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# *Pythium phragmitis* sp. nov., a new species close to *P. arrhenomanes* as a pathogen of common reed (*Phragmites australis*)

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During a study on the occurrence and pathogenicity of oomycetes in the reed-belt (*Phragmites australis*) of Lake Constance (Germany), a new *Pythium* resembling the important cereal pathogen species complex *P. arrhenomanes*/*P. graminicola* was consistently isolated from necrotic mature reed leaves and reed rhizosphere samples. The new species proved to be significantly more aggressive towards reed leaves and seedlings *in vitro* than related species. It is characterised by filamentous, inflated sporangia and plerotic oospores with usually more than one antheridium. ITS and *cox* II sequence data indicate this new species shares a common ancestor with *P. arrhenomanes*, but the sequence differences are clearly consistent with a divergence of the two taxa and with *P. phragmitis* being a distinct species. ITS 1 and 2 of 15 isolates of the taxon consistently differed from *P. arrhenomanes* by 13 positions. Sequence analyses of the *cox* II gene confirmed the new species' phylogenetic position. This paper gives a formal description of the taxon as *P. phragmitis* sp. nov., providing information on morphology, ecology and pathogenicity in comparison to related species. As indicated by the close association to *Phragmites australis*, the high aggressiveness towards reed leaves and seedlings, and the abundance in the investigated stands, *Pythium phragmitis* might act as a reed pathogen of considerable importance, in particular under flooding situations.

## INTRODUCTION

Common reed (*Phragmites australis*, *Poaceae*), a large perennial wetland grass, is a major component of littoral plant communities of freshwater lakes in the Northern Hemisphere, forming large natural monocultures with important ecological functions (Haslam 1972). Within the last 50 years, dieback of reed stands has been a periodically recurring threat to littoral ecosystems of many European freshwater lakes (Brix 1999). Lake Constance, one of Europe's largest inland water bodies, is also affected by the decline, and its reed belt dynamics have been investigated in great detail (e.g. Ostendorp, Dienst & Schmieder 2003, Dienst, Schmieder & Ostendorp 2004). Adverse effects of lake water eutrophication, organic acid toxicity, wave action, water level regulation, insect attack, algal mats, and low genetic diversity have repeatedly been suggested as possible reasons for the decline (Ostendorp 1989, Brix 1999). A negative impact of extreme floods on reed stand health due to impaired oxygen supply of rhizomes and submerged shoots has already been observed in the 19th century (Honsell 1879). It currently seems to be accepted as a major factor in Lake Constance reed dieback, and is under discussion in the context of global warming, and of subsequent environmental changes on a more local scale (Ostendorp *et al*. 2003, Dienst *et al*. 2004).

The fungal community associated with reed has quite extensively been investigated, with a main focus on endophytic and saprobic fungi (e.g. Wirsel *et al*. 2001, Wong & Hyde 2001). Some fungal endophytes were found to have beneficial effects on reed performance *in vitro* (Ernst, Mendgen & Wirsel 2003). However, a contributing role of fungal pathogens in the dieback of reed has only rarely been addressed (e.g. Bán, Fischl & Virányi 1996). Similarly, while free water of freshwater lakes was shown to harbour large numbers of propagules of several oomycete species (e.g. Hallett & Dick 1981), information on their occurrence and pathogenicity in reed stands is scarce. Plant diseases caused by oomycetes are often encountered under water-logged or wet soil conditions as found in littoral ecosystems such as reed belts, as high soil water contents will facilitate zoospore dispersal and mediate disease spread. In particular, species of the genus *Pythium* are economically significant soilborne pathogens with worldwide distribution, causing root and fruit rot, pre- or post-emergence seedling damping-off, or fine root disorders of numerous different host plants (van der Plaats-Niterink 1981). Cereals and grasses, such as wheat, maize, sugar-cane or *Lolium* spp. are

among the major hosts of this genus, and a particular group of *Pythium* spp., the *P. graminicola*/*P. arrhenomanes* species complex (Hendrix & Papa 1974), is specifically associated with diseases of such gramineous host plants (Hendrix & Campbell 1973). Up to now, *Pythium* spp. are not known to be substantially involved in diseases of natural, unmanaged plant communities, and rather cause yield losses in agricultural systems. However, much knowledge has accumulated within the past decade on the possible involvement of *Pythium* spp. in plant diseases or seedling losses in natural ecosystems, and their potential influence on plant community composition (e.g. Mills & Bever 1998, Packer & Clay 2000, Nechwatal & Oßwald 2001). In this study, extensive investigations on the occurrence of *Pythium* spp. in *P. australis* stands of Lake Constance, Germany were carried out in order to assess their potential influence on reed vitality and performance, and their role in reed dieback phenomena. During the course of the survey, isolates of an unknown *Pythium* sp. close to *P. arrhenomanes* with a unique combination of sporangial and oospore characteristics, and sequence data were repeatedly obtained from reed rhizosphere and leaf samples. This paper describes this species as *Pythium phragmitis* sp. nov., gives details on its morphology, physiology, ecology, and pathogenicity in comparison to similar species, and provides molecular evidence to support its status as a distinct species.

## MATERIAL AND METHODS

### *Sampling sites and procedures*

Soil samples for the recovery of *Pythium* spp. were taken from the rhizosphere of *P. australis* growing in the reed belt of the Lake Constance littoral between April and October 2003, and in December 2004. Sampling site 1 (Egg) is located on the southern shore of Bodan peninsula (Überlinger See, 9° 11′ 18″ E, 47° 41′ 53″ N), and surrounded by mixed alluvial forests. Site 2 (Reichenau) is located on the southern side of the Reichenau dam (Untersee, 9° 06′ 04″ E, 47° 41′ 15″ N). Both stands are considered heavily affected by flood induced reed dieback (Ostendorp *et al.* 2003). In total, 15 soil samples were taken from permanently flooded ($n=9$) or from drier sites ($n=6$) within the extension of the reed belt in stand 1. In stand 2, three samples from flooded reed sites were taken. Soil was collected in clean plastic bags, brought to the laboratory, and stored cool (6 °C) until further use. Soil samples were subjected to a standard bait test using oak leaflets (*Quercus robur*, greenhouse plants; Nechwatal & Oßwald 2001), young reed seedlings (*in vitro* grown from seed) or grass leaf blades (boiled for 10 min) as baits. *Ca* 0.25 l of each soil sample was flooded with deionised water, and several baits were spread over the water surface. Infected baits showing discolouration after *ca* 3–5 d of incubation at 19 ° were blotted dry on sterile filter paper, cut into segments, and plated onto a selective agar medium inhibiting growth of fungi other than oomycetes (16 g agar, 3 g $CaCO_3$, 100 ml V8 juice, 900 ml $H_2O$ dest., amended with 25 mg $l^{-1}$ benomyl, 50 mg $l^{-1}$ PCNB, 10 mg $l^{-1}$ rifampicin, 200 mg $l^{-1}$ ampicillin, 0,05 ml $l^{-1}$ nystatin, [PARPN], Tsao 1983). Plates were incubated at 19 ° in the dark. Developing cultures were transferred to V8 agar plates (V8A, 100 ml V8 juice, 16 g Agar, 3 g $CaCO_3$, 900 ml $H_2O$ dest.) for further identification and maintenance. For the isolation from symptomatic reed plants, several mature leaves showing yellowing or necroses were collected from the same location (site 1) on three occasions during June 2004. Symptoms usually were found on those parts of the blades that had become inundated due to rising summer water levels. Leaves were thoroughly washed under running tap water, and surface disinfected with 70 % ethanol (1 min). Small segments (*ca* 4 mm diam) from the margins between healthy and diseased tissue were plated onto PARPN, and further processed as described above.

### *Growth and morphology*

For the assessment of growth rates isolates of *Pythium phragmitis* and related *Pythium* spp. (Table 1) were grown on 20 ml corn meal agar (CMA, van der Plaats-Niterink 1981), malt extract agar (MEA, with 2 % malt extract and 2 % agar), and V8A in 90 mm Petri dishes, and incubated at 6, 15, 19, 25, 30, 34, 37 and 40 ° for 3 d after the onset of hyphal growth. Colony morphology was recorded after incubation for 6 d at 19 ° in the dark. Investigations on sporangial development and germination behaviour were made on discs (diam 5 mm) cut from the edge of a culture actively growing on V8A or CMA, and floated in demineralised water (DW) or non-sterile soil extract water (SEW) for 24 h at 20 ° or 6 °. Oogonial, antheridial, and oospore characteristics were determined after several days of incubation at 20 ° in the dark on cultures prepared of V8A and CMA. Dimensions of 25 mature oogonia/oospores chosen at random were recorded at 320× magnification with the light microscope.

### *Sequence analysis*

In order to determine the phylogenetic relationship of the new species to those already known, sequence analyses of the ITS regions of the rDNA repeats and the cytochrome oxidase II (*cox* II) gene were performed and data compared to those of related species. These sequences were either generated during this study or obtained from GenBank. For DNA isolation, mycelial material was scraped off from agar plates and extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All sequence editing and aligning was carried out using BioEdit, version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

**Table 1.** *Pythium* species and isolates used in this study.

| *Pythium* sp. | Clade[a] | Internal ID | Other ID | ITS GenBank accession No. | Location, country, year of isolation | Isolated from[b] | Source[c] |
|---|---|---|---|---|---|---|---|
| *P. phragmitis* | B1e | P13 | CBS 117104 | AY594259 | Egg, D, 2003 | *Phragmites australis*, s | UKN |
| | | P40 | – | – | Egg, D, 2003 | *P. australis*, s | UKN |
| | | P42 | – | – | Egg, D, 2003 | *P. australis*, s | UKN |
| | | P52 | – | – | Egg, D, 2003 | *P. australis*, s | UKN |
| | | P55 | – | – | Egg, D, 2003 | *P. australis*, s | UKN |
| | | P58 | – | – | Egg, D, 2003 | *P. australis*, l | UKN |
| | | P59 | – | – | Egg, D, 2004 | *P. australis*, l | UKN |
| | | P61 | – | – | Egg, D, 2004 | *P. australis*, l | UKN |
| | | P62 | – | – | Egg, D, 2004 | *P. australis*, l | UKN |
| | | P63 | – | – | Egg, D, 2004 | *P. australis*, l | UKN |
| | | P64 | – | – | Egg, D, 2004 | *P. australis*, l | UKN |
| | | P65 | – | – | Egg, D, 2004 | *P. australis*, l | UKN |
| | | P69 | – | – | Egg, D, 2003 | *P. australis*, s | UKN |
| | | P71 | – | – | Reichenau, D, 2004 | *P. australis*, s | UKN |
| | | P73 | – | – | Reichenau, D, 2004 | *P. australis*, s | UKN |
| *P. arrhenomanes* | B1e | – | CBS324.62 | AY858635 | USA, 1962 | *Zea mays* | CBS |
| | | – | CBS430.86 | – | NL, 1986 | *Z. mays* | CBS |
| | | – | OPU480 | – | Japan, 2001 | *Oryza* sp. | OPU |
| | | P54 | – | AY743661 | Konstanz, D, 2003 | *P. australis*, s | UKN |
| *P. graminicola* | B1d | – | 70406 | – | Stuttgart, D, 1997 | *Rhapis* sp. | BBA |
| *P. myriotylum* | B1c | – | OPU715 | – | Japan, 2004 | *Phaseolus* sp. | OPU |
| | | – | CBS162.68[d] | – | USA, 1962 | *Chrysanthemum* sp. | CBS |
| *P. torulosum* | B1a | – | OPU511 | – | Japan, 1989 | *Agrostis* sp. | OPU |
| *P. vanterpoolii* | B1e | – | OPU512 | – | Japan, 1989 | *Agrostis* sp. | OPU |

[a] Phylogenetic clade according to Lévesque & De Cock (2004).
[b] s, soil sample; l, leaf sample.
[c] BBA, Federal Biological Research Centre for Agriculture and Forestry – Microbiology, Berlin (Helgard Nirenberg); CBS, Centraalbureau voor Schimmelcultures, Utrecht; OPU, Osaka Prefecture University, Japan (Motoaki Tojo); UKN, Universität Konstanz, Phytopathology, Konstanz, Germany.
[d] Isolate CBS 162.68 is deposited at CBS as *P. aristosporum*.

### *ITS rDNA*

PCR amplification of ITS1, 5.8S and ITS2 regions was performed with primer pair ITS4 (White *et al*. 1990) and ITS6 (Cooke & Duncan 1997). Direct sequencing of the PCR products was carried out by MWG Biotech (Ebersberg, Germany), and boundaries of the ITS1, 5.8S and ITS2 regions determined according to Cooke *et al*. (2000). Sequence entries of *Pythium* spp. from clade B1e of Lévesque & de Cock (2004) and *P. graminicola* (B1d) were retrieved from GenBank. *P. aphanidermatum* (clade A) was used as an outgroup. Sequence data were analysed and neighbour-joining phylogenetic analyses conducted using the programs DNADIST and NEIGHBOR from the PHYLIP package (v. 3.5, Felsenstein 1993), as described in Cooke *et al*. (2000). Kimura-2-parameter distances were calculated, with a transition/transversion ratio of 2.0. Tree topology was tested with 1000 bootstrap trials using SEQBOOT and CONSENSE (Felsenstein 1993). Trees were drawn using TreeView (Page 1996).

### cox *II gene DNA*

To confirm the ITS sequence results and to separate *Pythium phragmitis* from its closest relative *P. arrhenomanes*, additional analysis of the *cox* II gene was performed. Fragments were generated using the primers FM35 and FM58 as described by Martin (2000). Using these primers, direct sequencing was carried out by MWG Biotech. All *cox* II sequence data from *P. arrhenomanes* and *P. aristosporum* available in GenBank were used for phylogenetic analysis, as described for rDNA ITS regions. The sequence for *P. arrhenomanes* strain CBS 324.62 was newly generated in this study. Sequences were trimmed to a length of 563 bp to match the length of most of these GenBank entries. Again, *P. aphanidermatum* was used as an outgroup.

## ***Pathogenicity***

Pathogenicity of five isolates of *Pythium phragmitis* was evaluated in comparison to that of five closely related species, i.e. *P. arrhenomanes* (four isolates) *P. graminicola*, *P. myriotylum*, *P. torulosum*, and *P. vanterpoolii* (one isolate each) (Tables 3–4).

### *Pathogenicity towards reed seedlings*

Pathogenicity towards reed seedlings was assessed in a small-scale soil infestation test. Petri dishes (60 mm diam) were filled with *ca* 2.5 g autoclaved, moist wheat kernels (*ca* 25 kernels). These were inoculated with a V8 agar disc colonised with the *Pythium* sp. to be tested. When the culture completely covered the wheat kernels (depending on growth rate),

**Table 2.** Morphological and growth features of *Pythium* spp. examined.

| *Pythium* sp. (clade[a]) | No. of isolates | (a) Culture morphology (b) Aerial mycelium (on V8A) | Growth rate (V8A) at 30 °C [mm $d^{-1}$][b] | Maximum temp. for growth (°C) | Oospore diam (μm)[b] | Oospore state | Oospore abortion[c] |
|---|---|---|---|---|---|---|---|
| *P. phragmitis* (B1e) | 15 | (a) no specific pattern (b) cottony | 30.9 (29.5–33) | 40 | 25 (23.5–26.5) | plerotic | − |
| *P. arrhenomanes* (B1e) | 3 | (a) no specific pattern (b) cottony, loose | 25.7 (23–27.0) | <40 | 32 (31.5–32.5) | plerotic | ++ |
| *P. arrhenomanes* P54 (B1e) | 1 | (a) no specific pattern (b) dense-cottony | 36 (n/a) | >40 | n/a | n/a | n/a |
| *P. vanterpoolii* (B1e) | 1 | (a) no specific pattern (b) cottoy-appressed | 13.5 (n/a) | 30 | 20 (n/a) | plerotic | − |
| *P. graminicola* (B1d) | 1 | (a) no specific pattern (b) scarce | 25 (n/a) | >40 | 23.5 (n/a) | plerotic/aplerotic | ++ |
| *P. myriotylum* (B1c) | 2 | (a) no specific pattern (b) cottony, loose | 46.5 (39–54) | >40 | 25.5 (25–26.5) | aplerotic | + |
| *P. torulosum* (B1a) | 1 | (a) stellate–rosette (b) none | 16 (n/a) | 37 | 17.5 (n/a) | plerotic | − |

[a] Phylogenetic clade according to Lévesque & De Cock (2004).
[b] If applicable, mean and range of isolate means is given.
[c] −, nil or rare; +, occasional; ++, frequent.
n/a, not applicable.

the Petri dishes were filled with a non-sterile mixture of sand and potting soil, watered, and 10 reed seedlings (2 wk old) were planted into the soil. Control plants grew on a non-inoculated wheat/substrate mixture. Seedling experiments were performed in duplicate. Number of dead plants was noted after 4 d incubation at 19 ° under natural light.

### *Pathogenicity towards reed and maize leaves*

Six month old greenhouse-grown reed and 4 wk old maize plants (*Zea mays*) were used for the assessment of the pathogenicity towards mature leaves. For each isolate, seven leaves of approximately the same age (i.e. the same position on the culm) were collected, clipped on base and apex (length *ca* 12–15 cm), and placed in glass Petri dishes containing moist filter paper. Clipped edges were sealed with paraffin wax to avoid infection through these large wounds. Leaf blades were inoculated with a disc (4 mm diam) taken from the margin of an actively growing V8A culture of each of the *Pythium* spp. tested. A drop of a 0.05% skimmed milk solution was applied to the agar to facilitate adhesion to the leaf surface. Controls received uncolonised V8A plugs. Experiments were conducted in triplicate for each test plant. Leaf lesion length (longitudinal extension) was recorded after 3 d (maize) or 6 d (reed) of incubation at 19 °.

## RESULTS

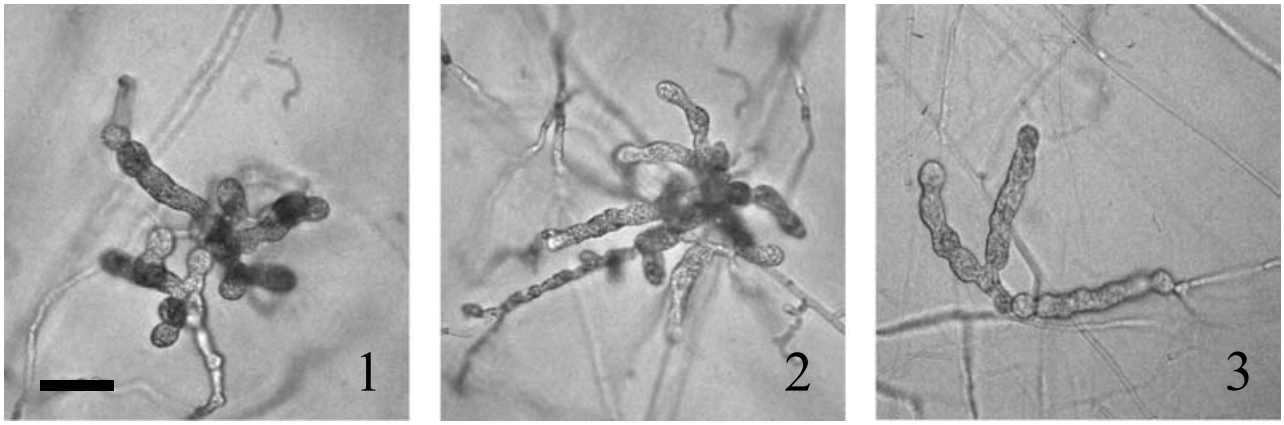
### *Distribution*

In total, 13 isolates of *Pythium phragmitis* were obtained from site 1. The species was recovered from 6 out of 9 soil samples from flooded sites, but was not found in any of the soil samples from drier sites. It was also readily isolated from all symptomatic mature reed leaves collected in stand 1, revealing 7 additional isolates. In site 2, *P. phragmitis* was isolated from 2 out of 3 flooded soil samples (Table 1). Isolation from soil was successful during the whole sampling period (April–December). The species was exclusively caught with young reed seedlings as baits, while it was never recovered from other grass blades or oak baits.

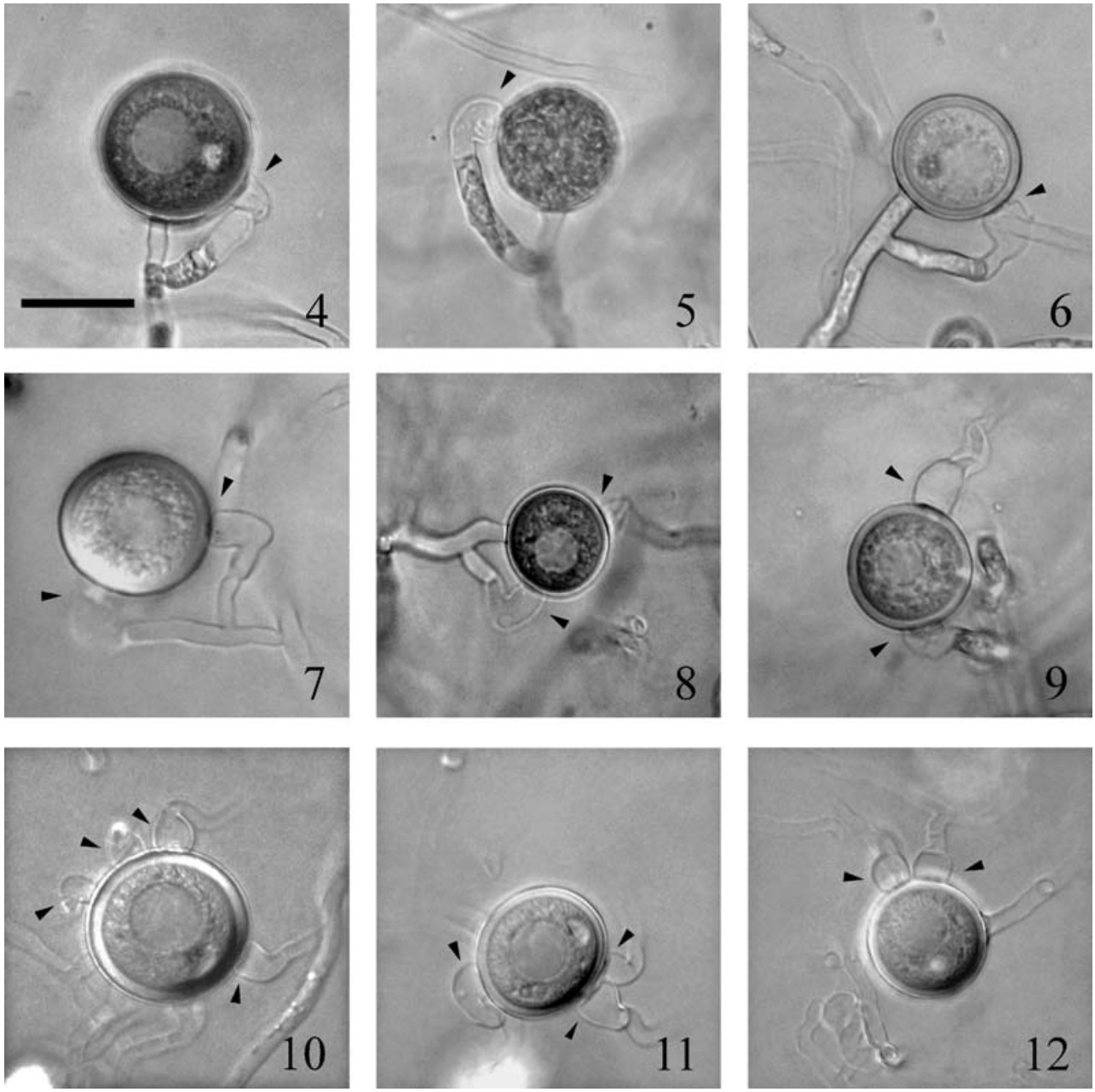
### *Growth and morphology*

All isolates of *Pythium phragmitis* with an optimum growth temperature of 30 ° on all agar media. Maximum growth temperature *ca* 40 °. Growth rates at 30 ° on V8A in comparison to other species are given in Table 2. Colonies without a specific growth pattern, showing various amounts of dense or loose cottony aerial mycelium on V8A, CMA and MEA.

*Main hyphae* to 7 μm wide. Hyphal swellings or chlamydospores not observed. *Sporangia* not observed on solid agar, but readily produced in water or SEW culture, consisting of irregularly lobulate, inflated filamentous (*ca* 10–15 μm wide) and knot-like, branched elements (Figs 1–3). *Zoospore* release observed only in very few isolates under the conditions applied, with low numbers of zoospores being released. *Oogonia* abundantly produced in single culture, strictly globose, smooth-walled, and borne terminally. Mean oogonial diameter of six isolates ranging from 23.5 to 26.5 μm (mean 25 μm, Table 2). *Antheridia* usually monoclinous, often also diclinous, usually crook-necked, 1–5 (8) per oogonium, making broad apical contact to the oogonium. Antheridial cells measuring *ca* 10–11 × 6–7 μm (means of six isolates). *Oospores* single, plerotic, completely filling the oogonium, and oospore diameters not significantly different from oogonial diameters. Oospore walls up to 2.5 μm thick (Figs 4–12). Levels of oospore abortion low in all isolates.




**Figs 1–3.** Sporangia of *Pythium phragmitis* consisting of irregularly lobulate, inflated filamentous elements. Bar = 40 μm.




**Figs 4–12.** Oogonia, oospores and antheridia (arrowheads) of *Pythium phragmitis*. **Figs 4–6.** Oogonia/oospores with single, monoclinous antheridia. **Figs 7–9.** Oogonia/oospores with two mono- or diclinous antheridia. **Figs 10–12.** Oogonia/oospores with two or more, mostly diclinous antheridia. Bar = 20 μm.

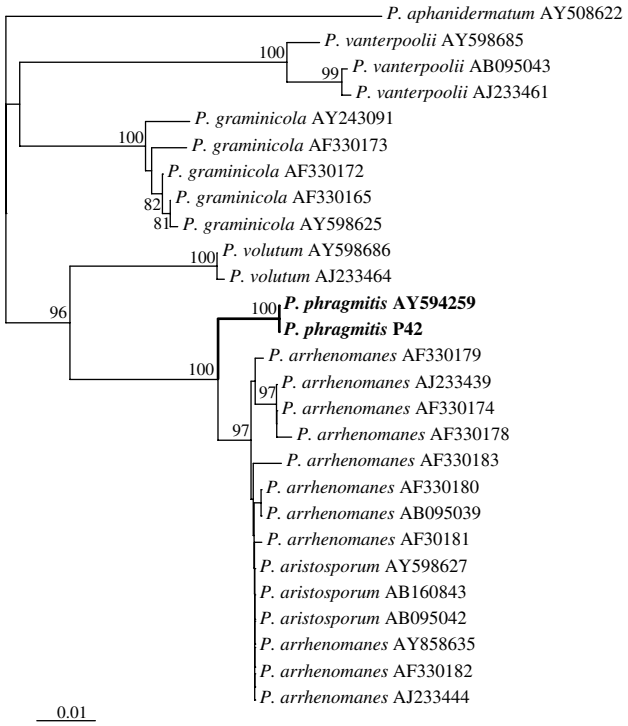



**Fig. 13.** Phylogenetic tree of *Pythium* spp. from clade B1e of Lévesque & de Cock (2004), including *P. phragmitis*, and *P. graminicola* constructed after distance-based analysis of ITS1, 5.8S and ITS2 regions of the rDNA. If available, GenBank accession numbers are given. Numbers at the branches indicate the percentage of bootstrap values after 1000 replications (values below 50% not shown). *P. aphanidermatum* was used as an outgroup. Bar = number of nucleotide substitutions per site.

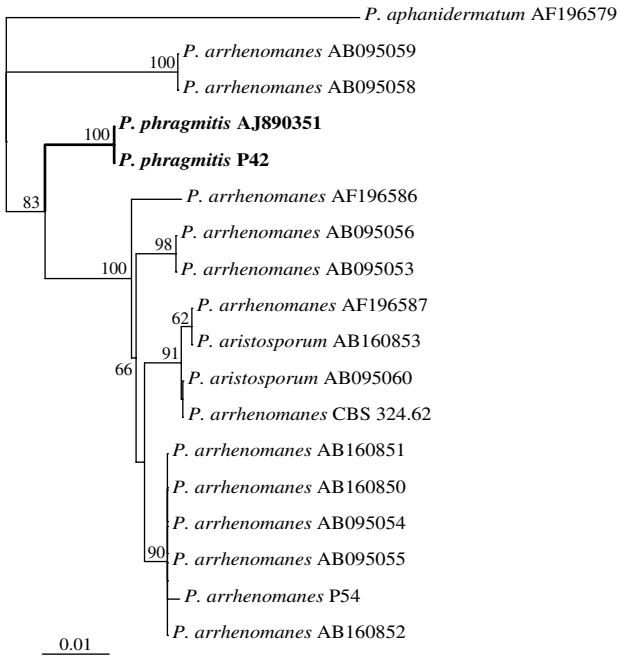



**Fig. 14.** Phylogenetic tree of *Pythium arrhenomanes*, *P. aristosporum* and *P. phragmitis*, constructed after distance-based analysis of sequences of the cytochrome oxidase II gene. If available, GenBank accession numbers are given. Numbers at the branches indicate the percentage of bootstrap values after 1000 replications (values below 60% not shown). *P. aphanidermatum* was used as an outgroup. Bar = number of nucleotide substitutions per site.

### *Sequence analysis*

#### *ITS sequences*

All 15 isolates of *Pythium phragmitis* had identical ITS sequences with the length of the complete ITS1, 5.8S and ITS2 being 796 bp. The sequence has been submitted to GenBank (AY594259). BLAST searches indicated the species' close relatedness to *P. arrhenomanes*. The sequence was 98% identical to most GenBank database entries for this species (e.g. AY858635 = CBS 324.62, ex-type strain of *P. arrhenomanes*), corresponding to 13 bp difference. Two more 98% matches were observed with 3 entries for *P. aristosporum* and with another *P. graminicola* (AY099310), while it was clearly different from that of most other *P. graminicola* entries (e.g. AY598625 = CBS 327.62, 89% identity). Neighbour-joining phylogenetic analysis of the ITS sequence data confirmed the distinctness of the new species from *P. arrhenomanes* (Fig. 13) with high bootstrap values.

#### cox *II gene DNA*

There was no sequence diversity within the *cox* II sequence of 15 *Pythium phragmitis* isolates, and the sequence has been submitted to EMBL (AJ890351). Similarly, all but two *P. arrhenomanes* GenBank entries for this gene differed by only 0.86%. In contrast, sequence divergence between the type strains of *P. phragmitis* and *P. arrhenomanes* was 3.1% (17 positions). Phylogenetic analysis confirmed that *P. phragmitis* isolates did not cluster within the majority of *P. arrhenomanes* isolates, but on a separate branch, supported by high bootstrap values (Fig. 14).

### *Pathogenicity*

#### *Pathogenicity towards reed seedlings*

All isolates of *P. phragmitis* were pathogenic on *Phragmites* seedlings. Sixty to 100% of the plantlets infected with this species were dead after 4 d of incubation (Table 3). The isolates of *P. graminicola* and *P. myriotylum* were equally aggressive towards reed seedlings. Mortality caused by *P. arrhenomanes* in our tests was lower (30–70% after 4 d, Table 3). *P. vanterpoolii* and *P. torulosum* caused minor damage to the seedlings. Controls did not show any damage, nor plant death during the course of the experiment.

#### *Pathogenicity towards reed and maize leaves*

All isolates of *Pythium phragmitis* caused extensive necroses on both maize and reed leaves after 3 or 6 d

**Table 3.** Reed seedling mortality caused by *Pythium phragmitis* and related species. Mean and range of two replicate experiments is given, with each experiment consisting of 10 seedlings per isolate.

| *Pythium* sp. (no. of isolates tested) | Mean seedling mortality (range) (%) |
|---|---|
| *P. phragmitis* (5) | 79 (70–88) |
| *P. arrhenomanes* (4) | 58 (43–70) |
| *P. vanterpoolii* (1) | 15 (0–30) |
| *P. graminicola* (1) | 75 (70–80) |
| *P. myriotylum* (1) | 100 |
| *P. torulosum* (1) | 5 (0–10) |
| Control | 0 |

incubation, respectively. On reed, they were consistently more aggressive than isolates of *P. arrhenomanes*, *P. graminicola*, *P. myriotylum*, *P. torulosum* and *P. vanterpoolii* in all tests. Necroses caused by *P. phragmitis* were significantly larger on this plant species (Table 4). *P. phragmitis* was readily re-isolated from infected reed leaves. In maize, no such clear-cut differences in virulence were observed, and *P. phragmitis*, *P. arrhenomanes*, and *P. graminicola* were equally aggressive (Table 4). Mock inoculated control leaves did not show any lesions.

**Table 4.** Lesions caused by *Pythium phragmitis* and related species on leaves of reed and maize. Mean lesion length (SEM) of three replicate experiments is given, with each experiment consisting of seven leaves per isolate. Means within each plant species followed by the same letter are not significantly different ($P < 0.05$, Kruskal–Wallis test, Dunn's Multiple Comparison Test).

| Plant species | *Pythium* sp. (no. of isolates tested) | Mean lesion length (cm) | SEM |
|---|---|---|---|
| Reed | *P. phragmitis* (5) | 1.8a | 0.12 |
| | *P. arrhenomanes* (4) | 0.6b | 0.05 |
| | *P. vanterpoolii* (1) | 0.2cd | 0.12 |
| | *P. graminicola* (1) | 0.4bc | 0.07 |
| | *P. myriotylum* (1) | 0.2cd | 0.05 |
| | *P. torulosum* (1) | 0.1d | 0.03 |
| | Control | 0.0d | 0.0 |
| Maize | *P. phragmitis* (5) | 4.9a | 0.29 |
| | *P. arrhenomanes* (4) | 4.3a | 0.27 |
| | *P. vanterpoolii* (1) | 1.7b | 0.45 |
| | *P. graminicola* (1) | 4.6a | 0.26 |
| | *P. myriotylum* (1) | 1.9b | 0.48 |
| | *P. torulosum* (1) | 1.1b | 0.25 |
| | Control | 0.0b | 0.0 |

## TAXONOMY

**Pythium phragmitis** J. Nechwatal, **sp. nov.**

*Etym.*: Named after its potential host plant, *Phragmites australis*

Coloniae crescentes celeres in agaris 'V8A', 'MEA' et 'CMA'. Crescunt in omnibus agaris inter 5 et 40 °C, optime ad 30 °, cum incrementum radiatum quotidianum 30.5 mm in agaro 'V8A'. Coloniae pubescentes, cum mycelio aerio, sine ordinatione distincto in omnibus agaris. Hyphae hyalinae, non-septatae, primariae ad 7 µm latae. Chlamydosporae vel inflationes hypharum non observatae. Sporangia formata abundantia in cultura aqua submerso, terminalia aut intercalaria, filamentosa et inflata, cum multis nodibus, plerumque ramosa, in medio 10–15 µm lata. Zoosporae raro formatae ad 20 aut 6 °. Culturae homothallicae, oogoniis et oosporis abundantibus in agaro 'V8A' vel 'CMA'. Oogonia terminalia, globosa, cum paries nonornatus, in medio 25.9 µm (22.0–30.0 µm) in diametro. Antheridia 1–5 (8) per oogonio, monoclina vel diclina, clavata, saepe curvata (9–12 × 5–8 µm), apices cum oogonia late coniuncta. Oosporae singularia, globosae, pleroticae, paries 1.5–2.5 µm crassus.

Typus: **Germany**: Konstanz/Egg, 9° 11′ 18″ E, 47° 41′ 53″ N, isol. ex solo rhizosphaerae ad *Phragmites australis* l, July 2003, *J. Nechwatal*, UKN P13 (dried culture) – holotypus; CBS 117104 – ex-type culture.

*Colonies* fast growing on V8A, MEA and CMA; growth observed between 5 and 40 ° on all agar media, optimum at 30 °, with daily growth of 30.5 mm on V8A. Colonies cottony, with aerial mycelium, without a distinct growth pattern. Hyphae hyaline, non-septate, up to 7 µm wide. *Chlamydospores* or hyphal swellings not observed. *Sporangia* abundantly produced in water culture, terminal or intercalary, consisting of inflated filamentous and knot-like, branched elements, 10–15 µm wide. *Zoospores* rarely formed at 20 or 6 °. Cultures homothallic, oogonia and oospores abundantly produced in V8A or CMA. Oogonia terminal, globose, smooth-walled, 22–30 µm (mean 25.9 µm) diam. Antheridia 1–5 (8) per oogonium, monoclinous or diclinous, clavate, crook-necked, 9–12 × 5–8 µm, making broad apical contact with oogonia. Oospores single, globose, plerotic; wall 1.5–2.5 µm wide.

## DISCUSSION

This study reports on the presence of a new *Pythium* sp. in reed stands of Lake Constance. *Pythium phragmitis* is a fast growing, homothallic species with inflated filamentous sporangia belonging to the *P. graminicola* complex (Hendrix & Papa 1974), or clade B1e of Lévesque & de Cock (2004), respectively. Although closely related to the species in these groups, *P. phragmitis* can still be easily distinguished by its unique combination of morphological characters (Table 2), pathogenicity (Tables 3–4), and DNA sequence data.

*P. arrhenomanes*, an economically important cereal pathogen, is the new species' closest relative, showing 98 or 97 % identity in the ITS and *cox* II sequences, respectively. As indicated by literature data (Drechsler 1928, Rands & Dopp 1934, van der Plaats-Niterink 1981, Kröber 1985), and comparison to reference strains (Table 2), it is distinguished from *P. phragmitis* by several traits: it usually has larger oogonia/oospores, more antheridia per oogonium (15–20) which are usually diclinous, and shows either high levels of oospore abortion or completely fails to form oogonia

and oospores. Although growth rate of *P. arrhenomanes* in our study was very variable (a fact that has also been reported by Rands & Dopp 1934), three out of four strains grew markedly slower than *P. phragmitis* (Table 2). Furthermore, all isolates regardless of their origin and age were significantly less aggressive towards reed as compared to *P. phragmitis* (Tables 3–4), indicating different virulence levels of the two species. High degrees of intraspecific variability in growth rate, morphology and virulence have already been described to occur in *P. arrhenomanes* in the past. In the 1930s, three varieties of *P. arrhenomanes* have been erected (Waterhouse 1968), and Rands & Dopp (1934) considered *P. arrhenomanes* to be a highly variable species, suggesting several 'morphologic strains'. Chen & Hoy (1993) studied 40 isolates of the species and found a large proportion of them to have smaller oogonia and less antheridia (as in *P. phragmitis*) than originally reported. Based on ITS-RFLP differences, three molecular *P. arrhenomanes* subgroups were erected. However, these were not consistent with oospore diameter data and no further taxonomical reference is being made to them. After *in silico* digests with the respective enzymes, we could rule out a potential congruence of any of these groups with *P. phragmitis*. Matsumoto *et al.* (2000), in contrast, suggested that *P. irregulare* isolates showing consistent ITS sequence and morphological divergence probably are distinct species. In the case of *P. phragmitis*, *cox* II sequences corroborated the ITS results, clearly placing the taxon distinct from *P. arrhenomanes*. Therefore, at present knowledge and as evidenced by our morphological and sequence data, we propose *P. phragmitis* to represent a new species distinct from *P. arrhenomanes*. Different levels of virulence towards reed as observed in our tests add further weight to this taxonomical implication.

*P. aristosporum* is considered a species very close to *P. arrhenomanes*. It is only distinguished by the number and origin of the antheridia (van der Plaats-Niterink 1981). Three out of four ITS GenBank entries for this species (AB095042, AY598627, AB160843) were identical to *P. arrhenomanes* (e.g. AY858635, ex-type), so that it remains doubtful whether *P. aristosporum* is a distinct species. Although it seems generally accepted not only in the genus *Pythium* that ITS sequence identity does not prove conspecificity (Lévesque & de Cock 2004), the fact that *cox* II sequences also placed *P. aristosporum* within *P. arrhenomanes* (Fig. 14) cast doubt on this taxon being a distinct species. According to BLAST searches with ITS sequence data produced in our study, and Arthur W. A. M. de Cock (pers. comm.), the *P. aristosporum* isolate studied here (CBS 162.68, Table 1), is a misidentified *P. myriotylum* with which it also shared growth rate, temperature maximum and oospore characteristics (Table 2). Similarly, in BLAST searches the fourth GenBank entry for *P. aristosporum* (AF290844) found its closest match in *P. myriotylum*.

*P. graminicola*, another related grass pathogen (clade B1d) has also been considered difficult to separate from *P. arrhenomanes* in the past, due to overlapping morphological characters (Chen & Hoy 1993, Gilbert, Cother & Nicol 1995). However, it could be clearly distinguished from this species by molecular evidence (Chen & Hoy 1993). Although *P. graminicola* also shares several morphological traits with *P. phragmitis* (oogonial diameter, number and origin of antheridia, growth temperatures), ITS and *cox* II sequence differences readily separate these species (89 and 94 % identity, respectively). A single ITS 98 % match of *P. phragmitis* with *P. graminicola* (AY099310) is due to the misidentification of this isolate (Motoaki Tojo, pers. comm.). In addition, our observations as well as Kröber (1985) proved the presence of a significant number of aplerotic oospores in *P. graminicola* (Table 2).

*P. vanterpoolii* also clusters within clade B1e of Lévesque & de Cock (2004). It differs considerably from *P. phragmitis* as to its morphology (Table 2), pathogenicity (Tables 3–4) and DNA sequence. It has sporangia consisting of rather irregularly catenulate globose, unbranched elements, its oogonia and oospores are smaller, with less antheridia, and it reaches its maximum growth temperature at 30 °C (Table 2). ITS sequence similarity to *P. phragmitis* is 89 %. Similarly, *P. volutum* from clade B1e differs by morphology (long antheridial stalks coiling around oogonial stalks), growth temperature (maximum 31 °C; van der Plaats-Niterink 1981) and ITS sequence (92 % identity). *P. torulosum* (clade B1a) and *P. myriotylum* (clade B1c), two more grass associated species from the *P. graminicola* complex also differ distinctly from *P. phragmitis* in growth, morphology (Table 2; van der Plaats-Niterink 1981), pathogenicity (Tables 3–4) and ITS sequence (89 % and 88 % identity, respectively).

The new species *P. phragmitis* possibly plays an important role in reed stand ecology. A number of factors have been proposed as possible causes for the dieback of reed stands in European lakes (Ostendorp 1989, Brix 1999). A current study on the mechanisms of reed belt area losses in Lake Constance emphasised the importance of water level variation, and flooding of reed plants during high water levels was suggested to be among the main causes for the decline (Ostendorp *et al.* 2003, Dienst *et al.* 2004). The probability of a reed stand being severely damaged was shown to be significantly increasing with decreasing elevation relative to the mean water level. Although this could in part be explained by limited oxygen supply of shoots and rhizomes after flooding, other as yet unknown factors have been postulated to account for the damage observed (Ostendorp *et al.* 2003). Our results provide several strong lines of evidence for a close association of the proposed reed pathogen *P. phragmitis* with *P. australis*: (1) it was readily isolated from the majority of analysed reed rhizosphere soil samples; (2) it was exclusively caught with reed seedlings as baits

and not with oak leaflets or boiled grass leaves; (3) it was consistently isolated from necrotic mature reed leaves in the field; and (4) a high level of virulence towards reed was demonstrated in *in vitro* infection assays where it was significantly more aggressive than related species. Koch's postulates have been fulfilled for *P. phragmitis* on *P. australis* leaves. Furthermore, these results are indicative of a considerable degree of specificity towards a single host species, a trait not generally established in the genus *Pythium*. Given its high virulence even against mature reed leaves, *P. phragmitis* infection of reed inundated during flooding events may be of particular significance. Although *Pythium* spp. are generally considered to be root pathogens, once present in a reed stand they will easily disperse and disseminate by means of zoospores, and infect submerged parts of reed plants. This is in accordance with the observation that mature reed leaves readily became necrotic on flooded parts, i.e. those parts of the blades that came in contact to the source of inoculum during rising water levels. Experiments with young reed plants flooded with *P. phragmitis*-infested water *in vitro* showed that virtually every unfolded submerged leaf died within several days, while this did not occur in flooded but uninoculated controls (J.N. & A.W., unpubl.). Significant losses in assimilating leaf area will contribute to a decrease of carbohydrate storage in the rhizomes, reducing number and size of emerging shoots in the next growing period, and eventually resulting in a patchy appearance typical for declining reed stands (Brix 1999, Ostendorp *et al*. 2003).

Low rates of seedling establishment and the sensitivity of seedlings to submergence are well-established for *P. australis*, and seedling survival was suggested to be even lowered by lake water regulation, and the subsequent lack of periodically non-submerged natural seedbeds (Rea 1996, Mauchamp, Blanch & Grillas 2001). In Lake Constance, a largely non-regulated lake with natural water regimes, the rate of generative propagation in reed stands is likely to diminish due to the high susceptibility of seedlings to *P. phragmitis*. As indicated by our infection tests and baiting procedures (with reed seedlings used as baits), even natural *Pythium* loads in littoral soils can negatively influence seedling survival. The resulting failure of generative reproduction (i.e. failure of 'pulse recruitment', Clevering & Lissner 2000) could possibly prevent the re-colonisation of gaps, and subsequently lead to further opening of previously homogeneous reed stands. In addition, lack of generative offspring can reduce genetic diversity of reed on a landscape level, thus increasing a stand's vulnerability to biotic pests and long-term environmental changes (Rea 1996, Brix 1999, Clevering & Lissner 2000). For example, water depth is considered one of the main selective forces in mature *P. australis* populations (Clevering & Lissner 2000). As flooding events in Lake Constance are anticipated to occur more frequently and earlier during the growing period (Dienst *et al*. 2004), reed genotypes showing retarded shoot regrowth (Kühl *et al*. 1999) would possibly be able to escape negative effects of selective forces such as leaf submergence and *Pythium* infection. A higher incidence of generative propagation within reed stands would allow for natural selection in favour of such genotypes (Clevering & Lissner 2000).

The results of our study indicate that *P. phragmitis* is able to cause damage to Lake Constance reed stands and should be considered as a contributing factor to decline phenomena that have repeatedly been reported for this lake. Further research should elucidate the distribution of the species on a more regional scale and eventually clarify its possible role in reed stand dynamics in other European lakes. In this context, more insight into the origin and evolution of the taxon will be of particular interest.

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