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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

Isozyme analysis

Isozyme analysis was performed as described by Man in 't Veld et al. (2002).

ITS sequence analysis

DNA was isolated and the ITS region was amplified with primers ITS5 and ITS4 (White et al. 1990) as described before (Man in 't Veld et al. 2007a). Sequences were aligned using MUSCLE (Edgar 2004) under default settings. The result was edited manually in MEGA 4 (Tamura et al. 2007) to correct obvious mistakes in the automated alignment. An evolutionary model for the Bayesian analysis was selected by analyzing the dataset in jModeltest v0.1.1 (Posada 2008); the outgroup consisting of the two *Pythium* species was omitted from this analysis only. The general time reversible nucleotide substitution model with gamma distributed rate variation (GTR + G) received the highest score under both the Aikake Information Criterion (AIC) and the corrected AIC model.

Phylogenetic analysis was performed using Mrbayes v3.1.2 (Ronquist & Huelsenbeck 2003), using the GTR + G model. Two simultaneous analyses were run, with three heated and one cold chain each. The temperature was set to 0.08 in order to obtain acceptable rates of swapping between the cold and heated chains. The analysis was run for 2.5 million generations, with a sampling frequency of every 100th generation. All other settings were left at their default values. The first 10 % of samples were discarded as burnin.

Analysis of the data in Tracer v.1.5 (Rambaut & Drummond 2009) showed good convergence and mixing after the burnin period.

Results

Morphology of Phytophthora gemini

Widely spaced catenulate and clustered intercalary hyphal swellings occasionally occurred both in agar and water culture (Fig 2A–D). All *P. gemini* strains formed sporangia abundantly in sterile filtered pond water. Sporangial shapes vary from obovoid, to ellipsoid, ovoid and obpyriform (Fig 2E and F), often with a tapered base (Fig 2E). Sporangia have a conspicuous basal plug and are non-papillate with a wide exit pore (Fig 2J and L). Sporangia discharge their zoospores quickly after formation and in older cultures only empty sporangia remain (Fig 2D, H, I and L). Internal proliferation was not observed. Secondary sporangia may form by sympodial branching of the hypha directly below the terminal, primary sporangium (Fig 2F, I and L). The septum that delineates the secondary sporangium from the mycelium frequently forms in the hyphae carrying the primary sporangium, before the sympodial branchpoint, resulting in a sporangium with intercalary base (Fig 2K and L). This characteristic has not been reported for any other *Phytophthora* species, at least not to our knowledge. Sporangia are persistent on the hyphae, that is non-caducous (Fig 2G an H). Sporangiophores are undifferentiated from vegetative hyphae.

Sporangial dimensions are summarized in Table 1. Sexual structures were not produced in single culture (not homothallic) nor in mating tests using *Phytophthora cambivora* CBS356.78 (A1), *Phytophthora cryptogea* PD20032149 (A1) or *P. cambivora* CBS376.61 (A2) as tester strains nor when the different *P. gemini* isolates were grown together. In addition, the type strain of *P. gemini* CBS123381 was paired with *Phytophthora capsici* CBS111332 (A1) and *P. capsici* CBS128.23 (A2), but again no sexual structures were observed. Chlamydospores were not observed. Minimum temperature for growth lies beneath 3 °C, optimum temperature for growth between 24 °C and ~27 °C for all isolates tested. Maximum temperature at which growth was observed was 33 °C for all isolates tested (Fig 3).

Isozyme analysis

Two enzyme systems, MDH and IDH, which comprised altogether four putative loci, were used to characterize strains of *P. gemini* and *Phytophthora inundata* (Table 1). Both enzyme systems generated clearly interpretable bands at two loci, *Idh-1*, *Idh-2* (Fig 4). At *Mdh-2* only the patterns of *P. gemini* were clearly interpretable. At *Mdh-1* a long smear was visible which started at different positions in *P. inundata* compared to *P. gemini*. Different isozyme alleles were present in *P. inundata* and *P. gemini* at all four loci (Table 1). At *Mdh-2* clear three-banded patterns were visible in *P. gemini*. In *P. inundata* three-banded patterns were visible as well, but faint and smeary, differing however in mobility from those in *P. gemini*.

Phylogenetic analysis

Phylogenetic analysis of the ITS region of representatives of all *Phytophthora* clades as assigned by Cooke et al. 2000 also included some marine *Halophytophthora* spp. and this showed

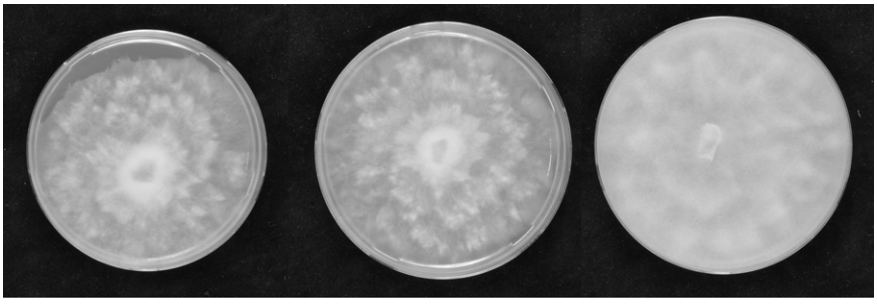

Fig 1 – Colony morphology on PDA of *P. gemini* strains CBS 123381, CBS123383, CBS268.85 after 1-week growth at 18 °C in the dark.

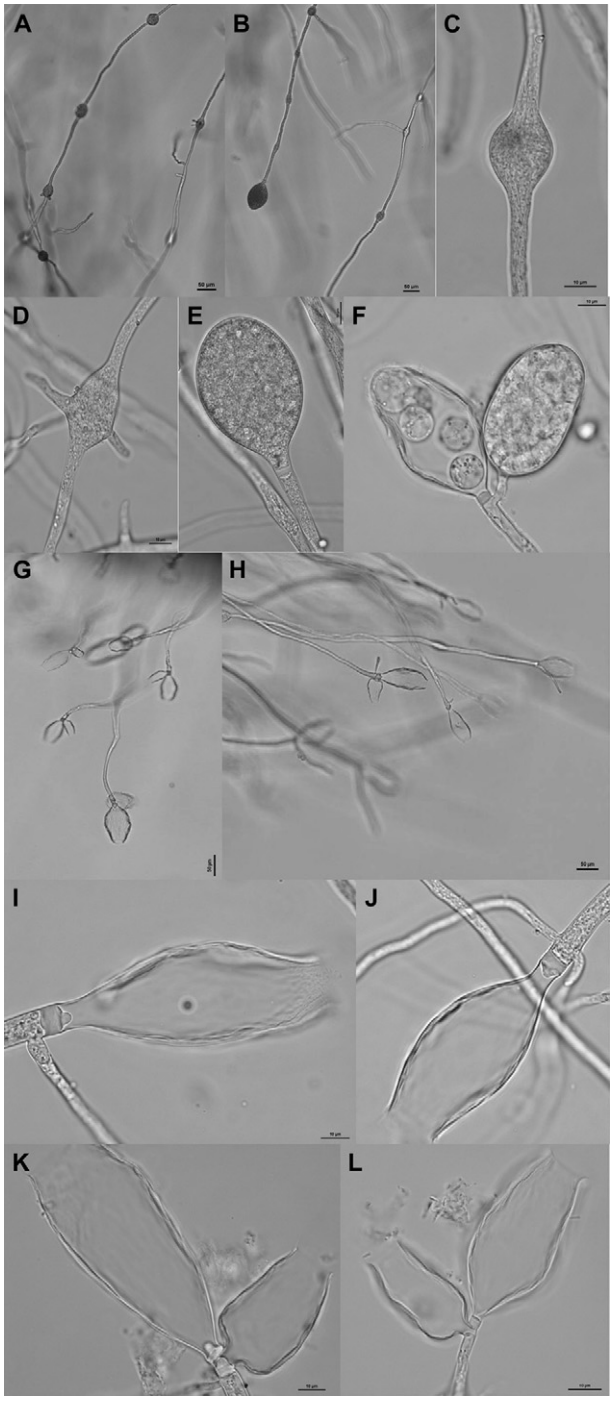

Fig 2 – Morphological structures of *P. gemini*. A: Catenulate hyphal swellings. B: Catenulate hyphal swellings with terminal sporangium. C: Intercalary hyphal swelling. D: Hyphal swelling at hyphal branchpoint. E: Ovoid sporangium with tapered base. F: External proliferation. G, H: Sporangiophores with empty sporangia and external proliferation, resulting in 'twins'. I, J: External proliferation, conspicious basal plug. K, L: Secondary sporangium with intercalary base.

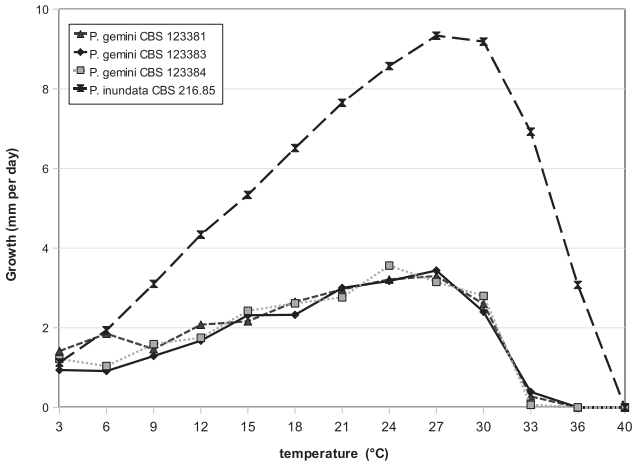

Fig 3 – Growth-temperature curves of *P. gemini* and *P. inundata* at temperatures ranging from 3 °C to 40 °C on CMA-oxoid agar, 2 d after inoculation.

that *Phytophthora gemini* is a clade 6 species (Fig 5). The sequence of *P. gemini* CBS123381 (GenBank FJ217680) was identical to that of *P. gemini* CBS 268.85 (GenBank FJ217679) except at position 128 where CBS123381 had a double base Y instead of T. The ITS sequence of *P. gemini* differs in length from two *Phytophthora inundata* strains, CBS216.85 and CBS215.85 (GenBank FJ217681, FJ217682) isolated from *Zostera marina* (807 *versus* 816) and at 26 positions including three deletions of one nucleotide, one deletion of two nucleotides and one deletion of four nucleotides. The two *P. inundata* strains both contained one double base Y at position 726, not present in *P. gemini*. *Phytophthora gemini* is phylogenetically most closely related to the as yet undescribed species *P.* sp. *personii* (Blair *et al.* 2008), but differs at 33 positions, including four insertions of one nucleotide, two deletions of one nucleotide, one deletion of two nucleotides and one deletion of four nucleotides in all *P. gemini* strains. The ITS sequence of four other marine species, *Halophytophthora avicenniae*, *Halophytophthora batemanensis*, *Halophytophthora polymorphica* and *Halophytophthora vesicula* were very distantly related to *P. gemini* and grouped in a separate clade. Considering the fact that *H. vesicula* is the type species of *Halophytophthora*, and the sequence that

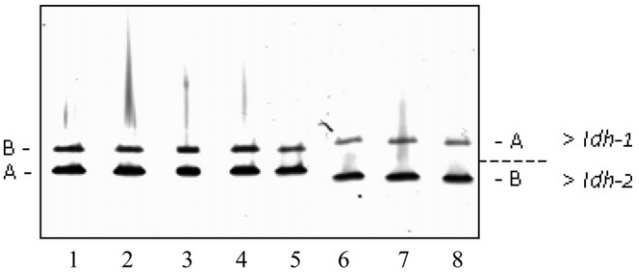

Fig 4 – Isozyme patterns of *Idh*-1 and *Idh*-2 isozymes generated by *P. gemini* (lanes 1–5) and *P. inundata* (lanes 6–8). 1: CBS 123381 5: CBS 268.85. 2: CBS 123384 6: CBS 217.85. 3: CBS 123383 7: CBS 216.85. 4: CBS 123382 8: CBS 215.85.

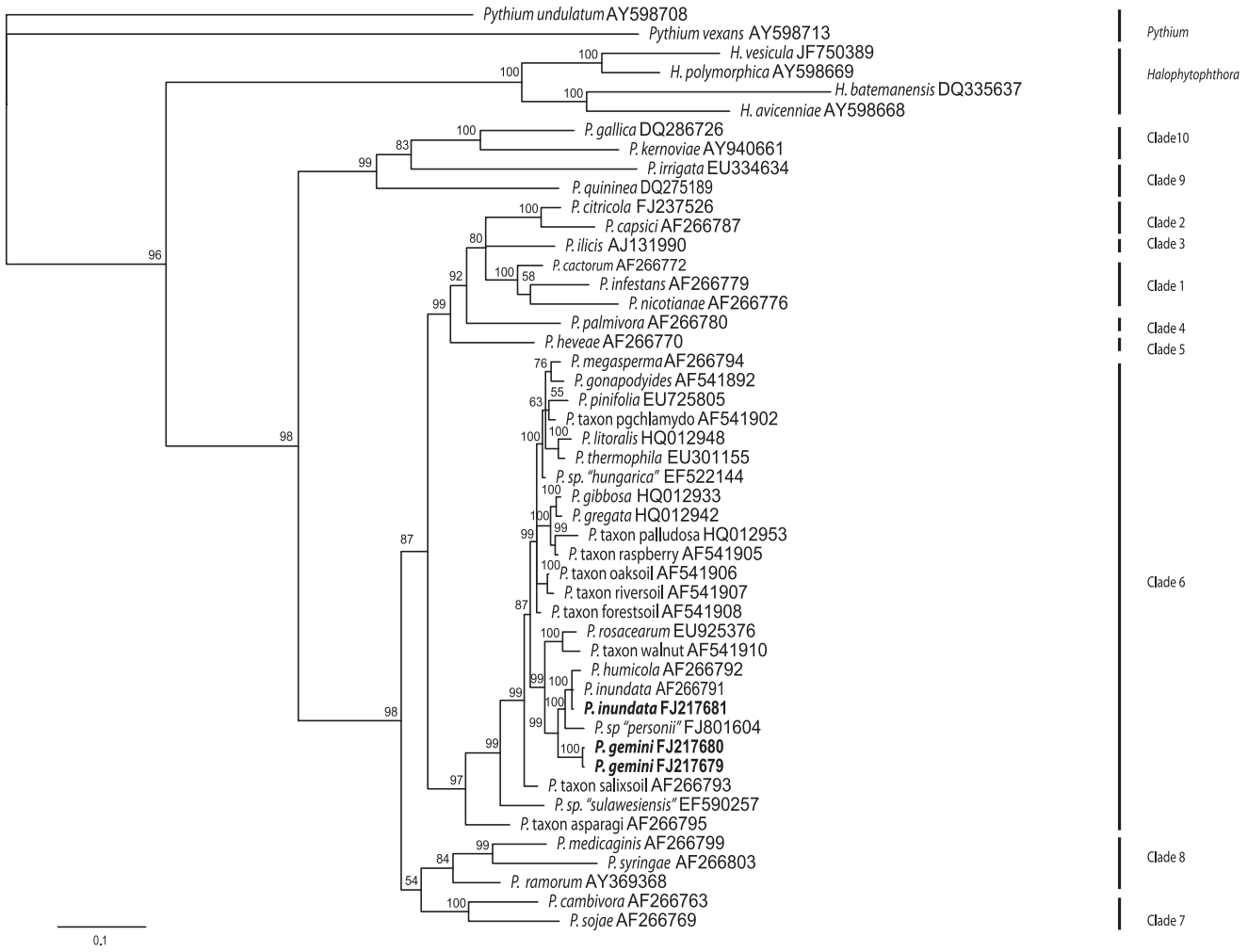

Fig 5 – Phylogenetic tree of *Phytophthora* species based on rDNA – ITS sequences, inferred from Bayesian analysis. Numbers above the branches represent Bayesian posterior probabilities, presented as percentages. Scale bar indicates number of substitutions per site.

we used was obtained from the type strain of this species, this clade was assigned to *Halophytophthora*.

Phytophthora gemini Man in 't Veld, K. Rosendahl, Brouwer & de Cock – Mycobank 519967; Figs 1–3.

Coloniis in agaro dextrosis Solani tuberosi (PDA) forma chrysanthema, nonnunquam obducta per mycelio aeri. Temperaturae crescentiae, optima 24–27 °C et maxima 33 °C. Vesiculae ex hyphis inflatis oriundae numerosae in agaro, catenulatae cum spatio lato, aliquando aggregatae. Sporangia forma ovoidea, obpyriforma, saepe cum basi contracto, nonpapillata, non caduca; longitudo et latitudo sporangiorum in medio 52–89 × 30–59 μm, longitudo-latitudo ratio1.6:1. Hyphae sporangiorum progenerantiae unum sporangium vel duo sporangia. Sporangia primaria terminales, sporangia secondaria producta per prolificam externam, septum separatum sporangium novum productum sub zonarium ramosum. Chlamydosporae nullae. Species veri similis sexus sterilis. Regio 'rDNA ITS' producta sequentiam unicam; et unica combinatio alleles isozymarum ad locos *Idh-1*, *Idh-2*, *Mdh-1*, *Mdh-2*.

Colony morphology in PDA chrysanthemum pattern, sometimes obscured by aerial mycelium. Optimum growing temperature 24–27 °C and maximum growing temperature 33 °C. Hyphal swellings numerous on agar, rounded catenulate, widely spaced, sometimes clustered. Sporangia are ovoid, obpyriform, often with tapered base, non-papillate, not caducous; dimensions average 52–89 × 30–59 μm, length-breadth ratio 1.6:1. Sporangiogenous hyphae developing one or two sporangia. First sporangia terminal, subsequent sporangia formed by external proliferation, whereby the septum delineating the new sporangium may be formed below the branching point. Chlamydospores are not produced. It is probably a sterile species. The sequence of the 'rDNA ITS' region is unique and at *Idh-1*, *Idh-2*, *Mdh-1* and *Mdh-2* unique isozyme alleles were present.

Holotype: The Netherlands, Lake Grevelingen, from *Z. marina*, CBS123381, dried culture, in CBS-KNAW, Fungal Biodiversity Centre, Utrecht, The Netherlands. Living culture ex-type CBS 1233381.

Etymology: geminus is twin, referring to two sporangia on one hypha.

Discussion

We have shown that *Phytophthora* strains, isolated from decaying leaves and seeds of *Zostera marina*, did not form a homogeneous group, but actually consisted of two species, *Phytophthora gemini* sp. nov. and *Phytophthora inundata*. The new species *P. gemini* is characterized by the possession of catenulate hyphal swellings, by its unique combination of terminal sporangia and secondary sporangia formed by external proliferation, by the lack of internal proliferation, by its optimum and maximum growing temperature and also by the possession of unique isozyme alleles (Table 1, Fig 4) and ITS sequence (Fig 5) and was therefore described as the new species *P. gemini*. Parenthetically, the formation of new sporangia below a terminal sporangium has also been reported in the Oomycete genus Sclerophthora (Payak & Renfro 1967). De Cock (1986) tentatively identified some of the isolates included in this study as *Phytophthora japonica* (Ito & Nagai) Waterhouse (Waterhouse 1974) and did not distinguish between the *P. gemini* and *P. inundata* isolates. *Phytophthora japonica* was originally described as *Pythiomorpha oryzae* (Ito & Nagai 1931), and indeed, at least superficially, seems to be morphologically similar to *P. inundata* and *P. gemini* with large non-papillate sporangia, and occurrence of intercalary hyphal swellings. *P. japonica*, a pathogen of rice (*Oryza sativa*) differs from *P. gemini* by its ability to induce production of gametangia in cultures of opposing mating type (Waterhouse 1958), a characteristic not observed for *P. gemini* that appears to be sterile. Hyphal swellings for *P. japonica* were described as occurring at irregular intervals (Waterhouse 1974), whereas they occur at widely spaced, regular intervals in *P. gemini* (Fig 2A, B). Furthermore, features typical for *P. gemini*, such as pronounced basal plugs and secondary sporangia directly below the terminal sporangium have not been described for *P. japonica*. To our knowledge, no isolates remain of this species, and it has not been included in any molecular study, making comparison difficult.

Two *P. gemini* ITS haplotypes were present in the population differing at position 128. Sequence analysis of the ITS region indicated that *P. inundata* and *P. gemini* are close neighbours belonging to the same clade 6 (Cooke *et al.* 2000) differing, however, at 26 positions. Another closely related species in ITS clade 6 is *Phytophthora humicola*. This species is more closely related to *P. inundata* than to the new species *P. gemini* and differs from both forementioned species by its homothallism. Several new clade 6 species *Phytophthora gibbosa*, *Phytophthora gregata*, *Phytophthora litoralis*, *Phytophthora thermophila* and *P.* taxon *palludosa*, were recently described by Jung *et al.* (2011) and *Phytophthora rosacearum* was recently described by Hansen *et al.* 2009. Phylogenetic analysis of the *ITS* sequence indicates that all these species are distinct from *P. gemini* (Fig 5).

A new ITS clade 6 species, *P.* sp. *personii* awaits description (Blair *et al.* 2008), but has a different ITS sequence. More putative new species, yet to be described, are known to belong to clade 6, including e.g. *P.* taxon *pgchlamydo* (Brasier *et al.* 2003a), *P.* sp. ex *Asparagus* (Saude *et al.* 2008), *P.* sp. *hungarica*, *P.* sp. *sulawesiensis* and *P.* sp. *sylvatica*. More putative new species, yet to be described, are known to belong to clade 6, including e.g. *P.* taxon *pgchlamydo*, *P.* taxon oaksoil, *P.* taxon riversoil, *P.* taxon forestsoil and *P.* taxon salixsoil, *P.* taxon Raspberry, *P.* taxon Walnut (Brasier *et al.* 2003a), *P.* sp. ex *Asparagus* (Saude *et al.* 2008), *P.* sp. *hungarica* and *P.* sp. *sulawesiensis*. Blasting of the ITS sequence of *P. gemini* in GenBank revealed that ITS sequences of all these putative new species differed from that of *P. gemini*, and were more distantly related to *P. gemini*.

In addition to *P. gemini*, a second species was isolated from *Z. marina* and identified as *P. inundata* Brasier, Sanchez–Hernandez & Kirk, described as a pathogen of trees and shrubs in wet or flooded soils (Brasier *et al.* 2003b). The ITS sequence of this group was almost identical to GenBank AF 266791 belonging to the ex-type IMI 389751 of *P. inundata*, except for the double base (Y) at position 726. All sequences determined by Brasier *et al.* (2003a, 2003b) were uniform. Also the morphology is similar to the original description, showing non-papillate sporangia, internal proliferation, a optimum growing temperature of ~30 °C and a maximum growing temperature of ~38 °C. Mating tests did not produce sexual structures and our strains are probably sterile. Partial or complete sterility is a frequently occurring character among clade 6 species (Brasier *et al.* 2003a, 2003b; Jung *et al.* 2011).

Isozyme analysis, using *Idh-1* and *Idh-2* and *Mdh-2* has been used before successfully to delineate *Phytophthora porri* and the later described *Phytophthora brassicae* (Man in 't Veld *et al.* 2002), and *Phytophthora fragariae* and *Phytophthora rubi* (Man in 't Veld 2007). Mining genomic sequences of *Phytophthora ramorum* and *Phytophthora sojae* revealed one mitochondrial and one cytoplasmatic form for MDH, resulting in two loci (Man in 't Veld *et al.* 2007b). In the genome of *Phytophthora infestans* one mitochondrial and one cytoplasmatic form of IDH were present (PITG-07056-1 and PITG-04441.1), justifying the assignment of two loci (*Idh-1* and *Idh-2*). A similar situation exists with both enzymes in *P. gemini* and *P. inundata*. Both enzymes revealed a lack of gene flow between *P. gemini* and *P. inundata*, indicating that the two species are reproductively isolated.

Interestingly, both the *P. gemini* as well as two *P. inundata* strains, although smeary and less clear, had three-banded patterns at *Mdh-2*, differing in mobility however. The middle band is the so-called heterodimeric band which indicates outcrossing. Double bases present in the ITS in both species probably indicate heterozygosity as well. Although mating experiments in the laboratory were unsuccessful, the heterozygous strains may have been involved in outcrossing in their recent past. Alternatively, three-banded patterns could theoretically also be the result of gene duplication (Man in 't Veld *et al.* 2007b). Incidentally, two sequences of *P. gemini* differ at one position indicating that the population may not be clonal.

Initially the newly described *P. gemini* was expected to be closely related phylogenetically to other marine Oomycetes belonging to the genus *Halophytophthora* (Ho & Jong 1990), but, instead, it appeared to be very distantly related to this group (Fig 5). The new species *P. gemini* is also not related to another aquatic species *Phytophthora irrigata*, because the ITS sequence of this species is located in clade 9 (Fig 5).

As proposed by Mayer (1942), species diverge by geographical isolation (allopatric) or, in the case of pathogens, by a change in host (sympatric e.g. *P. infestans* – *Phytophthora mirabilis* – *Phytophthora ipomoeae*) and hence it is highly unlikely that two closely related species are able to diverge if they share a common host in the same geographical area. *Phytophthora inundata* has been found before in several European countries but it has also been reported from South America (Brasier *et al.* 2003b) and recently from Australia (Burgess *et al.* 2009). *Phytophthora inundata* seems to have dispersed panglobally from its unknown centre of origin. In 2006 one *P. inundata* strain, identified by its ITS sequence, was isolated by baiting from soil in a creek, demonstrating that this species has been continuously present for at least 21 y in the delta area, whereas *P. gemini* has been continuously present for at least 14 y (table 1). Further studies on the genetic diversity within this new species and sampling in other areas may offer insight whether the new species is a hitherto overlooked species that naturally occurs in the delta area, or if it's a more recent introduction, possibly introduced in lumber water, transported by ships without cargo from all over the world.

The occurrence of *Phytophthora* spp. in marine environments has received relatively little attention in the literature; this may be the result of lower sampling in this habitat. Höhnk (1953) isolated *Phytophthora* from a branch on the beach of the North Sea island Wangerooge and from a soil sample in the Baltic Sea near Kiel. Weste *et al.* (1982) reported the isolation of a *Phytophthora* isolate from leaf baits on mangrove roots in Australia. The isolate was identified as *Phytophthora nicotianae* based on morphological characters and a high maximum growing temperature. A recent survey on Hainan island by Zeng *et al.* (2009) mentions the discovery of several marine *Phytophthora* isolates, that still await further characterization. Some of the isolates included in our study were previously reported and studied by De Cock (1986). The *ITS* sequences of *Halophytophthora* isolates in our study, which included the type strain of this marine genus, show that this genus forms a separate clade with a basal position to *Phytophthora* (Fig 5) The relationship of these *Halophytophthora* isolates to *Phytophthora*, and presence of marine isolates in *Phytophthora* raise questions about the habitat of the common ancestor of these two genera, and about the frequency of migration events between fresh water and marine environments.

The fact that *P. inundata* has now been recovered from both marine and fresh water seems to indicate that some species are able to survive in both habitats, this would imply that migration events from land to water or *vice versa* could be quite common. Alternatively it may indicate a very recent migration event that has not yet resulted in clearly detectable speciation. Comparisons between fresh and saltwater strains of *P. inundata* on salt tolerance and other characters could provide more insight in this issue. A survey for other *Phytophthora* species in marine environments and elucidation of their phylogenetic relationships could also provide valuable information on this subject.

In the delta formed by the rivers Meuse and Scheldt in the province of Zealand in The Netherlands extended tidal flats and salt marshes are present in the transition zone between fresh and salt water. The salt marshes are only inundated by springtide whereas the tidal flats and lower parts of the salt marshes are inundated twice a day by the tides from the North Sea. Due to the presence of vegetation, fine branched creek systems develop within the salt marshes. The population of the local marsh vegetation is composed of a dense carpet of unique salt tolerant plants like *Aster tripolium*, *Atriplex portulacoides*, *Limonium vulgare*, *Spartina anglica* and *Salicornia europea*, which have a distinct distribution along the elevational gradient (De Leeuw *et al.* 1994). Within these intertidal ecosystems, dense mats of the seagrasses *Z. marina* and *Zostera noltii* can be present within the creeks intersecting these tidal marshes as well as on the lower parts of the tidal flats. Seagrass meadows have been identified as belonging to the most valuable ecosystems in the world (Constanza *et al.* 1997) as these ecological key systems offer many ecosystem services e.g. several fishes like herring, three-spined stickle-back and garfish deposit their eggs on the leaves and fields of seagrass provide shelter for young eels and garfishes. The plants are also a source of food for swans, geese and ducks. Nevertheless, these valuable ecosystems are declining on a global scale, including those in The Netherlands (Orth *et al.* 2006; Lotze *et al.* 2006).

It is well-known that within The Netherlands the population of *Z. marina* as well as *Z. noltii* has been suffering a massive decline since the 1930s of the former century (Den Hartog 1987). Whereas seagrass was once so abundant that local people harvested it to use it as pillow filling, nowadays, in the same area, the seagrass is considered to be an endangered and protected plant species. Several potential causes have been proposed to have induced this decline, but hitherto the actual cause has never been identified beyond reasonable doubt. It has been speculated that the construction of dams, triggering a change in seacurrents, may have contributed to this decline. Another possibility could be that the water of the rivers at that time was starting to get increasingly polluted by run-off from agricultural fertilizer and probably also contained toxic chemicals from industry. The marine slime mold *Labyrinthula zosterae*, causal agent of wasting disease of seagrass in North-America (Muehlstein *et al.* 1991) has also been considered to be the cause of seagrass decline in The Netherlands (Westhoff & van Oosten 1991), but it is known that this organism can live side by side with seagrass without harming the seagrass population (endophytical). Although the taxonomy of *Labyrinthula* species is not so well defined, ten different recognized species exist in the literature (Muehlstein *et al.* 1991), and only *L. zosterae* is pathogenic to *Z. marina*. It is not clear whether a *Labyrinthula* species is involved in the decline of seagrass in The Netherlands. It is realistic to assume that there is probably not just one culprit for this decline and it may never be possible to reconstruct the chain of events that caused it. However, the involvement of *Phytophthora* was hitherto not considered to be a serious option. Both *P. inundata* and *P. gemini* were found coincidentally on *Z. marina* in two locations about 25 km apart. This may indicate that *Phytophthora* spp. are widespread in the delta area; the dispersal of sporangia and infected plant material is facilitated by the tides and, once established, *Phytophthora* spp. are able to spread rapidly. Years of isolation of *P. gemini* (Table 1) indicate that this species has been continuously present in the area for at least 14 y. Four *P. gemini* strains were isolated from rotting seeds of *Z. marina*, suggesting that *P. gemini* could have played a negative role in the recovery of *Z. marina*. Since

their pathogenicity on *Z. marina* has not yet been tested experimentally, it is currently not certain whether *P. gemini* and *P. inundata* were linked to the decline of *Z. marina*, or merely present as saprophytes on already dead plant material. Pathogenicity trials showed that *P. inundata* is a highly aggressive pathogen on young olive plants under waterlogged conditions (Sanchez-Hernandez *et al.* 2001; Brasier *et al.* 2003a, 2003b). Moreover, the genus *Phytophthora* contains primary pathogens, including *P. infestans* (Mont.) de Bary, causal agent of potato blight resulting in the potato famine in Ireland in the nineteenth century (Fry & Goodwin 1997) and *Phytophthora ramorum* Werres, de Cock & Man in 't Veld responsible for the death of hundreds of thousands of oaks in North-America (Rizzo *et al.* 2002) and hence the presumption is plausible that *P. inundata* and *P. gemini* could have played a role in the decline of *Z. marina*. Pathogenicity experiments are foreseen in the future in order to determine the relative contribution of each of the *Phytophthora* species involved in the decline of seagrass. In addition, surveys will be held in the whole delta area to explore the dispersal and the genetic diversity of the two *Phytophthora* species.

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