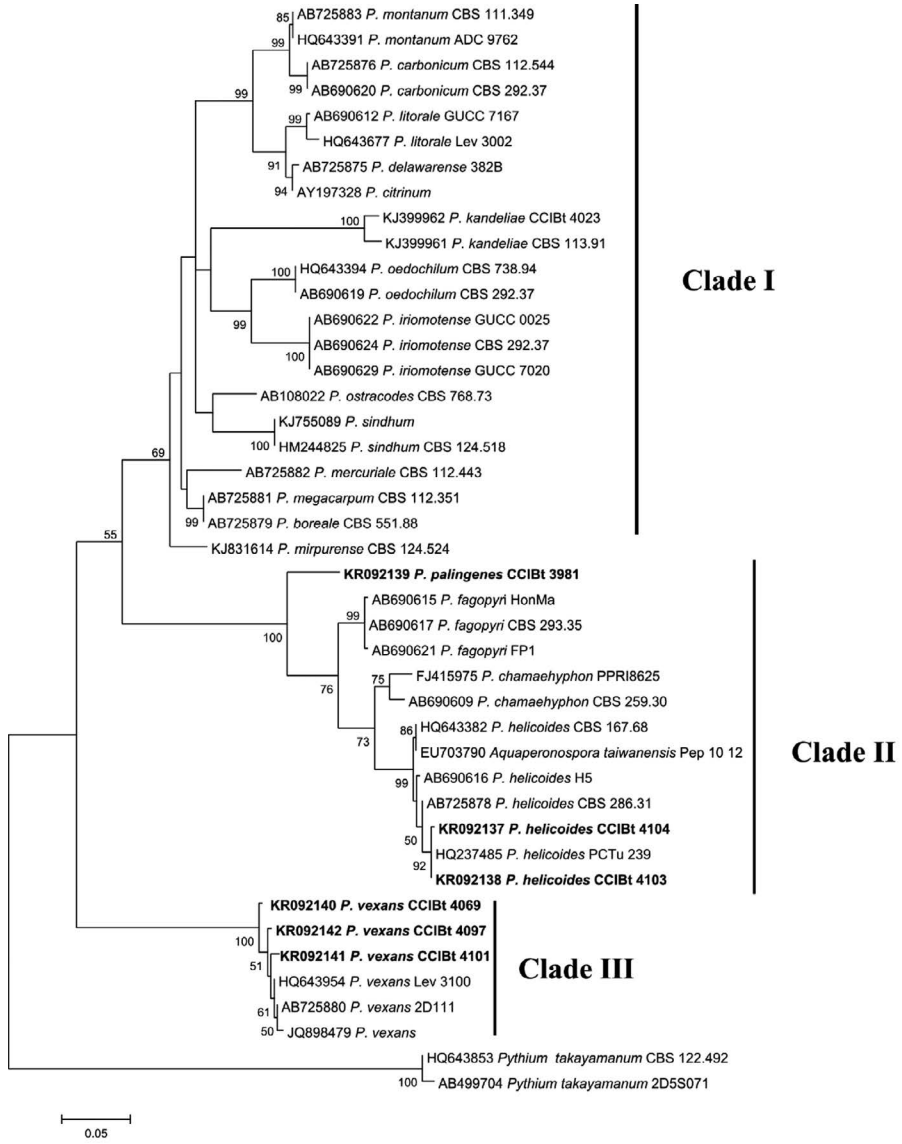

Fig. 2. Maximum likelihood tree inferred from complete ITS rDNA sequences of *Phytopythium* isolates. Numbers next to branches indicate bootstrap support (%) and the bar shows the number of substitutions per site. Only branches with > 50% of bootstrap support are shown.

sessile peduncle, intercalar or unilaterally intercalate, subglobose, 22.5-34.5 (av. 29.0) µm diam. Antheridia 1-3 per oogonium, monoclinous or diclinous, antheridial stalks and also vegetative hyphae wrapping around the oogonial stalk in a few turns, antheridial cells cylindrical, often wavy or irregular in contour. Oospores yellowish, aplerotic, subglobose, 20.0-28.5 (av. 24.5) µm diam., 1 per oogonium, smooth-walled, wall 1.5-3.0 (av. 2.0) µm in thickness.

Material examined: BRAZIL. São Paulo State: Cananéia: Parque Estadual da Ilha do Cardoso, Perequê river. From freshwater and soil samples 20/VIII/2012 and 06/XI/2012, on *Allium cepa* (onion) skin. Leg. & det. S. C. O. Rocha & C. L. A. Pires-Zottarelli (CCIBt 3981).

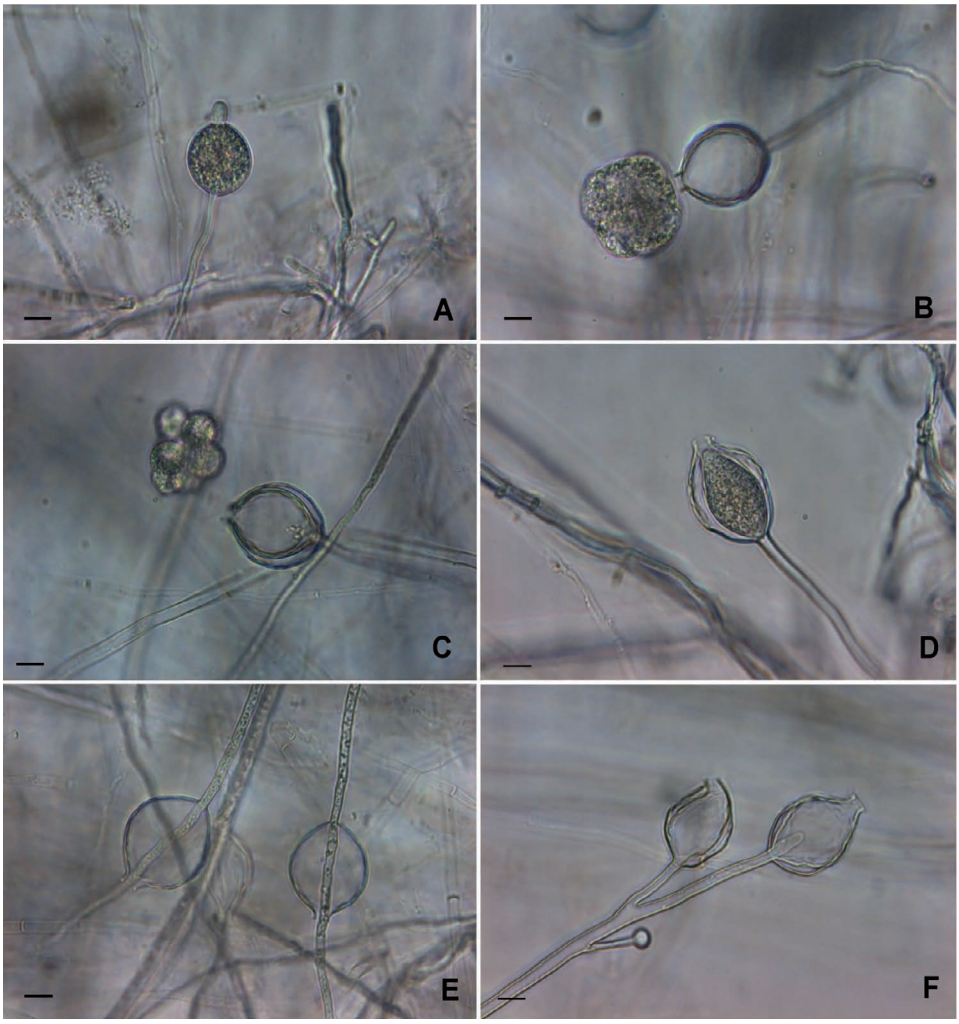

Fig. 3 A-F. *Phytopythium helicoides.* **A.** Zoosporangium with zoospore discharge tube. **B-C.** Zoospores development and discharge. **D-F.** Internally proliferation of the zoosporangia. Bar = 10 µm.

Culture growth: colony pattern radiate, without aerial mycelium. Growth of the isolates after 48 h at 23°C: *(i)* CCIBt 3981: on CMA without salt: 3.1 cm; on CMA with 0.5% of salt: 5.4 cm; on CMA with 1.0% of salt: 4.9 cm; on CMA with 1.5% of salt: 3.9 cm; on CMA with 2.0% of salt: 2.8 cm; on CMA with 2.5% of salt: 2.7 cm; on CMA with 3.0% of salt: 0.9 cm.

Remark: The characteristics of this isolate are in agreement with the original description of Drechsler (1930, 1941), van der Plaats-Niterink (1981) and Rocha *et al.* (2001). *Phytopythium palingenes* was originally described by Drechsler (1930, 1941) from discoloured roots of *Ambrosia trifida* L. collected near Delaplane, Virginia (USA) in August, 1926. Our specimen grew on onion skin, *Sorghum* sp. seeds and onto CMA culture medium. *P. palingenes* is strikingly similar to

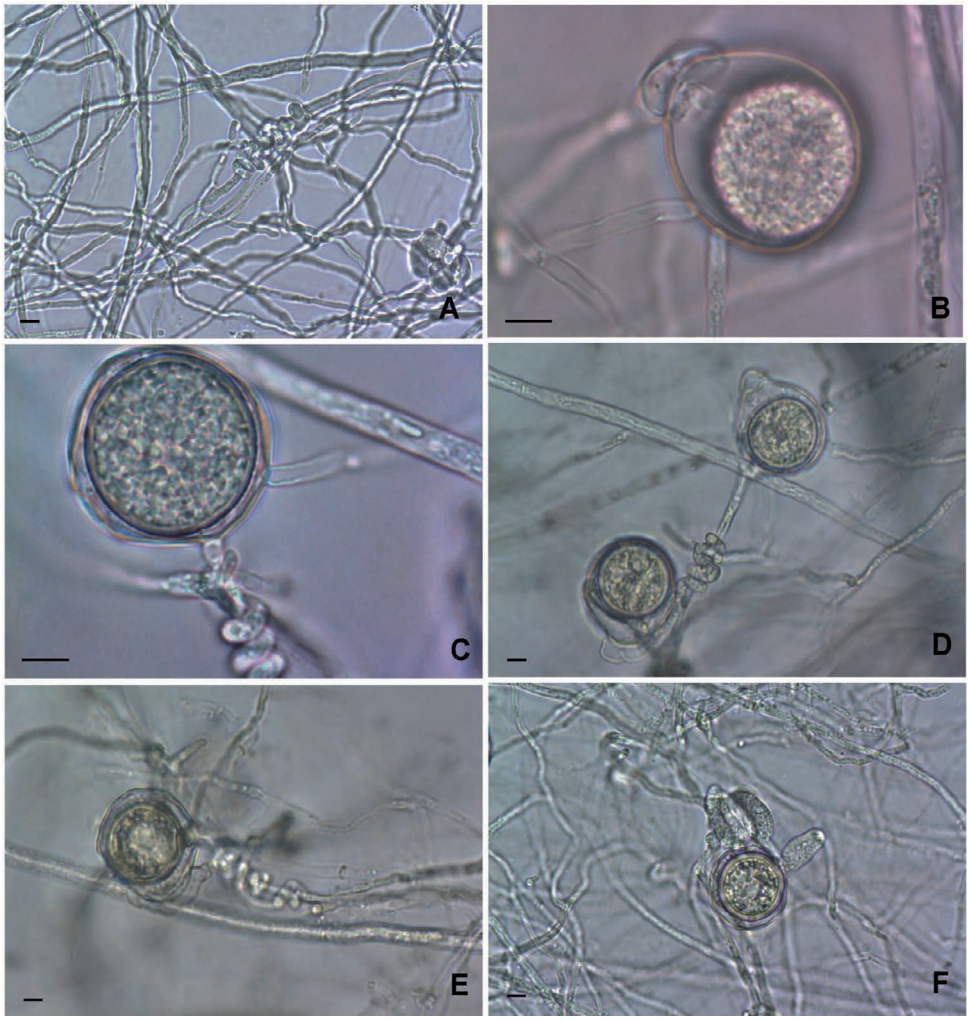

Fig. 4 A-F. *Phytopythium palingenes*. **A.** Mycelium with wrapping hyphae. **B.** Intercalary oogonium. **C-D.** Oogonium with antheridia wrapping around the oogonial stalk. **E.** Oogonia and wavy antheridial cell. **F.** Oogonium with cylindrical antheridial cells attached. Bar = 10 µm.

P. helicoides in morphology, although it presents cylindrical antheridial cells, which are often wavy or irregular, while *P. helicoides* has regular and non-furrowed antheridia (van der Plaats-Niterink 1981). *Phytopythium palingenes* was recently transferred to *Phytopythium* by de Cock *et al.* (2015) based solely on morphological characteristics of the described specimens because no living culture is available in public culture collections.

Phytopythium vexans (de Bary) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque, Persoonia 34: 37. 2015. **Fig. 5 A-F**

≡ *Pythium vexans* de Bary, J. R. Agric. Soc. Engl. 12: 255. 1876.

≡ *Ovatisporangium vexans* (de Bary) Uzuhashi, Tojo & Kakish., Mycoscience 51 (5): 360. 2010.

Fig. 5 A-F. *Phytopythium vexans*. **A-B.** Zoospore development. **C.** Intercalary zoosporangium with zoospore discharge tube. **D.** Oogonium with monoclinous antheridium. **E-F.** Oogonia with antheridia. Bar = 10 µm.

= *Pythium complectens* M. Braun, J. Agric. Res. 29: 415. 1924.
= *Pythium allantocladon* Sideris, Mycologia 24 (1): 27. 1932.
= *Pythium ascophallon* Sideris, Mycologia 24 (1): 29. 1932.
= *Pythium polycladon* Sideris, Mycologia 24 (1): 32. 1932.
= *Pythium euthyhyphon* Sideris, Mycologia 24 (1): 34. 1932.
= *Pythium piperinum* Dastur, P Indian Acad. Sci. (11): 803. 1935.

Description: Mycelium well-developed. Zoosporangia terminal or intercalary, globose, subglobose and ovoid 25.0-37.5 × 25.0-35 (av. 29.75 × 29.37) µm. Encysted zoospores, spherical 10.0-12.5 (av. 11.75) µm diam., differentiating into an evanescent vesicle. Oogonia terminal or intercalary, ovoid or globose, 16.25-22.5 (av. 18.5) µm diam. Antheridia large bell-shaped, monoclinous or rarely diclinous, 1 per oogonia. Oospores aplerotic, 12.5-18.75 (av. 14.4) µm diam., 1 per oogonia, smooth-walled, wall 1.5 µm in thickness.

Material examined: BRAZIL. São Paulo State: Cananéia: Parque Estadual da Ilha do Cardoso, Perequê river. From leaf samples of *Laguncularia racemosa* 07/XI/2012 (0.05% salinity), on *Sorghum* sp. seeds. Leg. & det. A. L. Jesus, A. V. Marano & C. L. A. Pires-Zottarelli (CCIBt 4069, 4097, 4101).

Culture growth: colony pattern radiate, without aerial mycelium. Growth of the isolates after 48 h at 23°C: *(i)* CCIBt 4069: on CMA without salt: 5.8 cm; on CMA with 0.5% of salt: 6.2 cm; on CMA with 1.0% of salt: 5.4 cm; on CMA with 1.5% of salt: 4.5 cm; on CMA with 2.0% of salt: 3.5 cm; on CMA with 2.5% of salt: 2.0 cm; on CMA with 3.0% of salt: 0,7 cm. *(ii)* CCIBt 4097: on CMA without salt: 6.7 cm; on CMA with 0.5% of salt: 7.3 cm; on CMA with 1.0% of salt: 6.0 cm; on CMA with 1.5% of salt: 5.0 cm; on CMA with 2.0% of salt: 4.6 cm; on CMA with 2.5% of salt: 3.5 cm; on CMA with 3.0% of salt: 2.0 cm.

Remark: The characteristics of the specimens are in agreement with the description of van der Plaats-Niterink (1981). *P. vexans* was originally described by de Bary (1896), and was frequently reported from soil and plants in several countries (van der Plaats-Niterink 1981). In Brazil, it was firstly reported by Carvalho (1965), isolated of root rot of *Strelitzia* sp. Our specimen grew well on *Sorghum* sp. seeds, onion skin and onto solid CMA culture medium.

DISCUSSION

All isolates of *Phytopythium helicoides*, *P. palingenes* and *P. vexans* showed the morphological features typical of these species and clustered together with other isolates of the species in Clades II and III of Baten *et al.* (2015). As the result of this study, *P. palingenes* is again available in culture, its sequences deposited in GenBank and is included for the first time in phylogenetic analyses. Although *P. palingenes* is strikingly similar in morphology to *P. helicoides*, our LSU and ITS phylogenies clearly showed that both taxa are separate species.

All morphological characteristics of *Phytopythium helicoides* matched with the description of *Aquaperonospora taiwanensis*, and as expected, both species clustered together in a well-supported clade, as shown in Figs 1 and 2. Hence, *Aquaperonospora taiwanensis* should be considered a synonym of *Phytopythium helicoides*, as previously suggested by Marano *et al.* (2014a).

The salinity tests indicated that all isolates are able to tolerate a wide range of salinities, even in the case of specimens isolated from different conditions, such

as substrate for hydroponically grown crops. Up to date, there are no studies testing the tolerance of *Phytopythium* spp. to different salinity ranges, except for *P. kandeliae* that was recently transferred from the genus *Halophytophthora* and whose ability to tolerate a wide range of salinity was already documented (Marano *et al.* 2014b).

Our results contribute to the knowledge of Peronosporales in general and particularly of the genus *Phytopythium* in Brazil.

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