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Pythium litorale sp. nov., a new species from the littoral of Lake Constance, Germany

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Abstract

A description is given of *Pythium litorale* sp. nov., a new species from reed stands in Germany. *Pythium litorale* was among the most abundant species when the oomycete community of littoral soils of Lake Constance was studied. It was consistently isolated from flooded as well as from drier reed sites. The species is characterized by subglobose, papillate and internally proliferating sporangia, globose hyphal swellings, the absence of oogonia in single culture and a high optimum growth temperature. It proved to be nonpathogenic to *Phragmites australis*, the predominating plant in the investigated sites. Molecular analysis of ribosomal DNA internal transcribed spacer regions placed *Pythium litorale* in a clade together with its closest relatives *Pythium megacarpum*, *Pythium boreale*, *Pythium montanum* and *Pythium carbonicum*. The generic status of this basal clade in *Pythium* is currently under discussion, as it possibly represents a separate genus that is distinct from *Pythium*, and shares several characteristics with *Phytophthora*.

Introduction

The oomycete genus *Pythium* consists of more than 120 recognized species (Dick, 1990), many of which are soil-borne plant pathogens with a worldwide distribution, causing fruit, root or collar rot and damping-off of seedlings (Hendrix & Campbell, 1973). Most *Pythium* spp., including the pathogens, can also live as mere saprophytes in plant debris or soil. Plant diseases caused by *Pythium* spp. are often encountered under wet or flooded soil conditions, as the primary infection structures of these organisms are swimming, biflagellate zoospores. While traditionally, *Pythium* taxonomy has been basically relying on morphological features like size and shape of sporangia or oogonia (Van der Plaats-Niterink, 1981; Dick, 1990), molecular tools have now come to be widely used, and well established for species definition, delineation and identification. Supported by molecular evidence, several new *Pythium* species from agricultural and natural sites have been described recently (e.g. Nechwatal & Oßwald, 2003; Paul, 2003; Ko *et al*., 2004). Lévesque & De Cock (2004), in an extensive study on the phylogeny of 116 *Pythium* species and varieties, divided the genus into 11 major clades (A–K) based on sequence analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. Similar to the assignment of *Phytophthora* to clades 1–10 by Cooke *et al*. (2000), this molecular classification scheme is likely to become an important reference tool in the future of *Pythium* taxonomy. Molecular data also provided evidence for a polyphyletic origin of the genus *Pythium*, because of the phylogenetic positioning of a particular clade of species, the *Pythium vexans* clade (Cooke *et al*., 2000) or clade K of Lévesque & De Cock (2004). This clade is basal to all other *Pythium* species, and its members are considered 'border species' between *Pythium* and *Phytophthora* as some of them share several characters with the latter genus, such as the production of elicitins (Panabières *et al*., 1997). They were repeatedly suggested to be placed into a new genus (Briard *et al*., 1995; Cooke *et al*., 2000; Lévesque & De Cock, 2004).

During a study on the occurrence and diversity of oomycetes in *Phragmites australis* (Cav.) Trin. ex Steud. stands of Lake Constance, Germany, an unknown *Pythium* sp. from clade K of Lévesque & De Cock (2004) was consistently isolated from permanently flooded as well as from drier reed sites. Its unique combination of morphological and molecular characteristics indicated that it is only distantly related to any known species of the genus. This paper gives a formal description of this species as *Pythium litorale* sp. nov., and provides molecular support of its status as a distinct species.

Material and methods

Soil samples for the isolation of *Pythium* spp. were taken from reed rhizosphere soils (10–20 cm below surface) in the

reed belt of Lake Constance littoral between April and October 2003. The sampling site is located on the southern shore of Bodan peninsula (Überlinger See, 9°11′18″E, 47°41′53″N). In total, 14 soil samples were taken (eight from permanently flooded, and six from dry sites). Young oak leaflets were used as baits and processed as described previously (Nechwatal & Oßwald, 2003). Isolates were morphologically compared with keys and descriptions of known *Pythium* spp. (Waterhouse, 1968; Van der Plaats-Niterink, 1981; Dick, 1990). Seven isolates of the proposed new species, three from flooded, four from dry sites, were recovered.

Colony morphology was recorded after incubation on 2% malt extract agar, potato carrot agar (PCA) (Van der Plaats-Niterink, 1981) and V8 agar (V8A) (Nechwatal *et al.*, 2005) for 6 days at 20 °C in the dark. Investigations on sporangial development and germination were made on discs cut from the edge of a culture actively growing on V8A, and floated in demineralized water for 24 h at 20 °C under natural light. For the observation of oospore and chlamydospore production, *Pythium* isolates on V8A (containing 20 mg L^{-1} β-sitosterol), PCA, carrot agar (Ribeiro, 1978) and on hemp seed halves were incubated for up to several months, and checked monthly. All seven available isolates were used for crossings with each other on opposite sides of 90 mm V8A plates to test the existence of different mating types. They were incubated at 20 °C in the dark for up to several months. In each isolate, 25 randomly selected structures were measured. For the assessment of growth rates, all of the unknown isolates were subcultured in two replicates on PCA (20 mL) on 90 mm Petri dishes, and incubated at 20, 25, 30, 35 and 37 °C for 5 days. Radial growth was measured daily along two lines intersecting the centre of the inoculum at right angles.

DNA from *Pythium* mycelium scraped off from V8A cultures was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR amplifications of ITS1, 5.8S and ITS2 regions were performed with primers ITS1, ITS2, ITS3, ITS4 and ITS6, as described (White *et al.*, 1990; Cooke & Duncan, 1997). Sequencing of the PCR products was carried out by MWG Biotech (Ebersberg, Germany). Basic local alignment search tool (BLAST) searches of the GenBank nucleotide database revealed the most closely related known species. Sequence entries of a representative set of *Pythium* spp. were retrieved from GenBank and aligned using ClustalX. Sequence data were analysed, and neighbor-joining phylogenetic analyses were conducted using the programs DNAdist and Neighbor from the PHYLIP package as described by Cooke *et al.* (2000). An unweighted pair-group method with arithmetic mean tree of the ITS1 sequences of all clade K species available in GenBank and the unknown isolates' sequence was constructed using DNAdist and Neighbor from the PHYLIP package.

Pathogenicity towards common reed, the predominating plant in the investigated sites, was tested by placing colonized V8A plugs of three isolates of the new species onto mature, greenhouse-grown reed leaves (six per isolate) as described earlier (Nechwatal *et al.*, 2005), and incubating these at 20 °C in sealed Petri dishes on moist filter paper for 7 days. Mock-inoculated leaves and leaves inoculated with the aggressive reed pathogen *Pythium phragmitis* sp. nov. (Nechwatal *et al.*, 2005) served as controls. The test was repeated twice per isolate.

Results

Morphological description

Colonies on all agar media had sparse aerial mycelium, showing a distinct rosette pattern on malt extract agar, and a faint radiate to chrysanthemum pattern on PCA and V8A. Optimum growth occurred at 30 °C on PCA, with the average daily growth being 13.3 mm. The maximum growth temperature was 35 °C. The main hyphae were up to 5 µm wide.

Sporangia (Fig. 1) formed in large numbers in water culture at 20 °C, terminal, (sub-)globose, broad ovoid or ob-pyriform; the mean length/breadth ratio was 1.07 (1.02–1.11). Mature sporangia measuring 22.8–32.2 × 20.8–29.1 µm (means of seven isolates, mean: 28.5 × 26.7 µm) usually with a conspicuous apical papilla or outgrowth were from 2–10 µm to up to 35 µm in length (Figs 1a–c). Papillae either developed into a discharge tube, with subsequent release of sporangium contents for zoospore development (Figs 1g and h), or eventually ramified and led to direct germination of the sporangium (Fig. 1d). After zoospore release, new sporangia developed by internal proliferation, either within (nested) or outside the empty sporangium (Figs 1e and f). Zoospore discharge occurred at room temperature. Encysted zoospores were 7.5–10.0 µm in diameter. Globose hyphal swellings (Fig. 1i) were frequently produced, in particular on mature carrot agar cultures, single, on average 28.8 µm (means of seven isolates: 28.0–30.1) in diameter, inserted terminally or intercalary.

Oogonia and oospores were not produced in of any of the isolates, in single or dual cultures paired with themselves.

Sequence analysis

Internal transcribed spacer-1, 5.8S and large parts of the ITS2 region of *Pythium litorale* were identical in all isolates. However, even after repeated reactions with different primer sets, the 3′-end of the ITS2 region of all *Pythium litorale* isolates could not be completely assessed, possibly because of an accumulation of Ts and Gs and subsequent template slipping during PCR. The sequence has been submitted to GenBank (DQ144637). According to BLAST searches,

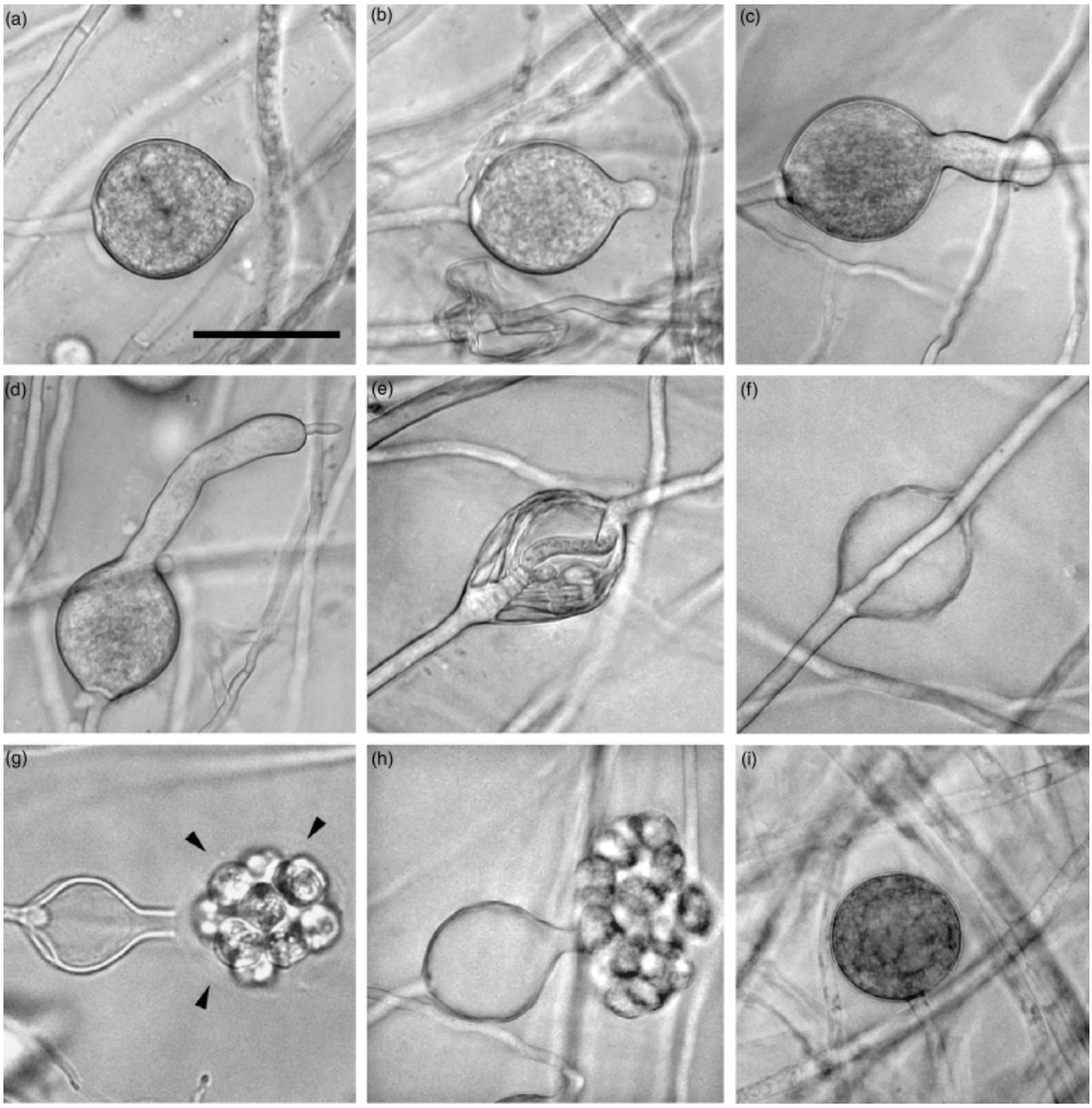

Fig. 1. Sporangia and hyphal swellings of *Pythium litorale*. (a–c) Sporangia produced in water culture showing different degrees of apical elongation. (d) Beginning of direct germination of sporangium. (e) Nested internal proliferation and external proliferation by hypha. (f) extended internal proliferation. (g and h) Zoospore development in vesicle (arrowheads) outside sporangium. (i) Hyphal swelling on carrot agar. Bar: 30 μm.

Pythium litorale is only distantly related to any known species of the genus. Its closest relatives are *Pythium boreale*, *Pythium carbonicum*, *Pythium megacarpum*, *Pythium montanum* and *Pythium ostracodes*, with sequence similarities in the ITS1 ranging from 68% to 78%, according to pairwise alignments. Phylogenetically, *Pythium litorale* groups with all of the above species in the basal *Pythium* clade K of Lévesque & de Cock (2004), supported by bootstrap values close to 100% (data not shown). Figure 2 shows the taxon's phylogenetic position within clade K.

Pathogenicity

Pythium litorale was nonpathogenic towards mature reed leaves in our tests. As in the mock-inoculated control leaves, none of the isolates tested caused any damage or

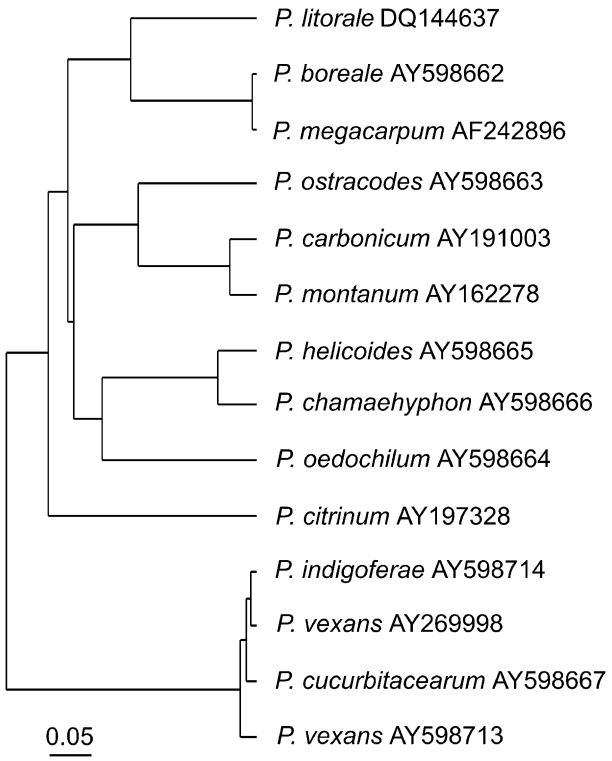

Fig. 2. Unweighted pair-group method with arithmetic mean tree for *Pythium* clade K including *Pythium litorale* constructed after distance-based analysis of representative ITS1 sequences of all clade K species available in GenBank. Scale bar unit: number of substitutions/site. ITS, internal transcribed spacer.

discolouration to the leaves. In contrast, inoculation with the aggressive reed pathogen *Pythium phragmitis* caused large leaf necroses in all cases, as reported earlier (Nechwatal *et al*., 2005).

Taxonomy

Pythium litorale J. Nechwatal, sp. nov.

Etymology: The epithet refers to the species' habitat on the littoral of Lake Constance

Coloniae crescunt in agaris 'MEA', 'V8A' et 'PCA' usque ad 35 °C, optime ad 30 °C, cum incrementum radiatum quotidianum 13.3 mm in agaro 'PCA' (12.6 mm ad 25 °C). Coloniae submersae, cum mycelio aerio restricto in omnibus agaris, cum ordinatione 'rosaceus' distinctum in agaro 'MEA'. Hyphae hyalinae, nonseptatae, primariae ad 5 µm latae. Sporangia formata abundantia in cultura liquida, terminalia, subglobosa, ovoidea vel ob-pyriformia, cum proliferatione interno vel externo. Sporangia in maturitate cum papilla aut appendice (longitudo 2–8 µm) apicali, 27–35 × 25–33 (medio 30.8 × 29.0) µm (sine papillae). Appendices apicales saepe formant rostra longas (ad 35 µm). Ex rostris germinatio directa sporangiorum saepe observata. Zoosporae formatae et dimissae ad 20 °C. Inflationes hypharum formata abundantia in agaro Dauci carotae in culturae maturae, 18–36 (medio 29.2) µm in diametro. Culturae heterothallicae vel steriliae, oogoniae et oosporae non observatae.

Typus: GERMANIA: Konstanz/Egg, (9°11′18″E, 47°41′53″N), isol. ex solo rhizosphaerae ad *Phragmites australis* (Cav.) Trin. ex Steudel, 07/2003, col. et isol. J. Nechwatal. Ex-type culture (UKN P03) held at CBS Utrecht, NL (CBS 118360).

Holotype: Isolate UKN P03 (dried culture), derived from living culture UKN P03.

Discussion

Pythium litorale belongs to molecular clade K of Lévesque & de Cock (2004). It shares several morphological characteristics with other species from that clade, such as growth pattern, ovoid, papillate, internally proliferating sporangia and the rather high optimum temperature for growth (Lévesque & de Cock, 2004). However, in contrast to all other related species, *Pythium litorale* seems to be heterothallic or sexually sterile according to present knowledge, as no oogonia or oospores could be observed in single or dual cultures under the conditions applied, even after prolonged incubation. Mating-type distinction within *Pythium litoralis* or the identity of a suitable mating strain therefore cannot be definitely clarified yet. In addition to zoospore releasing sporangia *Pythium litorale* regularly produces hyphal swellings on solid agar, a trait not described for any known clade K species. However, the similar-sized 'sporangia' described for *Pythium boreale*, *Pythium carbonicum* and *Pythium megacarpum* might in fact be hyphal swellings, too, as zoospore release was not observed (Duan, 1985; Paul, 2000, 2003). The main distinctive features of *Pythium litoralis* in comparison with its closest relatives are given in Table 1.

According to molecular data, the sequence similarity of *Pythium litorale* is low even with its closest relatives, and the new species can be considered only distantly related to any *Pythium* sp. known so far. It shares only 78% identical positions in the ITS1 region with *Pythium montanum*, a species described recently from forest soils (Nechwatal & Oßwald, 2003). Similarly, all other related species have very low degrees of sequence identity in the ITS1, ranging from 68% to 73%.

In the past, clade K species have repeatedly been suggested to belong to a separate genus, distinct from *Pythium* (Briard *et al*., 1995; Cooke *et al*., 2000; Lévesque & De Cock, 2004). Based on analyses of the rDNA clade K is basal in *Pythium*, and when data for *Pythium* and *Phytophthora* are considered, the former genus becomes polyphyletic because of clade K (Lévesque & De Cock, 2004). In addition, several

Table 1. Morphological features of *Pythium litorale* in comparison with related species

	Pythium sp.					
	P. litorale	*P. montanum*	*P. megacarpum*[*]	*P. carbonicum*[†]	*P. citrinum*[‡]	*P. boreale*[§]
Mean growth rate PCA, 25 °C (mm day^{-1})	12.6	5.8	9	5	11	ND
Sporangia, mean length × breadth (μm)	28.5 × 26.7	24.0 × 21.1[¶]	–	–	24.2[‖]	–
Oogonia, mean diameter (μm)	–	24.3[¶]	28.0	26.0	27.6	22.5
Oospores, mean diameter (μm)	–	18.7[¶]	26.8	25.7	24.9	22.2
Oospore state	–	Aplerotic[¶]	Plerotic	Plerotic	Plerotic	Plerotic
Hyphal swellings, mean diameter (μm)	28.8	–	13.0–36.0[‖]	25.6[‖]	–	22.0

[*]Data from Paul (2000).
[†]Data from Paul (2003).
[‡]Data from Paul (2004).
[§]Data from Duan (1985).
[¶]Data from Nechwatal & Oßwald (2003).
[‖]Given as diameter.
ND, not determined; PCA, potata carrot agar.

'*Phytophthora*-like' features in some species, such as elicitin production (Panabières *et al*., 1997) or hymexazol insensitivity (Ali-Shatayeh *et al*., 2003), place this group in an intermediate position between *Pythium* and *Phytophthora*.

The low degree of ITS sequence similarity among several clade K species has also been stressed by Lévesque & De Cock (2004), and may be considered indicative of an ancient origin of these taxa. Possibly, they were only rarely detected until the recent increase in interest in oomycete communities of natural and nonagricultural ecosystems. Two of the closest relatives of *Pythium litorale* were also described only recently, and were isolated from nonagricultural soils (Nechwatal & Oßwald, 2003; Paul, 2003). Finer levels of phylogenetic resolution within clade K will be achieved not before more of its members have been isolated. Therefore, any of the 'border species' (Panabières *et al*., 1997) of this clade will be of particular interest for a possible reassessment of its taxonomic status. The present study, apart from *Pythium litorale*, also revealed three more undescribed clade K species (J. Nechwatal, unpublished data). Another closely related isolate was reported from a Hungarian river bank (J. Bakonyi, pers. comm.). Although these taxa were isolated only sporadically, their presence emphasizes the potential wide distribution of this group in natural or seminatural ecosystems other than agricultural land.

In the investigated littoral soils in the reed belt of Lake Constance, *Pythium litorale* was the most abundant species in drier sites, constituting 25% of all isolates from this habitat. In permanently flooded sites, 12% of all isolates were *Pythium litorale*. Despite its ubiquity in the investigated area, it was nonpathogenic towards reed, the predominating plant in these sites. As reed belt plant communities are natural monocultures with only limited numbers of plant species other than *Phragmites australis*, and therefore with no alternative host plant of considerable abundance, *Pythium litorale* is likely to be a saprophytic species able to feed on plant litter of diverse origin. Otherwise it would probably not have been able to establish the high abundance observed in our study. However, nothing is known about its potential pathogenicity on other plants. Future studies should not only unravel the generic status of *Pythium* clade K as a whole, but should also investigate the possible impact of *Pythium litorale* and related species on agricultural and nonagricultural plants.

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