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# *Phytophthora gemini* sp. nov., a new species isolated from the halophilic plant *Zostera marina* in the Netherlands

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ABSTRACT

Eight strains belonging to the Oomycete genus *Phytophthora* were isolated from *Zostera marina* (seagrass) in The Netherlands over the past 25 y. Based on morphology, isozymes, temperature–growth relationships and ITS sequences, these strains were found to belong to two different *Phytophthora* species. Five strains, four of them isolated from rotting seeds and one isolated from decaying plants, could not be assigned to a known species and hence belong to a new species for which we propose the name *Phytophthora gemini* sp. nov. Three strains were isolated from decaying plants and were identified as *Phytophthora inundata*, thereby expanding the known habitat range of this species from fresh to brackish-saline areas. The possible role of both *Phytophthora* species in the decline of *Z. marina* in The Netherlands and the evolutionary significance of the presence of *Phytophthora* species in marine environments are discussed.



## Introduction

Over the past decades several *Phytophthora* strains were isolated from decaying leaves and seeds of *Zostera marina* plants in The Netherlands. These plants were growing in estuaries, connected by creeks to the Dutch part of the North Sea. *Z. marina* is a key ecological species in this marine ecosystem.

Due to variation of morphological characteristics within and overlap between species, *Phytophthora* species are notoriously difficult to identify using morphological data. Due to advances in molecular techniques in the last decades it has become more easy to determine the phylogenetic relationship of *Phytophthora* species and to determine species boundaries. Phylogenetic relationships between *Phytophthora* species were established based on ITS sequences (Cooke *et al.* 2000), but the resulting groupings in clades were not concordant with morphological groupings according to Waterhouse (1963) and Stamps *et al.* (1990). Nevertheless, sequence analysis of the ITS regions has been proven to be able to delineate *Phytophthora* species successfully with only some exceptions, *Phytophthora fragariae–Phytophthora rubi* and *Phytophthora infestans–Phytophthora mirabilis* (Cooke *et al.* 2000). However, isozymes and Cytochrome oxidase I sequences were able to distinguish *P. fragariae* from *P. rubi* (Man in 't Veld 2007). In later studies sequence analysis of several additional loci confirmed the initial groupings in eight clades (Kroon *et al.* 2004),

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whereas two new clades, clades 9 and 10, were added by Blair *et al.* (2008). Isozyme analysis has also been successfully used to delineate species of *Phytophthora* (Oudemans & Coffey 1991a, 1991b; Man in 't Veld *et al.* 2002; Man in 't Veld 2007) and to detect hybrids between different species (Man in 't Veld *et al.* 1998; Brasier *et al.* 2004; Man in 't Veld *et al.* 2007a).

In the last decade the number of newly described *Phytophthora* species has rapidly increased, including *Phytophthora irrigata* and *Phytophthora inundata*, two new species isolated from aquatic habitats in America and Europe (Hong *et al.* 2008; Brasier *et al.* 2003b).

In the present study we isolated two different *Phytophthora* species from *Z. marina*. The aim of our research was to characterize these *Phytophthora* spp. by morphology, isozyme genotyping using malate dehydrogenase (*Mdh-2*) and isocitrate dehydrogenase (*Idh-1* and *Idh-2*), and ITS sequence analysis. The first group of isolates could not be assigned to a known species and was shown to belong to a new species which is described here as *Phytophthora gemini* sp. nov.(for sake of convenience we refer to those *Phytophthora* isolates identified as this new species as *P. gemini* throughout the manuscript). The second group of isolates was identified as *P. inundata*, hitherto only known from fresh water habitats. The possible relations with marine *Halophytophthora* species as well as the possible role of *Phytophthora* species in the decline of seagrass are discussed.

## Materials and methods

Rotting leaves and seeds of *Zostera marina* were collected in the Grevelingen in the province of Zealand. Isolations were made on cherry decoction agar (CHA, Crous *et al.* 2009) and water agar (WA, Crous *et al.* 2009). Emerging colonies of *Phytophthora* were subcultured and stored on slants of V8 juice agar (Crous *et al.* 2009). The *Phytophthora* isolates studied here are listed in Table 1.

### *Morphology*

Colony morphology was compared on potato dextrose agar (PDA, Crous *et al.* 2009). Pieces of mycelial agar culture of the same size (5 mm in diameter) were used as inoculum; they were taken from actively growing colony margins of young cultures (3-d-old), in order to avoid delay in growth start and placed in the centre of the dish. Isolates were incubated at 18 °C in the dark. Colonies were photographed after 1 week of incubation.

Temperature–growth profiles were determined on cornmeal agar (CM-Oxoid-3, Basingstoke, Hampshire, England), by incubating the isolates in darkness at a range of different temperatures using a series of incubators set from 3 °C to 36 °C with increments of 3 °C, with an additional incubator set at 40 °C.

Inoculum plugs (5 mm diameter), from the edge of a young colony were transferred to the centre of a series of 13 Petri dishes that were incubated for one night at 18 °C. After confirming that all cultures showed some growth, one Petri dish was transferred to each of the aforementioned incubators. After an hour two perpendicular lines were drawn on the back of the Petri dish, intersecting beneath the inoculum plug. The margin of the colony was marked along these lines in all four directions. Radial growth was determined after 24 h, 48 h and 1 week.

Sporangium formation and morphology were studied on colonized hemp and pepper seeds in pond water: with tweezers ~five seeds were put on the margin of actively growing mycelium; when the seeds were covered by mycelium, they were transferred with tweezers to wateragar and sterile filtered pond water was poured on top of it; after ~5 h usually sporangia were formed. The production and morphology of sexual structures were studied on different agar media at 20 °C. Induction of oogonia and antheridia was examined on carrot piece agar (CPA, Kröber 1985) and CMA-Oxoid-3, Basingstoke, Hampshire, England by pairing the isolates with strains of known mating type from different *Phytophthora* spp. Strains used as mating partner were *Phytophthora cambivora* CBS356.78 (A1), *Phytophthora capsici* CBS111332 (A1), *Phytophthora cryptogea* PD20032149 (A1), *P. cambivora* CBS376.61 (A2) and *P. capsici* CBS128.23 (A2). In addition, all *Phytophthora gemini* isolates were paired with each other. For all structures studied, at least 25 measurements were made for each isolate.

**Table 1 – Reference numbers of *Phytophthora* strains used in this study, years of isolation, dimensions of sporangia, maximum growing temperature, isozyme profiles and GenBank accession numbers of *Phytophthora gemini* and *Phytophthora inundata*.**

| Strain | Year | Sporangia dimensions (μm) | | | $T_{max}$ °C | Isozyme loci | | | | GenBank |
|---|---|---|---|---|---|---|---|---|---|---|
| *Phytophthora gemini* | | | | | | | | | | |
| | | Range | Average | L/B | | *Idh-1* | *Idh-2* | *Mdh-1* | *Mdh-2* | |
| CBS123381 | 1998 | 60–88 × 30–40 | 77.8 × 39.4 | 1.9:1 | 33 | BB | AA | BB | CD | FJ217680 |
| CBS123382 | 1999 | 44–96 × 30–76 | 74.9 × 52.5 | 1.4:1 | n.a. | BB | AA | BB | CD | – |
| CBS123383 | 1999 | 60–90 × 36–70 | 77.4 × 52.5 | 1.5:1 | 33 | BB | AA | BB | CD | – |
| CBS123384 | 1999 | 56–100 × 32–60 | 78.1 × 45.1 | 1.7:1 | 33 | BB | AA | BB | CD | – |
| CBS 268.85 | 1985 | 42–72 × 20–48 | 62.2 × 36.9 | 1.7:1 | n.a. | BB | AA | BB | CD | FJ217679 |
| *Phytophthora inundata* | | | | | | | | | | |
| CBS 215.85 | 1985 | 40–56 × 28–40 | 46.4 × 36.0 | 1.3:1 | n.a. | AA | BB | AA | n.a. | FJ217682 |
| CBS 216.85 | 1985 | 56–64 × 44–56 | 58.7 × 48.5 | 1.2:1 | ~38 | AA | BB | AA | AB | FJ217681 |
| CBS 217.85 | 1985 | 48–72 × 40–48 | 56.0 × 42.3 | 1.7:1 | n.a. | AA | BB | AA | AB | – |

n.a.: not analyzed