Eur J Plant Pathol
https://doi.org/10.1007/s10658-018-1561-1

Multiple *Halophytophthora* spp. and *Phytophthora* spp. including *P. gemini*, *P. inundata* and *P. chesapeakensis* sp. nov. isolated from the seagrass *Zostera marina* in the Northern hemisphere

Willem A. Man in 't Veld · Karin C. H. M. Rosendahl · Patricia C. J. van Rijswick · Johan P. Meffert · Edu Boer · Marcel Westenberg · Tjisse van der Heide · Laura L. Govers

Accepted: 27 July 2018

Abstract A plethora of oomycetes was isolated mostly from *Zostera marina* but also from other halophilic plants originating from several locations including the Wadden Sea, Limfjord (Denmark), the Dutch Delta area (the Netherlands), Thau lagoon (France), Lindholmen (Sweden) and Chesapeake Bay (Virginia, U.S.). Based on ITS sequences, seven different groups could be distinguished. The largest group was assigned to *Phytophthora gemini* (Germany, Sweden, the Netherlands, U.S.). The *CoxI* sequences of all *P. gemini* strains were identical indicating that *P. gemini* is probably an invasive species in the Wadden Sea. A second group was identified as *P. inundata* (the Netherlands, Denmark), that was also isolated from the halophilic plants *Aster tripolium* and *Salicornia europaea*. Four strains, originating from Chesapeake Bay clustered in a monophyletic clade with high bootstrap support at the ITS as well as the *CoxI* loci. They are phylogenetically closely related to *P. gemini* and are considered to represent a new species described here as *Phytophthora chesapeakensis* sp. nov. In addition, *Salisapilea sapeloensis* was isolated from *Zostera noltii*. Eleven other strains belonging to three unidentified taxa, originating from the Wadden Sea, the Dutch Delta area and Thau lagoon, clustered each in a monophyletic clade with high bootstrap support at the ITS locus, including *Halophytophthora vesicula*, the type species of the genus *Halophytophthora*. Hence, these strains were considered to belong to the *Halophytophthora* sensu stricto group and probably represent three new *Halophytophthora* species, informally designated here as *Halophytophthora* sp-1, *Halophytophthora* sp-3 and *Halophytophthora* sp-4 sensu Nigrelli and Thines. *Halophytophthora* sp-2 was not detected in this study. In addition, *P. gemini* and *Halophytophthora* sp-3 were obtained by baiting from locations in the Wadden Sea and *Halophytophthora* sp-1 was obtained by baiting from the Delta area.

Keywords *Cytochrome oxidaseI* · Invasive species · ITS · Marine oomycete species · New species · Phylogeny

W. A. Man in 't Veld (✉) · K. C. H. M. Rosendahl · P. C. J. van Rijswick · J. P. Meffert · E. Boer · M. Westenberg
Dutch National Plant Protection Organization, National Reference Centre, Geertjesweg 15, 6706 EA Wageningen, Netherlands
e-mail: willemmanintveld@gmail.com

T. van der Heide · L. L. Govers
Department of Aquatic Ecology and Environmental Biology Institute for Water and Wetland Research (IWWR), Radboud University, Nijmegen, Netherlands

L. L. Govers
Conservation Ecology Group, Groningen Institute for Evolutionaire Life Sciences (GELIFES), University of Groningen, Groningen, Netherlands

Introduction

The sea grass, *Zostera marina* L. supports a major ecosystem along northern shores of North America and Eurasia, and hence is considered to be a key

Published online: 28 August 2018

ecological species. These seagrass meadows are located in areas along the coast and in saltmarshes in the Northern Hemisphere and are of high economic and ecological importance as they provide ecosystem services: seagrass meadows protect the coast line, promote marine biodiversity and biological productivity and provide food and shelter for organisms that cannot reproduce and survive without this vegetation. Seagrass meadows are a source of food for swans, geese and ducks and are considered to be a critical nursery area for certain shrimp and fish species.

In 2011 two *Phytophthora* species were isolated from rotting seeds of *Z. marina* in the Netherlands: the newly descibed species *P. gemini* and *P. inundata*, the latter known from wetlands all over Europe (Brasier et al. 2003a; Man in 't Veld et al. 2011). It is not known whether these two newly described *Phytophthora* species pose a threat to populations of *Z. marina*. *Phytophthora* species are often pathogenic and can cause considerable ecological and economic losses (e.g. *P. infestans*, *P. ramorum* and *P. cinnamomi* (Anderson et al. 2004)). However, recent experiments have shown that seed of *Z. marina* infected by *P. gemini* and/or *Halophytophthora* sp. had 6-fold reduced germination rates compared to non-infected seeds (Govers et al. 2016). In addition, seed infection by these oomycetes may negatively affect the quality of the germinated seedlings (Govers et al. 2017). These results indicate the association between oomycetes and *Z. marina* warrants further exploration.

The Wadden Sea situated to the north of the Netherlands is an important salt marsh nature reserve and a large tidal area where *Z. marina* was previously abundant. Worldwide, seagrass meadows have been declining in area since the 1930's, with the pathogenic fungus *Labyrinthula zosterae* being partly responsible (Muehlstein et al. 1991): the perennial, robust, long-leaved (< 1 m) form of *Z. marina* is now extinct in the Netherlands and the narrow-leaved, flexible, annual form survives only in some locations. The Wadden Sea population did not recover and more than 140 km^2 was permanently lost (Den Hartog 1987). Several other factors, mostly anthropogenic, were hypothesized to have contributed to the decline, including eutrophication by fertilizers from agricultural run-off, overfishing and seafloor habitat destruction by bottom trawling. In addition until the 1930s *Z. marina* was harvested in the Netherlands and elsewhere to be used in building dams, roofing material and as a pillow filling.

Pathogenic micro organisms (bacteria, viruses and fungi) of *Z. marina* are not well characterised (Sullivan et al. 2013). Our knowledge of oomycetes associated with marine plant species is limited due to undersampling of plants from marine ecosystems. Recently a systematic survey was conducted by collecting leaf litter and frozen algae debris along the Wadden sea shore at the German Bight from which oomycetes were isolated (Nigrelli and Thines 2013). Based on ITS and *Cox2* sequences they postulated that two, presumably new, *Halophytophthora* species, sp-1 and sp-2, were present in this area. Members of the genus *Phytophthora* were not sampled in that study.

Most of our knowledge concerning marine oomycetes comes from *Halophytophthora* species isolated from decaying leaves originating from trees growing in mangrove forests in the tropics. However, phylogenetic analysis based on SSU rRNA genes showed that the genus *Halophytophthora* is not monophyletic: except for the *Halophytophthora* sensu stricto group containing the type species *Halophytophthora vesicula*, some species cluster with *Phytopythium* whereas others seem to be more closely related to *Pythium* (Lara and Belbahri 2011). In a phylogenetic study of *Halophytophthora* species based on ITS sequences this conclusion was confirmed (Nigrelli and Thines 2013). Hence the concept of the *Halophytophthora* genus based on a *Phytophthora*-like morphology and its presence in marine ecosystems may require revision. It is expected that only *Halophytophthora* species that belong to the clade *Halophytophthora* sensu stricto belong to the genus *Halophytophthora*, and that all others species outside the clade will need to be assigned to other genera or possibly even to new genera, yet to be discovered (Marano et al. 2016).

Apart from *P. gemini* and *P. inundata* and some unidentified *Phytophthora* species closely related to, or identical to *P. lagoariana* from mangrove (Zeng et al. 2009), there are no other *Phytophthora* species described from marine ecosystems. The apparent paucity of *Phytophthora* species in these habitats may be due to the fact that there have been very few studies to identify oomycetes associated with the dominant host species. Thus the aim of this study was to survey *Z. marina* and other halophilic plants from several locations in Western Europe and the east coast of the

U.S. for the presence of oomycetes, and to identify them using DNA sequence analysis.

Materials and methods

Sampling and isolation

Isolates of *Halophytophthora* and *Phytophthora* spp. were obtained directly from host tissue and by baiting. Seeds and leaves of *Z. marina* and other halophilic plants originated from Denmark (Limfjord 56.913000 N, 8.993000 E), France (Thau lagoon 43.446510 N, 3.662817 E) Germany (Sylt 54.799000 N, 8.296000 E), the Netherlands (Grevelingen 51.553890 N, 3.965830 E; Oosterschelde 51.672000 N, 4.131000 E; Krabbendijke 5.1459000 N, 4.079000 E (these locations are part of the delta area); Uithuizen 53.459000 N, 6.666000 E; Schiermonnikoog 53.472000 N, 6.194000 E (these locations are part of the Wadden Sea), Sweden (Lindholmen, 57.703000 N, 11.939000 E), and the U.S. (Chesapeake Bay, Virginia, 37.567000 N, 76.101000 W (Fig. 1).

Isolates used in this study, their accession numbers, hosts, year of isolation, origin, ITS, *CoxI* and genotypes are listed (Table 1). *Halophytophthora* and *Phytophthora* species were isolated from seeds or leaves of *Z. marina* and other halophilic plants on ParpH cornmeal agar (Crous et al. 2009) supplemented with pentachloronitrobenzene (Sigma/P7626) 25 mg/L, pimaricin (Sigma/P-440) 0,0005%, ampicillin (Sigma/A-9393) 250 mg/L, rifampicin (Sigma/R-8626) 10 mg/L and hymexazol (Sigma/T-4014) 50 mg/L. Cultures were maintained on V8-agar slants (Crous et al. 2009).

Seeds were individually placed on ParpH selective growth medium in sterile culture plates containing 12 wells (one seed per well) with a growth area of 3.8 cm^2 per well. Seeds were incubated for 4 weeks, with a natural daylight cycle at room temperature (18–20 °C). The presence of *Phytophthora* spp. and/or *Halophytophthora* spp. was scored for each seed by identification based on colony morphology.

Small sections of leaves (0.5 × 0.5 cm) of *Z. marina*, *Z. noltii*, *Aster tripolium*, *Salicornia europaea* and *Spartina anglica* with dark-brown to black symptoms were placed on ParpH selective growth medium in 9-cm Petri plates, five pieces per Petri plate. Plates were incubated in the dark at room temperature and checked at least twice per week for the presence of *Phytophthora* spp. and/or *Halophytophthora* spp. Characteristic outgrowing colonies were transferred to cherry decoction agar, incubated at 21 °C in the dark, transferred to V8 slants and stored at 10 °C until further use (Crous et al. 2009).

Baiting of *Halophytophthora* and *Phytophthora* spp. was achieved by enclosing the following baits in a net bag (dimensions 30 × 20 cm, pore size 3 mm): one apple (cv. Granny Smith) pierced with 1.5 cm deep holes using an inoculating loop, five *Rhododendron* leaves (cv. Cunningham White) with four superficial cuts to the abaxial side of the midrib, and five leaves of *Prunus laurocerasus* with the same injuries as the *Rhododendron* leaves. The bags were attached to nautical buoys in the sea, sometimes in the vicinity of replanting experiments, sometimes arbitrarily. There were four net bait bags placed near Schiermonnikoog (Wadden Sea) and two net bait bags near Uithuizen (Wadden Sea) and Oosterschelde (delta area). After three weeks, isolations were made from lesions that developed on the bait. Sections of leaves, containing lesions, were placed on ParpH selective growth medium in 9-cm Petri plates, five pieces per Petri plate. Incubation was as described above.

Morphology

Formation and morphology of sporangia was studied on colonized seed of *Capsicum annuum*. Approximately five seeds were placed at the margins of actively growing colonies on CA at 21 °C in the dark. When the seeds were fully colonised, they were transferred to water agar, and just covered with sterile Petri's mineral solution; sporangia were usually produced after two days further incubation at room temperature in the light.

The dimensions of 91 sporangia were measured. *Capsicum annuum* seeds were transfered to glass slides, the mycelium was scraped off the seeds and covered with a drop of water, sealed with a cover slip, and examined at × 400 using a Leica DM LB2 microscope. Colony morphology was described on potato dextrose agar (PDA, Crous et al. 2009). Sections of CA agar with mycelia (5 mm in diameter) obtained from V8-agar slants were used as inoculum to inoculate PDA. In order to avoid delay in growth, agar plugs were taken from actively growing colony margins of 3-day old cultures and they were placed in the centre of the plate. Isolates

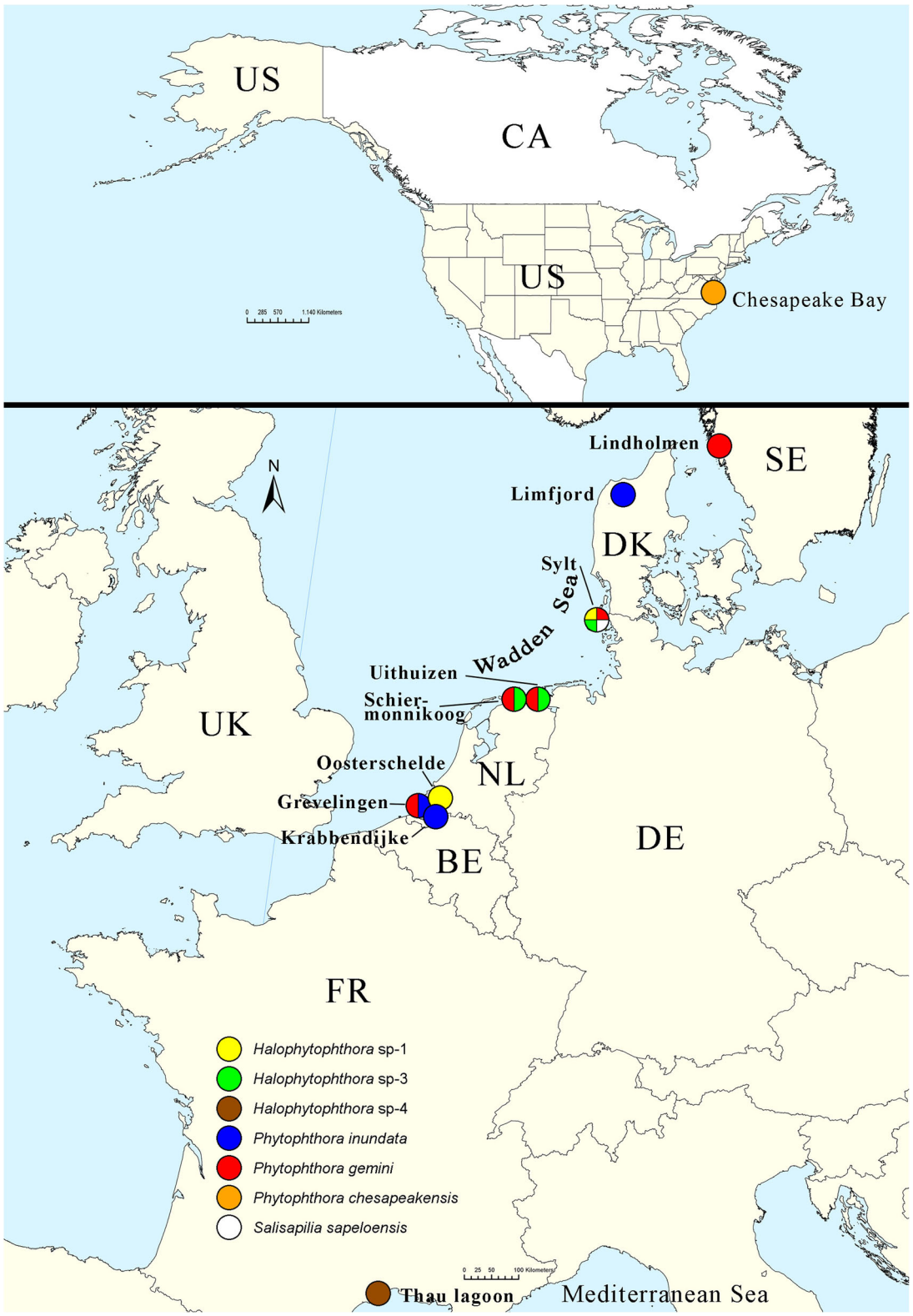

Fig. 1 Map showing the locations and distribution of *Halophytophthora* spp. and *Phytophthora* species collected in North America and Europe

Table 1 Isolates of *Phytophthora gemini*, *P. inundata*, *P. chesapeakensis*, *Halophytophthora* sp-1, *Halophytophthora* sp-3, *Halophytophthora* sp-4 and *Salisapilia sapeloensis*, strain number, year of isolation, host, origin and ITS and *CoxI* genotypes and Genbank accession numbers

Species	Isolate[a]	Year	Host	Origin	ITS genotype	GenBank accession no.	*Cox*I genotype	GenBank accession no.
P. gemini								
	CBS268.85*[b]	1985	*Zostera marina* (leaves)	Netherlands (Grevelingen)	D	FJ217679	–	
	CBS123381-T*	1998	*Zostera marina* (seeds)	Netherlands (Grevelingen)	A	FJ217680	A	KX197944
	CBS123382*	1999	*Zostera marina* (seeds)	Netherlands (Grevelingen)	B	KX172080	A	JX262931
	CBS123383*	1999	*Zostera marina* (seeds)	Netherlands (Grevelingen)	B	KX172081	A	KX197945
	CBS123384*	1999	*Zostera marina* (seeds)	Netherlands (Grevelingen)	D	KX172082	A	KX197946
	PD4764358	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	CBS140649	2014	*Zostera marina* (seeds)	Germany (Sylt)	C	KX172083	A	KX197947
	PD4225135	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	PD4225143	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	CBS140650	2014	*Zostera marina* (seeds)	Germany (Sylt)	D	KX172084	A	KX197948
	PD4282658	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	PD4097890	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	PD4097700–2	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	PD4285082–1	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	PD4285103	2014	*Zostera marina* (seeds)	Germany (Sylt)	D		A	
	PD4636647-B1	2015	*Zostera marina* (seeds)	Germany (Sylt)	C		A	
	PD4636647-B3	2015	*Zostera marina* (seeds)	Germany (Sylt)	C		A	
	CBS141308	2015	*Zostera marina* (seeds)	Sweden (Lindholmen)	A	KX172085	A	KX197949
	CBS141307	2014	*Zostera marina* (seeds)	Germany (Sylt)	C	KT986006	–	
	PD6234430	2015	*Zostera marina* (seeds)	Germany (Sylt)	C		–	
	PD6234481	2015	*Zostera noltii* (leaves)	Germany (Sylt)	C		–	
	PD4750706	2015	baiting	Netherlands (Uithuizen)	D		A	
	PD4750722	2015	baiting	Netherlands (Schiermonnikoog)	–		A	
	PD4750730	2015	baiting	Netherlands (Schiermonnikoog)	D		–	
P. inundata								
	CBS215.85*	1985	*Zostera marina* (leaves)	Netherlands (Grevelingen)	Y	FJ217682	–	
	CBS216.85*	1985	*Zostera marina* (leaves)	Netherlands (Grevelingen)	Y	FJ217681	V	KX197950
	CBS217.85*	1985	*Zostera marina* (leaves)	Netherlands (Grevelingen)	Y		V	
	PD4062073–1	2014	*Zostera marina* (seeds)	Denmark (Limfjord)	T		X	
	CBS141305	2014	*Zostera marina* (seeds)	Denmark (Limfjord)	T	KX172086	X	KX197951

Table 1 (continued)

Species	Isolate[a]	Year	Host	Origin	ITS genotype	GenBank accession no.	*Cox*I genotype	GenBank accession no.
	CBS141309	2014	*Aster tripolium* (leaves)	Netherlands (Krabbendijke)	T	KX172087	V	KX197952
	CBS141306	2014	*Salicornia europaea* (leaves)	Netherlands (Krabbendijke)	T	KX172088	V	KX197953
	CBS140656	2012	*Zostera marina* (soil)	Netherlands (Grevelingen)	C	KX172089	–	
P. chesapeakensis								
	CBS140652	2014	*Zostera marina* (seeds)	U.S. (Chesapeake Bay)	K	KX172090	H	KX172094
	CBS140653	2014	*Zostera marina* (seeds)	U.S. (Chesapeake Bay)	L	KX172091	F	KX172095
	CBS140655-T	2014	*Zostera marina* (seeds)	U.S. (Chesapeake Bay)	L	KX172092	H	KX172096
	CBS140654	2014	*Zostera marina* (seeds)	U.S. (Chesapeake Bay)	L	KX172093	H	KX172097
Halophytophthora sp-1								
	CBS140651	2014	*Zostera marina* (seeds)	Germany (Sylt)	a	KX364106	–	
	CBS141516	2015	*Spartina anglica* (leaves)	Germany (Sylt)	a	KX364107	–	
	PD4750693	2015	baiting	Netherlands (Oosterschelde)	a		–	
	PD6234318	2015	baiting	Netherlands (Oosterschelde)	a		–	
Halophytophthora sp-3								
	CBS140657	2014	*Zostera marina* (seeds)	Germany (Sylt)	b	KX364108	–	
	PD4097700–1	2014	*Zostera marina* (seeds)	Germany (Sylt)	b		–	
	CBS140648	2014	*Zostera marina* (seeds)	Germany (Sylt)	b	KT986007	–	
	PD4636647-C1	2015	*Zostera marina* (seeds)	Germany (Sylt)	c	KX364109	–	
	PD4750714–9	2015	baiting	Netherlands (Uithuizen)	b		–	
	PD4750730	2015	baiting	Netherlands (Schiermonnikoog)	d		–	
Halophytophthora sp-4								
	PD6234625	2015	*Zostera marina* (seeds)	France (Thau lagoon)	e	KX364110	–	
Salisapilia sapeloensis								
	CBS141515	2015	*Zostera noltii* (leaves)	Germany (Sylt)	–	KX364111	–	

[a] *CBS* Centraal Bureau voor Schimmelcultures, *PD* Plant protection Service; capital letters indicate *Phytophthora* genotypes, small letters indicate *Halophytophthora* genotypes

[b] * = Man in 't Veld et al. (2011)

were incubated at 21 °C in the dark. Colonies were photographed after 1 week of incubation.

Temperature-growth profiles were determined on PDA. Three replicate plates for each isolate were incubated in the dark at 10, 21, 25, 27, 29, 30, 32, 33, 35, 37, 38, 39, and 40 °C for *Phytophthora* and at 10, 18, 21, 24, 26, 28, 30, 32, 34, 35 and 36 °C for *Halophytophthora*. Agar plugs as described above were transferred to the centre of Petri plates. Two perpendicular lines were drawn on the back of the Petri plate, intersecting beneath the inoculum plug. The margin of the colony was marked in all four directions along the lines. Radial growth was measured after 5 days for *Phytophthora* and 7 days for *Halophytophthora* and mean growth rates were determined.

In order to test whether oogonia and antheridia could be induced in the strains to be tested, strains were paired on V8 agar with strains of known mating types of the different *Phytophthora* spp.: PD20017609 *P.* cryptogea A2, PD20032149 *P. cryptogea* A1, PD20032145 *P. cambivora* A1, PD20032146 *P. cambivora* A2, PD97/6360 *P. capsici* A1. Inoculum plugs (5 mm) of the strains to be tested and the tester strains were placed at a distance of 7 cm from each other on 9-cm Petri plates. After incubation in the dark at 21 °C, the strains to be tested were examined weekly for the presence of sexual structures (× 400) for a period of 7 weeks.

Seed testing

The extent (surface or internal) and incidence of *Z. marina* seed colonization by *Phytophthora* spp. was determined in a sample of 40 seeds. The seed was obtained from Chesapeake Bay, surface-sterilised by first washing in tap water for 2 min, followed by incubation in 70% ethanol for 12 min, rinsing twice in tap water and finally incubating in 0.5% hypochlorite solution for 30 min, followed by rinsing once in tap water for two minutes. The seeds were placed on ParpH selective growth medium in 9-cm Petri plates, five seeds per Petri plate, and incubated for 7 days under natural light conditions. Outgrowing colonies were transferred to CA and incubated at 21 °C for 7 days. The colonies were identified by ITS sequencing.

DNA isolation

Mycelium obtained from the margins of young CA cultures from a surface area of 1 cm^2 (approx. 30 mg) was placed in a 1.5 mL microcentrifuge tube (Superlock tubes; BIOzymTC, Landgraaf, The Netherlands) containing a stainless steel bead (4 mm diameter) and 300 μL of extraction buffer (0.02 M phosphate-buffered saline, 0.05% Tween T25, 2% polyvinylpyrrolidone, and 0.2% bovine serum albumin). The tube was placed in a bead mill (Mixer Mill MM300; Retsch, Aartselaar, Belgium) for 80 s at 1800 beats/min. The mixture was centrifuged for 5 s in a microcentrifuge at 16,100×*g* and DNA was isolated from 75 μL of the resulting supernatant. Automated DNA isolation was performed using a KingFisher Flex purification system (Thermo Fisher Scientific, Breda, The Netherlands) using a QuickPick SML Plant DNA kit from Bio-Nobile (Isogen Life Science, IJsselstein, The Netherlands) according to the manufacturer's protocol. Briefly, 5 μL of proteinase K and 50 μL of lysis buffer were added to the 75 μL of supernatant. After 30 min of incubation at 65 °C, 5 μL of MagaZorb Magnetic Particles and 125 μL of binding buffer were added. The particle-bound DNA was washed twice with 200 μL of wash buffer and DNA was eluted in 50 μL of elution buffer.

PCR and DNA sequencing

The complete nuclear rDNA ITS1–5.8S-ITS2 region was amplified using primers ITS1 and ITS4 (White et al. 1990). PCR was performed in a final volume of 25 μL containing 2 μL of isolated DNA, 0.3 μM primers, 0.08 mM dNTPs, 1.5 mM $MgCl_2$ and 0.8 U Taq DNA polymerase (Roche, Almere, The Netherlands) with the following PCR program: 94 °C for 30 s, then 40 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min and 30 s, with a final 72 °C for 5 min. Alternatively, the rDNA ITS1–5.8S-ITS2 region was amplified with primers ITS5 and ITS4 (White et al. 1990) in a final volume of 25 μL containing 2 μL of isolated DNA, 0.2 μM primers and 1X Bio-aCT Short mix form Bio-line (GC biotech, Alphen aan de Rijn, The Netherlands) with the following PCR program: 95 °C for 5 min, then 40 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min and 30 s, with a final 72 °C for 10 min.

Sequence analysis of *CoxI* was performed using COXF4N and COXR4N primers according to the protocol of Kroon et al. (2004). PCR was performed in a final volume of 25 μL containing 2 μL of isolated DNA, 0.4 μM primers, 0.2 mM dNTPs, 3.5 mM $MgCl_2$ and

1 U Taq DNA polymerase (Roche) with the following PCR program: 94 °C for 2 min, then 35 cycles of 94 °C for 1 min, 45 °C for 30 s, 72 °C for 1 min, with a final 72 °C for 10 min.

Gel electrophoresis was performed using 5 μL of PCR product and 1 μL 6× Bromopheno Blue loading solution (Promega, Leiden, The Netherlands). The electrophoresis was run using a 1.5% RESult LE General Purpose Agarose (BIOzymTC, Landgraaf, the Netherlands) gel with SYBR safe (Life Technologies, Bleiswijk, The Netherlands) staining to test for amplification. Bands were visualized in a GeneGenious gel imaging system (Syngene, Cambridge, United Kingdom). Amplicon sizes were estimated using a 1-kb Plus DNA ladder (Promega). PCR products were purified using the QIAquick PCR Purifcation Kit (Qiagen, Venlo, the Netherlands). Cycle sequence products were produced for both strands using BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer's instructions, and purified using the DyeEx 2.0 Spin Kit (Qiagen, Venlo, the Netherlands) and sequenced using a 3500 Genetic Analyzer (Life Technologies). Consensus sequences were generated from both trace files in Geneious R8 (Biomatters Auckland, New Zealand). Amplification primers were trimmed from the consensus sequence, and when needed, additional trimming was performed to obtain high quality consensus sequence.

Phylogenetic analyses

Alignments of the *Phytophthora* ITS and *CoxI* sequences respectively with selected sequences of other *Phytophthora* species from GenBank were made with MEGA5.05 (Tamura et al. 2011) using MUSCLE (Edgar 2004). The alignments containing these selected sequences from GenBank included two sequences of *Pythium aphanidermatum* and *Pythium splendens* as outgroups and were used to construct a phylogenetic tree using maximum likelihood interference applying the Tamura-Nei model (Tamura and Nei 1993) with 1000 bootstrap replicates.

Alignments of *Halophytophthora* ITS sequences with selected sequences of other *Halophytophthora*, *Pythium* and *Phytopythium* species from GenBank were made with MEGA5.05 (Tamura et al. 2011) using MUSCLE (Edgar 2004). The alignments containing these selected sequences of GenBank including *Salisipilia sapeloensis* as outgroup were used to construct a phylogenetic tree using maximum likelihood interference with MEGA5.05 (Tamura et al. 2011) applying the Tamura-Nei model (Tamura and Nei 1993) with 1000 bootstrap replicates.

Results

Phytophthora chesapeakensis Man in 't Veld, K. Rosendahl sp. nov. Figs. 2, 3, 4a. MycoBank MB 817847.

Holotype: Chesapeake Bay, U.S., isolated from seeds of *Zostera marina* by Patricia van Rijswick 2014, CBS140655, dried culture, in herb. CBS-KNAW, Fungal Biodiversity Centre, Utrecht, the Netherlands. Living culture ex-holotype CBS140655.

Colony morphology on potato dextrose agar (PDA) faintly chrysanthemum pattern. Optimum growing temperature on PDA approximately 27 to 30 °C with growth ceasing at approximately 38 °C. Hyphal swellings numerous on agar, rounded and mostly catenulate, sometimes widely spaced. All strains sporulated poorly. Sporangia were ovoid, non-papillate, non-caducous; dimensions 90.0–30.0 × 61.3–22.8 μm (average 57.2–40.2 μm), L/B ratio 1.42. Sporangiogenous hyphae developing one terminal sporangium, subsequently sporangia were formed by extended internal proliferation. Chlamydospores were not produced. Mating experiments were unsuccessful and sexual structures were not observed. It is probably a sterile species.

Etymology Chesapeakensis, after Chesapeake Bay, U.S., from where this species was first isolated.

Temperature-growth profiles

The isolates of *P. chesapeakensis* used in this study had an optimal temperature for growth between approximately 27 and 30 °C, with radial growth ceasing at approximately 38 °C (Fig. 4a).Temperature profiles for growth of *Halophytophthora* sp-3 strain CBS140657 and strain CBS149648 showed similar patterns with an

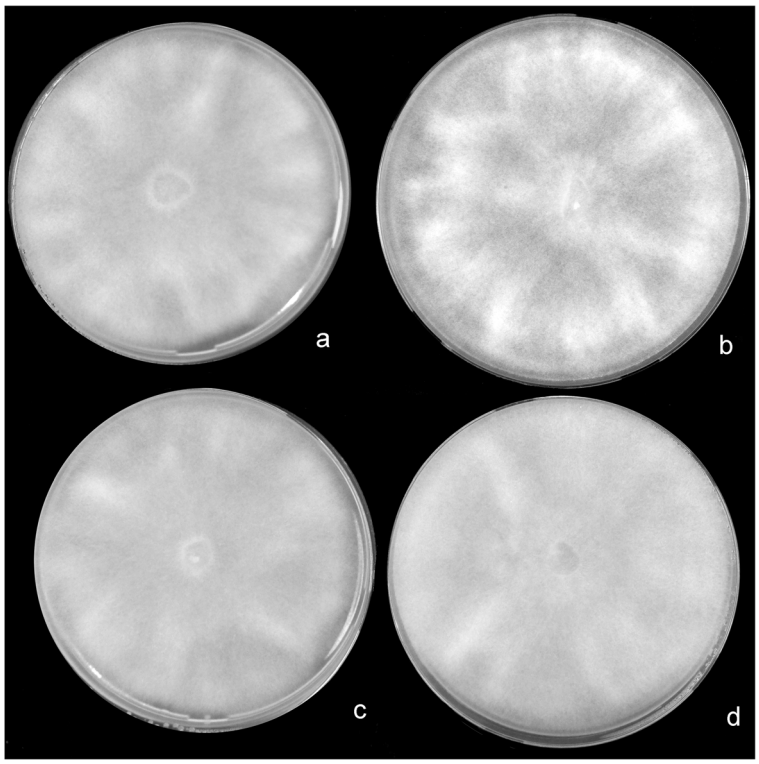

Fig. 2 Colony morphology of *Phytophthora chesapeakensis* strains CBS140652 (**a**), CBS140653 (**b**), CBS140654 (**c**), CBS140655 on potato dextrose agar (PDA) after one week of growth at 18 °C in the dark

optimum between approximately 20 to 25 °C, with radial growth ceasing at approximately 32 °C (Fig. 4b).

Seed testing

After the sterilization procedure only 27 of the 40 seeds (67.5%) from Chesapeake Bay germinated and of all 40 seed, four were internally colonised with *P. chesapeakensis*. *P. chesapeakensis* was only present in seeds that did not germinate. Thus 30.8% of the non-germinated seed (10.0% of all seed) were internally colonised with *P. chesapeakensis.*

Sequence and phylogenetic analysis

Sequence analysis of the ITS regions revealed the presence of three species of the genus *Phytophthora* and three of the genus *Halophytophthora* among the isolates collected from *Z. marina*. The majority of the isolates were *P. gemini*, previously described on *Z. marina* in the Netherlands (Man in 't Veld et al. 2011), and now found in the Wadden Sea at several locations near the island of Sylt, in the Kattegat near Lindholmen in Sweden (Table 1). ITS sequence variation of *P. gemini* occurred at only three nucleotide positions 47,128, and 700 where double bases mutated to single bases, depending on the position and on the isolate, resulting in four genotypes: A (T, Y, G), B (Y, Y, G), C (T, T, R) and D (T, T, G**).** All *P. gemini* strains contained identical *CoxI* sequences**.**

We identified *P. inundata* on two new hosts in the Netherlands, *A. tripolium* and *S. europaea,* and in Limfjord in Denmark on *Z. marina*. Sequence analysis of the ITS regions revealed three different nucleotides Y, C and T at position 726, depending on the isolate. The ITS and *CoxI* sequences of four isolates originating from Chesapeake Bay revealed unique sequences. Phylogenetic analysis indicated that these strains clustered in clade 6 and that they were closely related to *P. gemini*, *P. condilina*, *P.balyanboodja*, and *P. inundata*. (Figs. 5 and 6). They were assigned to the new species *P. chesapeakensis* described above. The ITS sequences of *P. chesapeakensis* differed from that of *P. gemini* at approximately 34 nucleotide positions, and the *CoxI* sequences differed at approximately 34 positions.

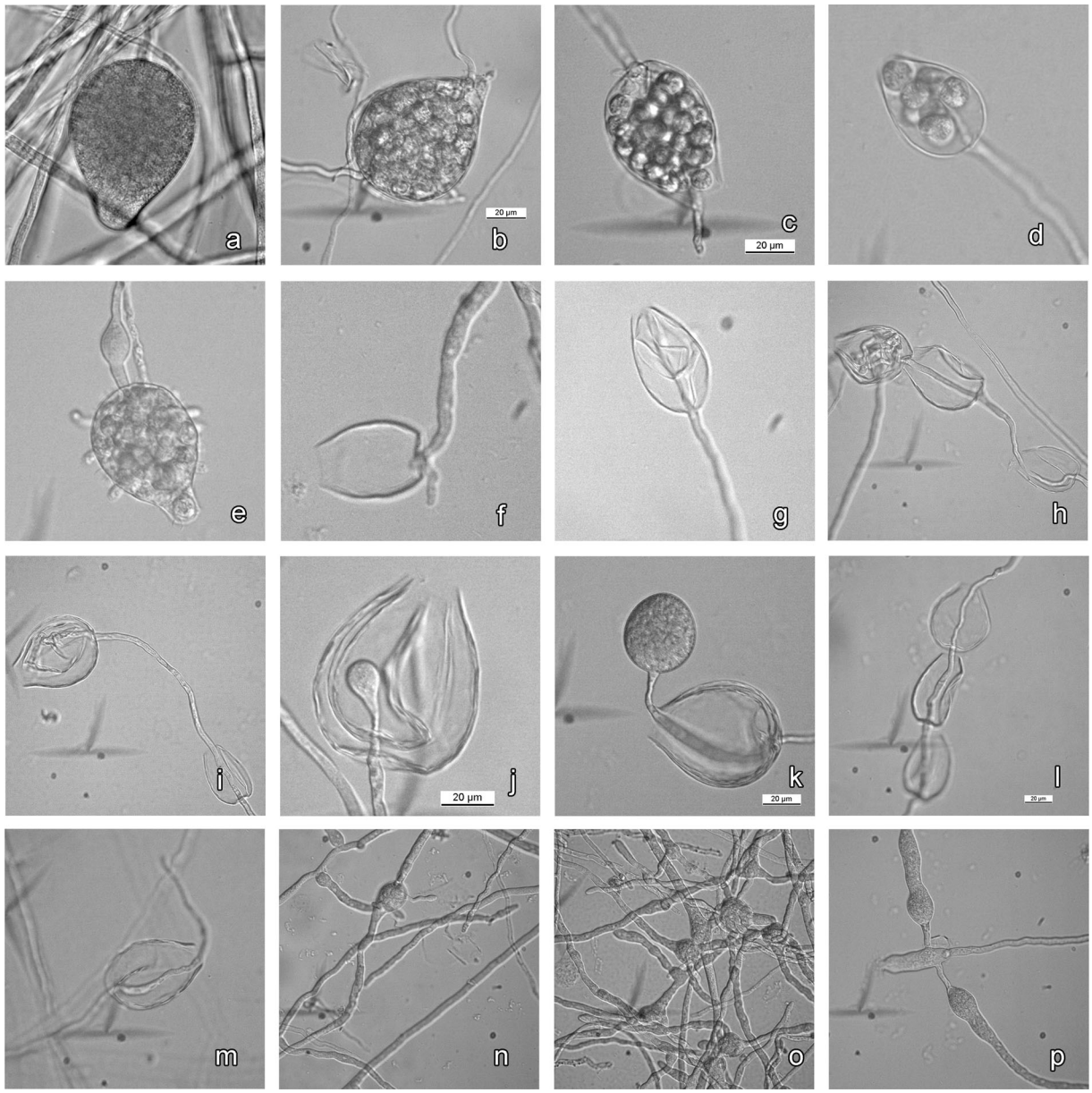

Fig. 3 Morphological structures of *Phytophthora chesapeakensis* sp. nov. **a**. Ovoid sporangium. **b**, **c**, **d**. Sporangium containing zoospores. **e**. Sporangium showing protuberances caused by germinating zoospores. f. Sporangium after release of zoospores. **g**, **h**, **i**, **j**, **k**, **l**, **m**. Extended internal proliferation of sporangia. **n**, **o**, **p**. Catenulate hyphal swellings

In addition, sequence analysis of the ITS regions revealed three unidentified *Halophytophthora* species (Fig. 7). The sequences of four isolates were identical to JX910903 provisionally assigned to *Halophytophthora* sp-1 (Nigrelli and Thines 2013). Another six ITS sequences were unique and provisionally assigned to *Halophytophthora* sp-3 continuing the numbering sensu Nigrelli and Thines (2013). The sequences of these isolates were more closely related to *Halophytophthora vesicula* (sharing four indels), than to *Halophytophthora* sp-1. The isolates originated from Germany and the Netherlands and differed at one nucleotide position in PD 4636647-C1 and two positions in PD4750730 from the other isolates. The ITS sequence of PD6234625 originating from France was unique and clustered in the *Halophytophthora* sensu stricto clade and was

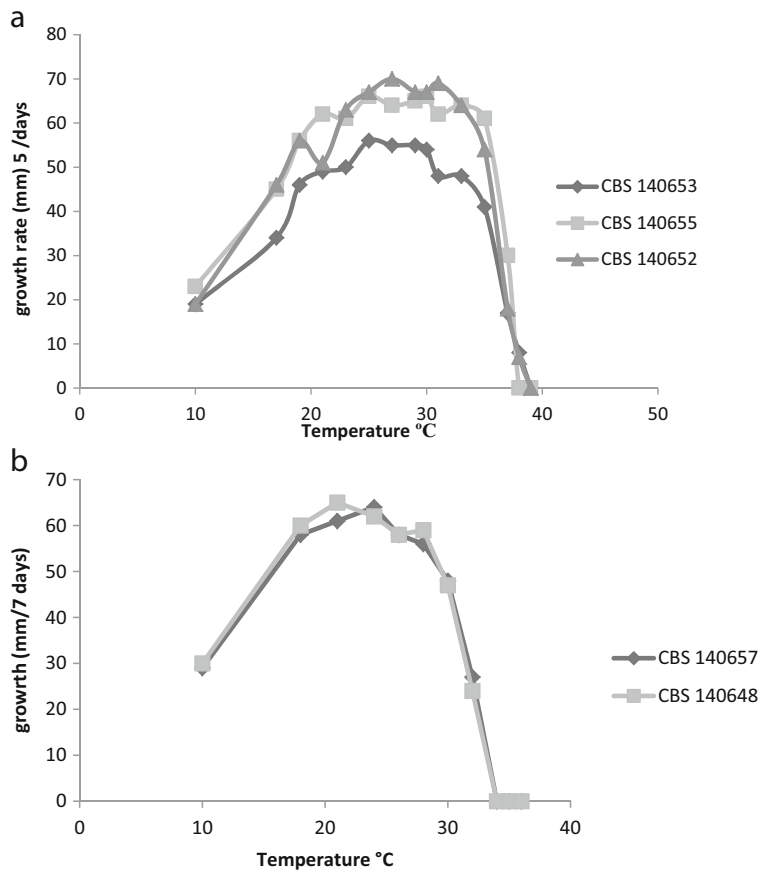

Fig. 4 **a** Temperature-growth profiles of *Phytophthora chesapeakensis* sp. nov. on potato dextrose agar (PDA), **b**. Temperature-growth profiles of *Halophytophthora* sp-3 strains CBS140657 and CBS140648 on potato dextrose agar(PDA)

provisionally assigned to *Halophytophthora* sp-4, continuing the numbering sensu Nigrelli and Thines (2013), sharing three indels with *Halophytophthora vesicula*. *Halophytophthora* sp-2 sensu Nigrelli and Thines (2013) was not found in this study.

Based on the ITS sequence strain CBS141515 was identified as *Salisapilia sapeloensis* and the sequence was identical to that of the type species (KX364111).

Discussion

The survey *Z. marina* in the Northern Hemisphere confirmed three *Phytophthora* species, including a new species described here as *P. chesapeakensis* sp. nov., and three unidentified species of *Halophytophthora* species. Phylogenetic analysis based on ITS and *CoxI* consistently differentiated these species and the four isolates of *P. chesapeakensis* grouped in a monophyletic clade. *P. gemini*, *P. inundata*, *P. humicola* and *P.* taxon *personii* were the most closely related. The new species differs from *P. gemini* in colony morphology, lack of twin sporangia, extended internal proliferation and variable growth rate, depending on the strain, and it can grow at a higher maximum temperature (38 °C). Both *P. gemini* and *P. chesapeakensis* are sterile species under laboratory conditions, as is the case for several clade 6 species (Brasier et al. 2003b).

Unexpectedly, *P. gemini* was the most abundant species. Although first isolated from *Z. marina* in the 1980s (Man in 't Veld et al. 2011), we have now confirmed it occurs in the Wadden Sea and in Lindholmen in Sweden, approximately 1000 km from The Netherlands.

The mode of dispersal over such large distances is likely by rafting on infected shoots of the host that carry zoospores in semi-persistent or persistent vesicles, as described for *H. vesicula* (Anastasiou and Churchland 1969). Identical *P. gemini* ITS genotypes (A and D) and *CoxI* genotype A were present in the Delta area of the Netherlands and in the Wadden Sea. This indicates these isolates of *P. gemini* are part of the same metapopulation. *P. gemini* contained several double bases in the ITS

Fig. 5 Maximum Likelihood phylogenetic tree showing *Phytophthora chesapeakensis* and related species based on ITS sequences generated in MEGA5.05. Bootstrap values are shown on the branches (values <50% are not shown)

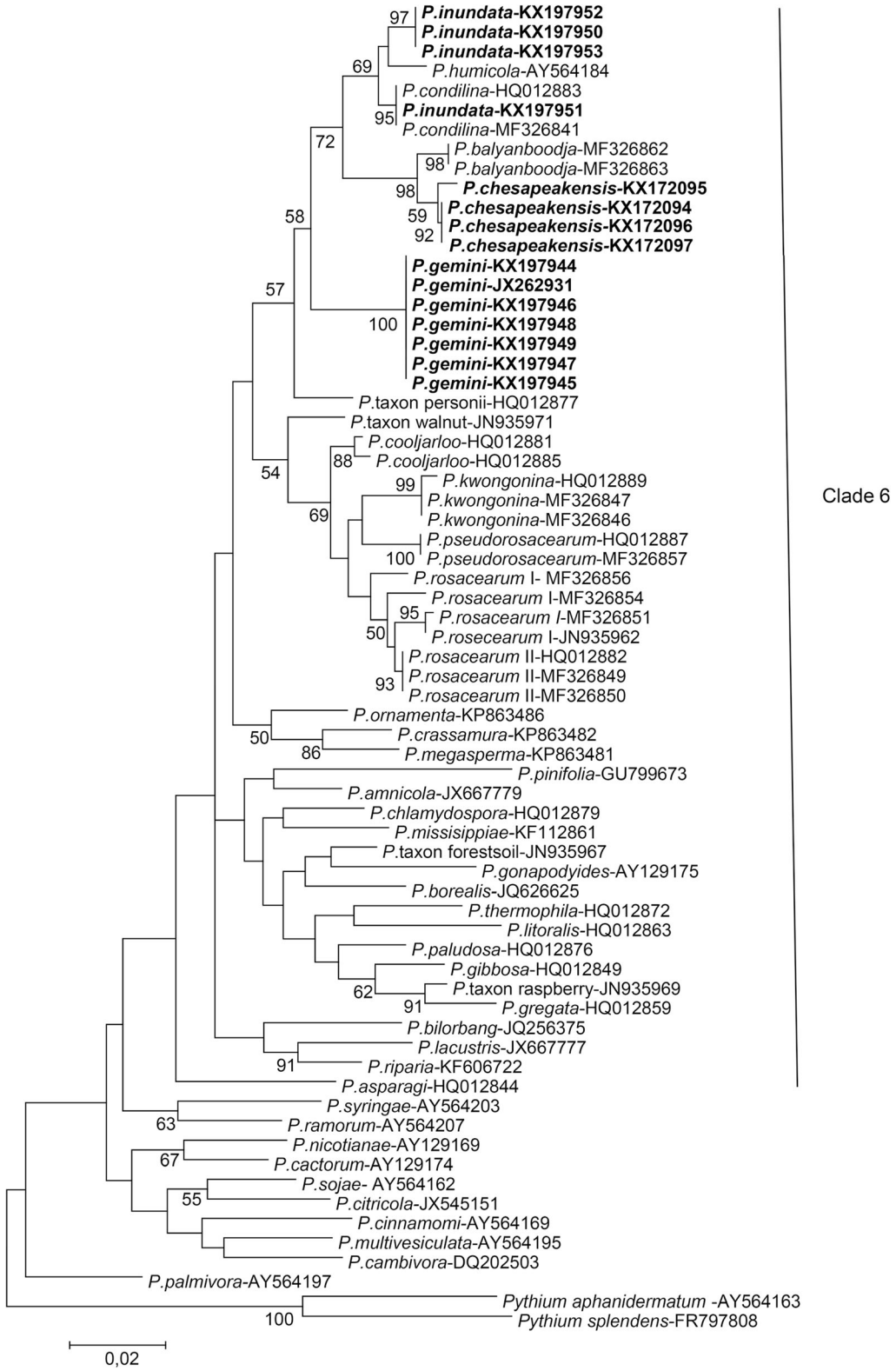

Fig. 6 Maximum Likelihood phylogenetic tree showing *Phytophthora chesapeakensis* and related species based on *CoxI* sequences generated in MEGA5.05. Bootstrap values are shown on the branches (values <50% are not shown)

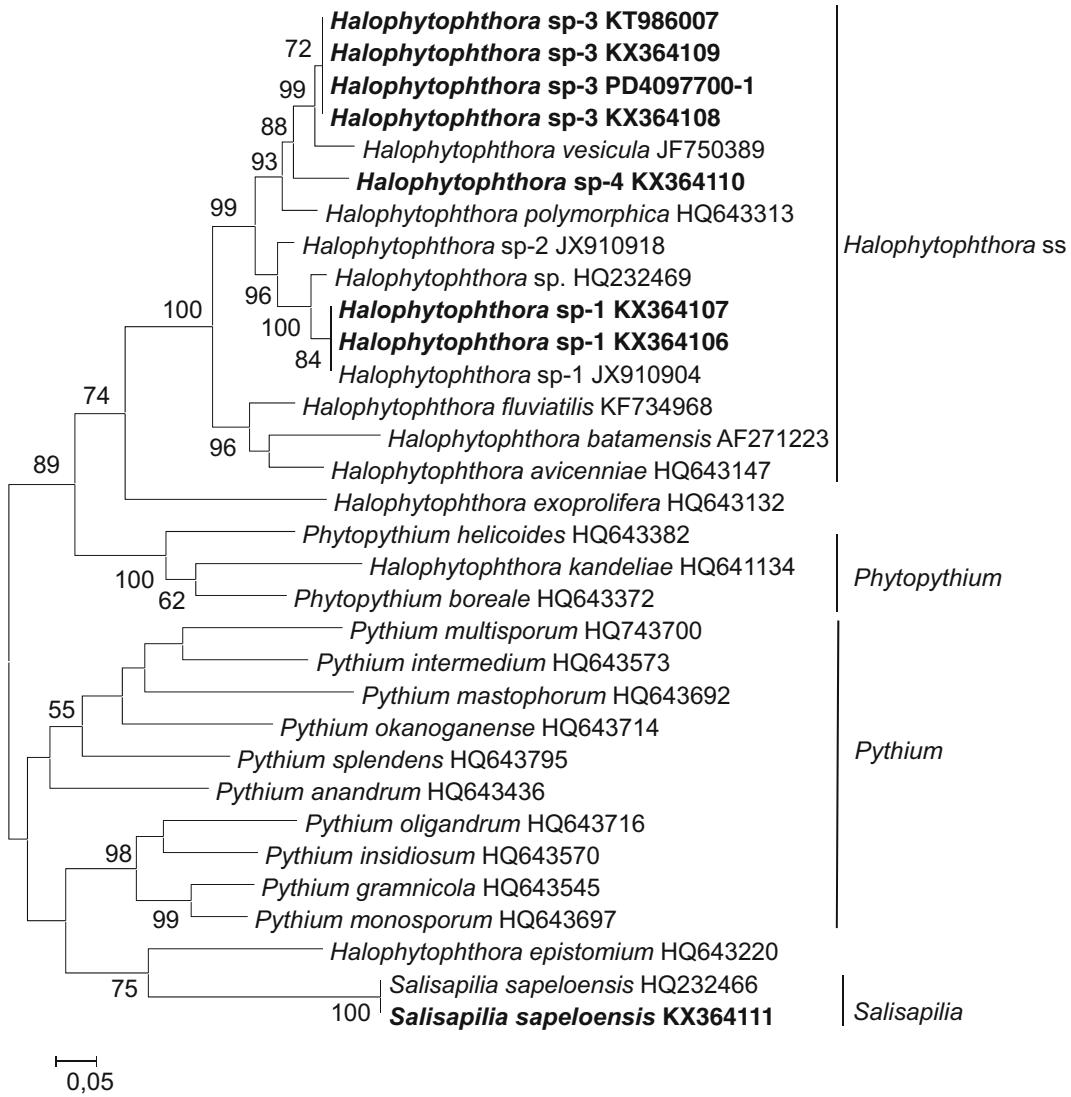

Fig. 7 Maximum Likelihood phylogenetic tree showing *Halophytophthora* spp. and related genera based on ITS sequences generated in MEGA5.05. Bootstrap values are shown on the branches (values <50% are not shown)

region, which possibly suggests a hybrid origin. In addition, heterozygous *Mdh-2* patterns, as demonstrated in five isolates of *P. gemini* (Man in 't Veld et al. 2011), and sterility support this hypothesis. This will be explored in the future.

Although there was some sequence variation in the ITS region of *P. gemini* in the Wadden Sea, indicating the population is not clonal, all *CoxI* sequences were identical. A resident species might be expected to have more variation in the *CoxI* sequence, particularly when this species was present over such a large area. Lack of variation may be due to a founder effect, resulting in a genetic bottleneck presented as reduced genetic variation (Mayr 1942). This strongly suggests that *P. gemini* was introduced in the Wadden Sea. Further evidence of introduction was found with *S. sapeloensis*. The isolate from the Wadden Sea had the same ITS sequence as the type strain isolated in a salt marsh near Sapelo island (Georgia, U.S.) (Hulvey et al. 2010). This may suggest introduction of isolates to the Wadden Sea, or vice versa. More research is needed to confirm this hypothesis. The Anthropocene has provided opportunity for novel disease outbreaks: for example *P. ramorum*, generally assumed to be an

introduced species in Europe and the U.S. from unknown origin, is currently destroying millions of oaks in California and Oregon (Grünwald et al. 2012). *P. gemini* could be introduced by ballast water from international shipping (Man in 't Veld et al. 2011). Ballast water, uploaded on a ship outside Bremerhaven (Wadden Sea), was analysed after 21 days with metabarcoding using *CoxI* and 55% of sequences were assigned to oomycetes (Zaiko et al. 2015). Indeed, inoculum increased during the journey, and considering the volume (>70 m^3) is highly likely to contain a genetically diverse population.

P. gemini and *Halophytophthora* sp-3 were obtained by baiting in the Wadden Sea near the island of Schiermonnikoog and Uithuizen (The Netherlands), where *Z. marina* is extinct. The baits were placed in the vicinity of *Z. marina* replanting experiments using seed from plants severely diseased with *P. gemini* and *Halophytophthora* sp-3. Thus it is possible that both species were introduced, and has implications for seagrass translocation efforts (Orth et al. 2006). Another possibility is that other halophilic plants (e.g. *S. anglica*, *A. tripolium*, *S. europaea*), growing towards the upper limits of the highest tides were infected, and diseased material may have been washed into the sea and provided a source of inoculum.

The genus *Halophytophthora* was erected to accommodate *Phytophthora* species originating from marine environments, but they are present in fresh water e.g. *H. fluviatilis* (Yang and Hong 2014), indicating this genus concept is not efficient. Moreover, *P. gemini*, *P. chesapeakensis* and *P. inundata* were isolated from the halolophyte *Z. marina*. It seems that, independent of *Halophytophthora* ssp. and *Salisapilia* spp., the phylogenetic cluster of species including *P. gemini*, *P. chesapeakensis*, *P. condilina*, *P.balyanboodja* and *P. inundata*, evolved in *Phytophthora* clade 6, and a common ancestor adapted to a marine lifestyle. Conversely, Marano et al. (2016) hypothesised that *Phytophthora* clade 6 species had a marine ancestor, supported by their phylogenetic relationship to brown algae, and that the majority of clade 6 species adapted to a terrestrial lifestyle. *P. inundata* is able to thrive in both sea and fresh water and is reported from six hosts in streams in Europe, Asia, Australia and the U.S. (Hüberli et al. 2013; Safaiefarahani et al. 2013). The isolates of *P. inundata* in our study may have originated from neighbouring terrestrial areas and been transported to the salt marshes by streams and rivers. Incidentally, phylogenetic analysis demonstrated that the *CoxI* sequence genotype X (KX197951) of *P. inundata*, originating from remote saltmarshes in Denmark, clustered in the *P. condilina* clade (Fig. 6). This is intriguing and suggests that these *P. inundata* strains have a hybrid history. More research is needed to clarify this. Also, *P. balyanboodja*, a recently described species from Australia (Burgess et al. 2018) is phylogenetically closely related to *P. chesapeakensis* (Figs. 5 and 6). *P. balyanboodja* has a high optimum temperature and maximum temperature for growth and survival similar to *P. chesapeakensis*, indicating both species are adapted to areas with high temperatures.

The decline of *Z. marina* affects other organisms in the ecosystem. Zoospores of *Halophytophthora* species can rapidly infect and spread through neighbouring plants (Leano et al. 1998; Newell et al. 1987). The cause of wasting disease of *Z. marina* in the 1930s in the U.S. was identified as *L. zosterae* (Muehlstein et al. 1991). In the Chesapeake Bay a continuing decline starting in the 1970s was blamed on eutrophication and environmental stress (Orth and Moore 1983). The Wadden Sea also had a history of decline in *Z. marina* caused by *L. zosterae* in the 1930's, during which more than 90% of the host population was eradicated.

Globally, attempts have been made to restore seagrass meadows by using seed (Govers et al. 2017), which risks dispersal of oomycetes in contaminated seed. It would be prudent to re-evaluate restoration experiments where seed from areas known to harbour *Halophytophthora* or *Phytophthora* spp. were used. Host resistance in *Z. marina* may emerge naturally allowing recovery of the population, but in the short term using copper sulphate treated seeds could be recommended (Govers et al. 2017). In the Govers et al. study, 99% of seeds in the Netherlands were infected with either *P. gemini* and *Halophytophthora* sp- 3, or both. Seed germination of *Z. marina* was reduced 6-fold by infection with *P. gemini* and *Halophytophthora* sp- 3. In our experiments we demonstrated that seed of *Z. marina* originating from Chesapeake Bay that were infected with *P. chesapeakensis* had internal coloonisation of the seed, as surface sterilization did

not remove disease. Furthermore, the seed did not germinate, implying these seed colonising pathogens can hamper establishment. This suggests that *P. gemini*, *Halophytophthora* sp- 3 and *P. chesapeakensis* are causing a seed disease and is supported by the observation that plants in neither the Wadden Sea nor Chesapeake Bay have any foliar symptoms (Robert Orth, personal communication).

Pathogenicity trials with these oomycetes need to be conducted to confirm Koch's postulates, and require sufficient clean seed for a healthy control. Since 99% of the seeds in the Wadden Sea is contaminated by either one or both species this is a problem, perhaps requiring production of seed in a protected environment. Experiments to clean the seeds with 0.2 ppm copper sulphate were successful in reducing infection by 86% (Govers et al. 2017). This method may allow provision of sufficient seed for pathogenicity trials. Pathogenicity trials may also identify host resistance. Both *P. gemini* and *Halophytophthora* sp-3 are widespread and preservation of *Z. marina* must ultimately rely on resistant host plants.

In conclusion, we have demonstrated many oomycetes are associated with seagrasses (*Z. marina* and *Z. noltii*) and other salt marshes plants (*S. anglica*, *A. tripolium*, *S. europeae*), including three *Phytophthora* species (*P. inundata*, *P. gemini* and *P. chesapeakensis*), three *Halophytophthora* species (*Halophytophthora* sp-1, 3 and 4) and *S. sapeloensis*. These oomycetes potentially endanger sea grass beds and declining salt marshes. Further research is needed to establish their impact sea grass populations.

Acknowledgements The authors thank Mike Coffey (US) for sequencing *Halophytophthora* sp-4 and Robert Orth (US), Eduardo Infantes, Per-Olav Moksnes, Louise Eriander (Sweden), Birgit Olesen, Flemming Gertz (Denmark), Matthijs van de Geest (France), Dick de Jong (The Netherlands) for their generous gift of *Z. marina* seeds and the Fieldwork Company (Jannes Heusinkveld, Remco de Nooij) for installation and collection of baits from the Dutch Wadden Sea area.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

References

Anastasiou, C. J., & Churchland, L. M. (1969). Fungi on decaying leaves in marine habitats. *Canadian Journal of Botany, 47*, 251–257.

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., & Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology & Evolution, 19*, 535–544.

Brasier, C. M., Sanchez-Hernandez, E., & Kirk, S. A. (2003a). *Phytophthora inundata* sp. nov., a part heterothallic pathogen of trees and shrubs in wet or flooded soils. *Mycological Research, 107*, 477–484.

Brasier, C. M., Cooke, D. E., Duncan, J. M., & Hansen, E. M. (2003b). Multiple new phenotypic taxa from trees and riparian ecosystems in *Phytophthora gonapodyides-P. megasperma* ITS clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. *Mycological Research, 107*, 277–290.

Burgess, T. I., Simamora, A. V., White, D., Wiliams, B., Schwager, M., Stukely, M. J. C., & Hardy, G. E. S. J. (2018). New species from *Phytophthora* clade 6a: evidence for recent radiation. *Persoonia, 41*, 1–17.

Crous, P. W., Verkley, J. Z., Groenwald, J. Z., Samson, R. A. (2009). Fungal biodiversity. CBS laboratory manual series. Utrecht, the Netherlands: CBS-KNAW fungal biodiversity Centre. 221 p.

Den Hartog, C. (1987). "Wasting disease" and other dynamic phenomena in *Zostera* beds. *Aquatic Botany, 27*(1), 3–14.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research, 32*, 1792–1797.

Govers, L. L., Man in 't Veld, W. A., Meffert, J. P., Bouma, T. J., van Rijswick, P. C. J., Heusinkveld, J. H. T., Orth, R. J., van Katwijk, M. M., & van der Heide, T. (2016). Marine *Phytophthora* species can hamper conservation and restoration of vegetated coastal ecosystems. *Proceedings of the Royal Society B, 283*(1837), 20160812. https://doi.org/10.1098/rspb.2016.0812.

Govers, L. L., van der Zee, E. M., Meffert, J. P., van Rijswick, P. C., Man in 't Veld, W. A., Heusinkveld, J. H., van der Heide T. (2017). Copper treatment during storage reduces *Phytophthora* and *Halophytophthora* infection of *Zostera marina* seeds used for restoration. *Scientific Reports*, 43172. https://doi.org/10.1038/srep43172.

Grünwald, N. J., Garbelotto, M., Goss, E. M., Heungens, K., & Prospero, S. (2012). Emergence of the sudden oak death pathogen *Phytophthora ramorum*. *Trends in Microbiology, 20*(3), 131–138.

Hüberli, D., Hardy, G. E. S. J., White, D., Williams, N., & Burges, T. I. (2013). Fishing for *Phytophthora* from Western Australia's waterways: a distribution and diversity survey. *Australasian Plant Pathology, 42*, 251–260.

Hulvey, J., Telle, S., Nigrelli, L., Lamour, K., & Thines, M. (2010). *Salisapiliaceae*–a new family of oomycetes from marsh grass litter of southeastern North America. *Persoonia, 25*, 109–116.

Kroon, L. P. N. M., Bakker, F. T., van den Bosch, G. B., Bonants, P. J. M., & Flier, W. G. (2004). Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology, 41*, 766–782.

Lara, E., & Belbahri, L. (2011). SSU rRNA reveals major trends in oomycete evolution. *Fungal Diversity, 49*, 93e100.

Leano, E. M., Vrijmoed, L. L. P., & Jones, E. B. G. (1998). Physiological studies on *Halophytophthora vesicula* (Straminipilous fungi) isolated from fallen mangrove leaves from Mai Po, Hong Kong. *Botanica Marina, 41*, 411–420.

Man in 't Veld, W. A., Rosendahl, K. C., Brouwer, H., & de Cock, A. W. A. M. (2011). *Phytophthora gemini* sp. nov., a new species isolated from the halophilic plant *Zostera marina* in the Netherlands. *Fungal Biology, 115*, 724–732.

Marano, A. V., Jesus, A. L., de Souza, J. I., Jerônimo, G. H., Gonçalves, D. R., Boro, M. C., Rocha, S. C. O., & Pires-Zottarelli, C. L. A. (2016). Ecological roles of saprotrophic *Peronosporales* (Oomycetes, Straminipila) in natural environments. *Fungal Ecology, 19*, 77–88.

Mayr, E. (1942). *Systematics and the origin of species*. New York: Colombia Univ. Press 340 p.

Muehlstein, L. K., Porter, D., & Short, F. T. (1991). *Labyrinthula zosterae* sp. nov., the causative agent of wasting disease of eelgrass, *Zostera marina*. *Mycologia, 83*, 180–191.

Newell, S. Y., Miller, I. D., & Fell, I. W. (1987). Rapid and pervasive occupation of fallen mangrove leaves by a marine zoosporic fungus. *Applied and Environmental Microbiology, 53*, 2464–2469.

Nigrelli, L., & Thines, M. (2013). Tropical oomycetes in the German bight – Climate warming or overlooked diversity? *Fungal Ecology, 6*(2), 152–160.

Orth, R. J., & Moore, K. A. (1983). Chesapeake Bay: An unprecedented decline in submerged aquatic vegetation. *Science, 222*, 51–53.

Orth, R. J., Carruthers, T. J. B., Dennison, W. C., Duarte, C. M., Fourqurean, J. W., Heck Jr., K. L., Hughes, A. R., Kendrick, G. A., Kenworthy, W. J., Olyarnik, S., Short, F. T., Waycott, M., & Williams, S. L. (2006). A global crisis for seagrass ecosystems. *BioScience, 56*, 987–996.

Safaiefarahani, B., Mostowfizadeh-Ghalamfarsa, R., & Cooke, D. E. L. (2013). Characterisation of *Phytophthora inundata* according to host range,morphological variation and multigene molecular phylogeny. *Phytopathologia Mediterranea, 52*(1), 46–65.

Sullivan, B. K., Sherman, T. D., Damare, V. S., Lilje, O., & Gleason, F. H. (2013). Potential roles of *Labyrinthula* spp. in global seagrass population declines. *Fungal Ecology, 6*, 328–338.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution, 10*, 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731–2739.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols, a guide to methods and applications* (pp. 315–322). San Diego: Academic Press.

Yang, X., & Hong, C. (2014). *Halophytophthora fluviatilis* sp. nov. from freshwater in Virginia. *FEMS Microbiology Letters, 352*(2), 230–237.

Zaiko, A., Martinez, J. L., Schmidt-Petersen, J., Ribicic, D., Samuiloviene, A., & Garcia-Vazquez, E. (2015). Metabarcoding approach for the ballast water surveillance–an advantageous solution or an awkward challenge? *Marine Pollution Bulletin, 92*, 25–34.

Zeng, H. C., Ho, H. H., & Zheng, F. C. (2009). A survey of *Phytophthora* species on Hainan Island of South China. *Journal of Phytopathology, 157*, 33–39.