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Phytophthora elongata (Peronosporaceae) is present as an estuarine species in Philippine mangroves

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Abstract

The genus *Phytophthora*, a group of hemibiotrophic oomycetes, is composed of almost 150 species, most of which are pathogens of terrestrial and freshwater plant species. Of the known taxa of *Phytophthora*, three species (*P. estuarina*, *P. gemini*, and *P. rhizophorae*) were only recorded in the estuarine or marine environment, while others were recently discovered to be present in these environments as saprotrophs, suggesting that more *Phytophthora* species might be present in marine or estuarine habitats. Thus, mangrove habitats of the Philippines were surveyed for additional *Phytophthora* species apart from the previously-reported species, *Phytophthora insolita*. As a result, *Phytophthora elongata*, which was reported as a pathogen of *Eucalyptus marginata* from Western Australia, was isolated from mangrove leaf litter in the coastal area of Cavite, Philippines, as the first Clade 2 species found in saline habitats. This suggests that among *Phytophthora* species there is the potential to rather easily evolve measures to deal with osmotic pressure, which supports the potential importance of mangroves as a cryptic habitat of *Phytophthora*.

Key words – Estuarine environment – *Halophytophthora* – Mangroves – Oomycetes – Peronosporaceae – *Phytophthora*

Introduction

The genus *Phytophthora*, a member of the family Peronosporaceae (Thines et al. 2009, Hulvey et al. 2010, Thines 2014), contains hemibiotrophic pathogens that have mostly been isolated from infested plants, freshwater, or soil (Kroon et al. 2012, Hyde et al. 2014). In molecular phylogenies, the genus can be divided into 8 to 10 clades with overlapping morphological characteristics (Cooke et al. 2000, Blair et al. 2008, Runge et al. 2011). Ho and Jong (1990) described *Halophytophthora* to accommodate *Phytophthora*-like species that are striving in an estuarine or marine environment and in subsequent years several filamentous oomycetes from estuarine or marine environments were added to the genus, rendering it increasingly heterogeneous (Hulvey et al. 2010). However, phylogenetic analyses using several markers strongly inferred the

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polyphyly of *Halophytophthora* (Hulvey et al. 2010, Lara & Belbahri 2011) and as a consequence some species were transferred to other genera, such as *Phytopythium* (*Phytopythium kandeliae*, basionym *H. kandeliae*) (Thines 2014), *Salisapilia* (*S. tartarea*, basionym *H. tartarea*) (Hulvey et al. 2010), and *Salispina* (*S. spinosa* and *S. lobata*, basionyms *Phytophthora spinosa* var. *spinosa* and *P. spinosa* var. *lobata*, respectively) (Guo et al. 2016). The primary concept of *Halophytophthora* as a genus containing marine or estuarine *Phytophthora*-like species is thus no longer applicable. Also, there are reports on the occurrence of genuine species of *Phytophthora sensu lato* in marine and estuarine systems (i.e. *P. estuarina*, *P. gemini*, *P. inundata*, *P. insolita* and *P. rhizophorae*) (Zeng et al. 2009, Man In'T Veld et al. 2011, Guo et al. 2016, Bennett & Thines 2017). Further, Preuett et al. (2016) demonstrated the survival of *P. ramorum* on media with varying salinity, supporting the conclusion of Duniway (1979) that *Phytophthora* spp. might generally have some tolerance for salinity. Because of the increasing evidence that oomycete species, including *Phytophthora* species, are common inhabitants of marine and estuarine environments similar to true fungi (Jones et al. 2015), it was the aim of the current study to survey mangrove habitats of the Philippines for the potential presence of additional species of *Phytophthora*.

Materials & Methods

Isolation and sporulation

Fallen senescent mangrove leaves from the coastal area of Cavite, Philippines were collected. Leaves were blot dried, cut into strips (~5.0 × 0.2 cm), and laid over onto clarified vegetable juice agar (VJ) (0.2 l V8® Juice, Campbell, 1.5% agar in 0.8 l distilled water or half-strength sea water) amended with 500 mg/ml Nystatin, 30 mg/ml Rifampicin or 0.5 mg/ml Streptomycin. Hyphae growing from the periphery of the leaf strips were cut and transferred to a new VJ agar until axenic. Agar blocks (~ 0.5 cm²) with mycelia from axenic cultures were either placed in 0 – 30 ppt aqueous sea salt solution, sterile or unsterile soil extract, or a combination of both for the production of sporangia and release of zoospores.

DNA extraction and Phylogenetic analyses

The strains BMYL-1217-1 and BMYL-1217-2 recovered from the leaf samples were incubated on VJ agar for 7 to 10 days at room temperature in the dark. After this, mycelia from the agar plates were harvested by tearing it off using forceps and homogenized in 2 ml reaction tubes with 600 µl extraction buffer (50 mM Tris pH 8, 200 mM NaCl, 0.2 mM EDTA, 0.5% SDS, 100 mg/ml Proteinase K, and 100 mg/ml glycogen) using a mixer mill (Retsch MM 200, Retsch GmbH, Haan, Germany). The homogenized lysate was incubated at 60 °C for 30 minutes with shaking at 850 rpm for 5 seconds in every 2 minutes in a Thriller® device (VWR peqlab, Erlangen, Germany). Subsequently, 600 µl of phenol-chloroform-isoamyl alcohol (25-24-1, Roti® Carl Roth, Karlsruhe, Germany) was added and tubes were centrifuged at 19,000 *g* for 10 minutes. Afterwards, 500 µl of the supernatant were transferred to a new tube and 5 µl of 20 mg/ml RNase A solution was added. Subsequently, the tubes were incubated at 37 °C for 30 minutes. Then 600 µl phenol-chloroform-isoamyl alcohol solution (25-14-1) were added and the tubes were centrifuged at 19,000 *g* for 10 minutes. The supernatant was transferred to a new tube and another 600 µl of phenol-chloroform-isoamyl alcohol solution (25-24-1) was added. This was followed by centrifugation at 19,000 *g* for 10 minutes. Afterwards, the supernatant was transferred to a new tube. Subsequently, 45 µl of 3 M sodium acetate at pH 5.3 and 1,000 µl of ethanol were added. Tubes were mixed carefully for 30 seconds and then incubated at –20 °C for 30 minutes. After incubation, DNA was pelleted at 6,000 *g* for 10 minutes and the supernatant was discarded. Ethanol (70% volumetric in water) was added to the DNA pellets and tubes were subsequently centrifuged at 6,000 *g* for 2 minutes. This step was repeated twice. Finally, DNA pellets were dried in a Thriller® at 60 °C for 10 minutes and 30 µl molecular grade distilled water was added to dissolve the purified DNA.

The DNA solutions were quantified using an IMPLEN Nanophotometer (Implen GmbH, Munich, Germany). Approximately 10 – 50 ng of DNA were used per PCR reaction for the amplification of the Internal Transcribed Spacers (ITS) and *cytochrome oxidase* 1 (*cox*1) regions using the primer pairs listed in Table 1. The PCR reaction mix contained 1× PCR buffer, 0.2 mM dNTPs, 2.0 mM MgCl, 0.8 µg BSA, 0.4 µM of each primer, 0.5 u *Taq* polymerase and 10 – 50 ng DNA.

Table 1 Primer pairs used in PCR amplification.

Loci	Primer name	Primer sequence (5' – 3')	Reference
ITS	ITS1-O	CGG AAG GAT CAT TAC CAC	Bachofer 2004
	LR0	GCT TAA GTT CAG CGG GT	Moncalvo et al. 1995
*cox*1	Oom*Cox1*_Levup	GCT TAA GTT CAG CGG GT	Robideau et al. 2011
	Oom*Cox1*_Levlo	CYT CHG GRT GWC CRA AAA ACC AAA	Robideau et al. 2011

Cycling conditions for the ITS region included an initial denaturation at 94 °C for 4 minutes, followed by 36 cycles of denaturation at 94 °C for 40 seconds, annealing at 55 °C for 20 seconds, and elongation at 72 °C for 60 seconds. Subsequently, a final elongation at 72 °C for 4 minutes was carried out. The cycling program for the *cox*1 region included an initial denaturation at 95 °C for 4 minutes, followed by 36 cycles of denaturation at 95 °C for 40 seconds, annealing at 51 °C for 40 seconds, and elongation of 72 °C for 60 seconds. Subsequently, a final elongation was done at 72 °C for 5 minutes. All PCR reactions were performed in an Eppendorf Mastercycler Pro Thermocycler equipped with a vapoprotect lid (Eppendorf AG, Hamburg, Germany).

PCR products were sent to the sequencing laboratory of the Senckenberg Biodiversity and Climate Research Centre, using the reverse primers of each amplified region. Obtained sequences were edited using Geneious, version 5.0.4 (Biomatters Ltd., Auckland, New Zealand). The edited sequences were uploaded to the TrEase webserver (http://www.thines-lab.senckenberg.de/trease/) (Mishra et al. unpublished) together with the other sequences obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/) and from the *Phytophthora* database (www.phytophthoradb.org) for sequence alignment and phylogenetic tree construction using Bayesian Inference (BI) with MrBayes (Ronquist et al. 2012) following the standard settings implemented in siMBa (Mishra and Thines 2014). MAFFT (Katoh et al. 2002) was used for alignments, choosing the G-INS-I algorithm for the ITS sequences and FFT-NS-1 (fast) for the *cox*1 sequences (applicable because of the lack of gaps in *cox*1 sequence alignments in the closely related species included in this study). Two additional phylogenetic methods were used alongside with Bayesian Inference (BI) as the primary phylogenetic tree. Maximum Likelihood (ML) inference was done using FastTree 2 (Price et al. 2010) and Minimum Evolution (ME) inference was done using MEGA version 6 or 7 (Tamura et al. 2013). For Bayesian inference, the 6 GTR substitution model was the chosen model and 1,000,000 generations with incrementally heated chains were calculated with sampling at every 10,000th tree and discarding the first 30% of the sampled trees. For Maximum Likelihood (ML) inference, the GTR substitution model was chosen and 1,000 bootstrap replicates were performed. For Minimum Evolution (ME) inference, the Tamura-Nei model was used as this is the most complex standard model offered by MEGA, version 6 and 7, performing 1,000 bootstrap replicates. All other settings were set to default. All phylogenetic trees were viewed in MEGA, version 6 or 7.

Results

The strains *Phytophthora* BMYL-1217-1 and BMYL-1217-2 were similar in morphology in both sporangia (i.e. shape, zoospore release, branching patterns, and proliferation) and gametangia with morphological features agreeing with the description of *P. elongata*. The morphological features of the strains are depicted in Figure 1. Both strains are homothallic, i.e. capable of producing antheridia and oogonia in the absence of an opposite mating type, and gametangia are

readily produced in vegetable juice agar media with or without seawater when incubated at room temperature. Antheridia are paragynous, and oogonia are spherical with aplerotic oospores, i.e. space is present between the oogonium wall and oospore wall (Figure 1). Sporangia were produced when agar blocks from 7 to 10 day-old cultures were placed in unsterile soil extract and incubated at room temperature. Sporulation was not observed in saline solutions. The shape of the sporangia is ovate to obpyriform. Sporangia were non-caducous and apapillate. Based on ITS and *cox*1 phylogenies (Figures 2 and 3, respectively), strains BMYL-1217-1 and BMYL-1217-2 belonged to the Clade 2 of *Phytophthora sensu lato*, and are grouped with the identical sequence from the ex-type culture of *P. elongata* (CBS 125799) with strong support in all analyses.

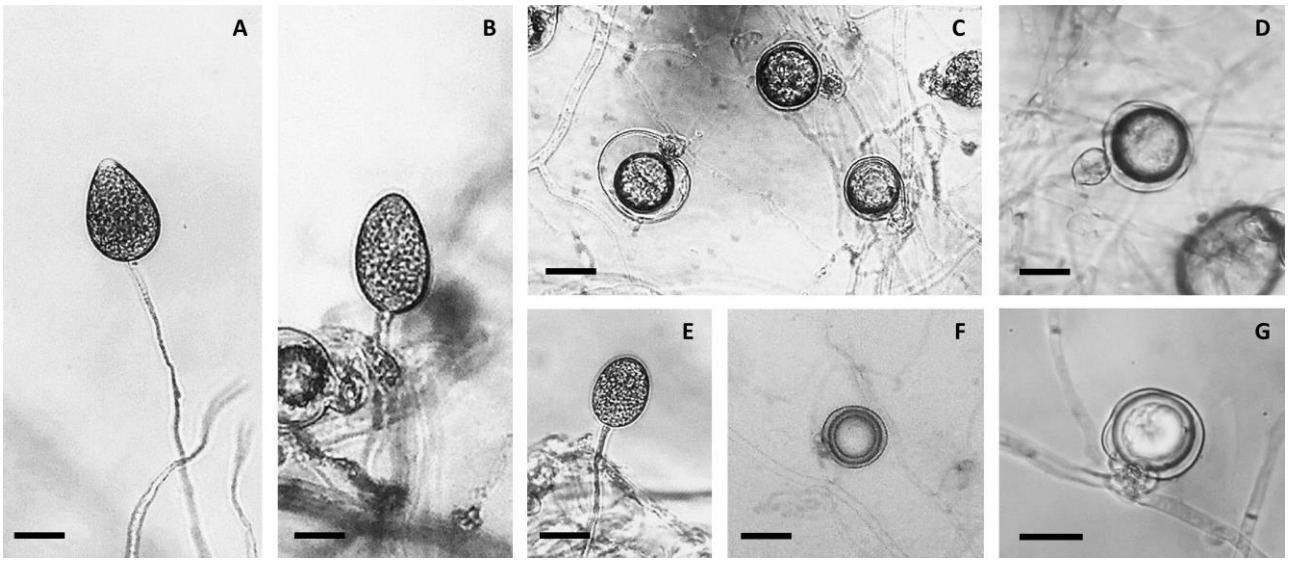

Figure 1 – *Phytophthora elongata.* BMYL-1217. A, B, E Sporangia. C, D, F, G Gametangia. Note the presence of the paragynous antheridia; Oospores, aplerotic. Scale bars = 20 µm.

Discussion

The knowledge of *Phytophthora* in the Philippines is limited and the majority of reports are from post-harvest fruits and agricultural fields. However, a single species has so far been reported as a saprotroph on mangrove leaves, *P. insolita* (Bennett & Thines 2017). The phytopathogenic *Phytophthora* spp. reported in the Philippines are listed in the review of Portales (2004), these include *P. cactorum*, *P. capsici*, *P. citrophthora*, *P. colocassiae*, *P. haveae*, *P. infestans*, *P. meadii*, *P. nicotianae*, *P. palmivora*, and *P. phaseoli*. Herewith, an additional species, *P. elongata*, is reported from an estuarine environment in the Philippines and as the first species of the Clade 2 group of *Phytophthora* in an estuarine environment.

Classical identification of *Phytophthora* is often based on the key of Waterhouse (1963) or the revised key of Stamps et al. (1990). With the advent of molecular phylogenetics, the Waterhouse and Stamps classification system of *Phytophthora* was revealed not to delineate monophyletic entities. Thus, a phylogenetic classification system with 8 to 10 clades is now widely applied (Cooke et al. 2000, Kroon et al. 2004). In this manuscript, the 10 clade classification system is followed as summarized in Kroon et al. (2012). Each of the proposed clades (1 – 10) of *Phytophthora sensu lato* does not possess obvious consensus morphology with clade-specific synapomorphies. Cooke et al. (2000) proposed that the morphology and phylogeny of the genus *Phytophthora* should be reanalysed and that naming one or more genera might be considered. A multigene phylogeny using both mitochondrial and nuclear DNA sequences representing 113 isolates from 48 species by Kroon et al. (2004) supported a division of *Phytophthora* spp. into 8 clades, while Blair et al. (2008) proposed a division of 10 well-supported clades. Runge et al. (2011) inferred a high degree of paraphyly of *Phytophthora* spp. when two downy mildew species (*Pseudoperonospora cubensis* and *Hyaloperonospora arabidopsidis*) were included in the dataset

of Blair et al. (2008). In their analysis, the downy mildews were placed within clade 4 of *Phytophthora*. Thus, Runge et al. (2011) seconded the proposal of Cooke et al. (2000) and concluded that it might be necessary to introduce at least six new genera within *Phytophthora sensu lato* (including clade 2) to resolve the paraphyly of the genus. However, none of the subsequent studies attempted to propose new generic names for some clades of *Phytophthora* due to the difficulty of finding synapomorphies.

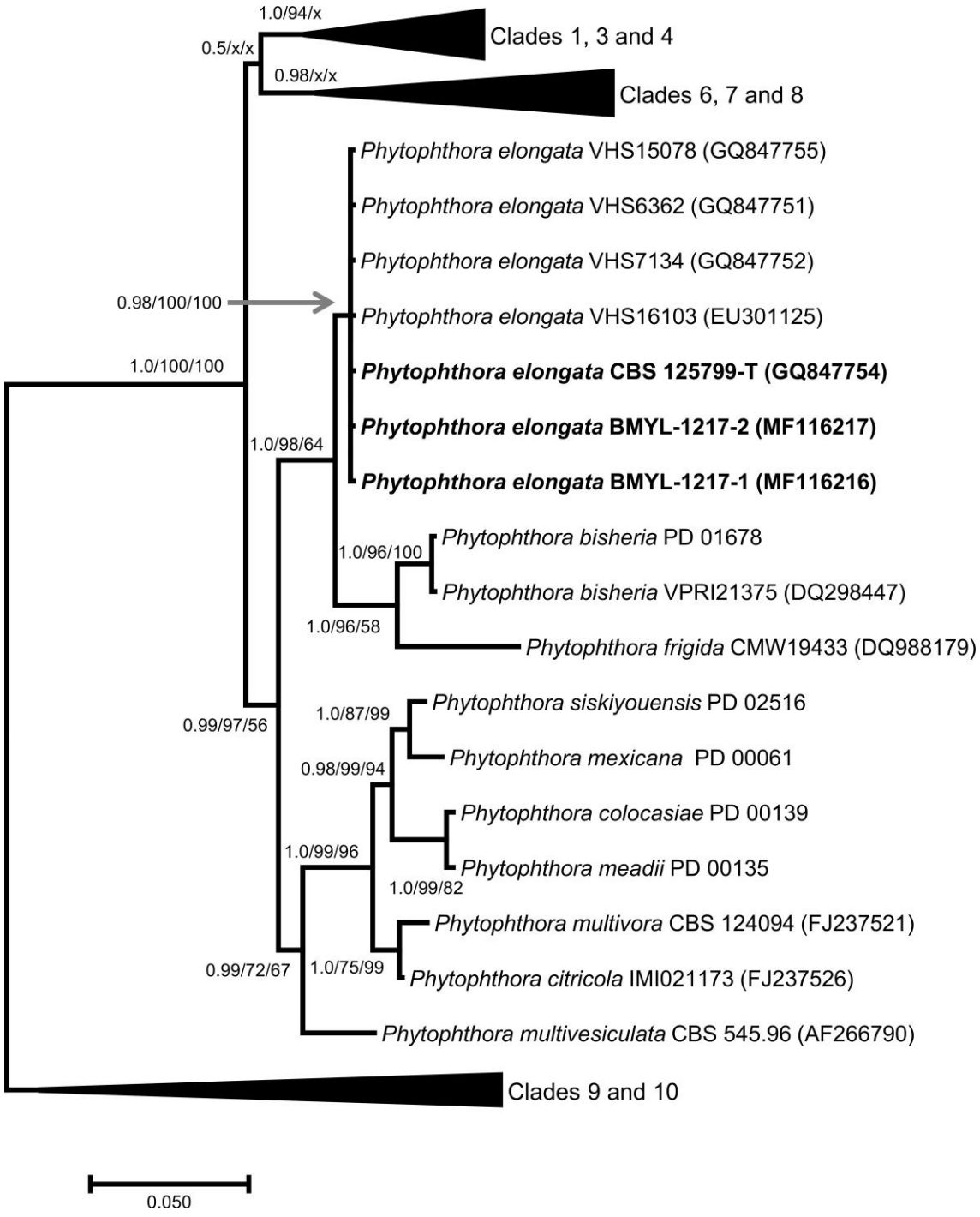

Figure 2 – ITS-based Bayesian phylogenetic inference of the genus *Phytophthora*. Support values from Bayesian inference, Maximum Likelihood, and Minimum Evolution, in the respective order. CBS 125799 (= VHS13482) is the ex-type culture of *P. elongata*, while the strains BMYL-1217-1 and BMYL-1217-2 are from the Philippines. The scale bar indicates the number of substitutions per site. (×) indicates support for an alternate topology in comparison to the tree derived from Bayesian inference.

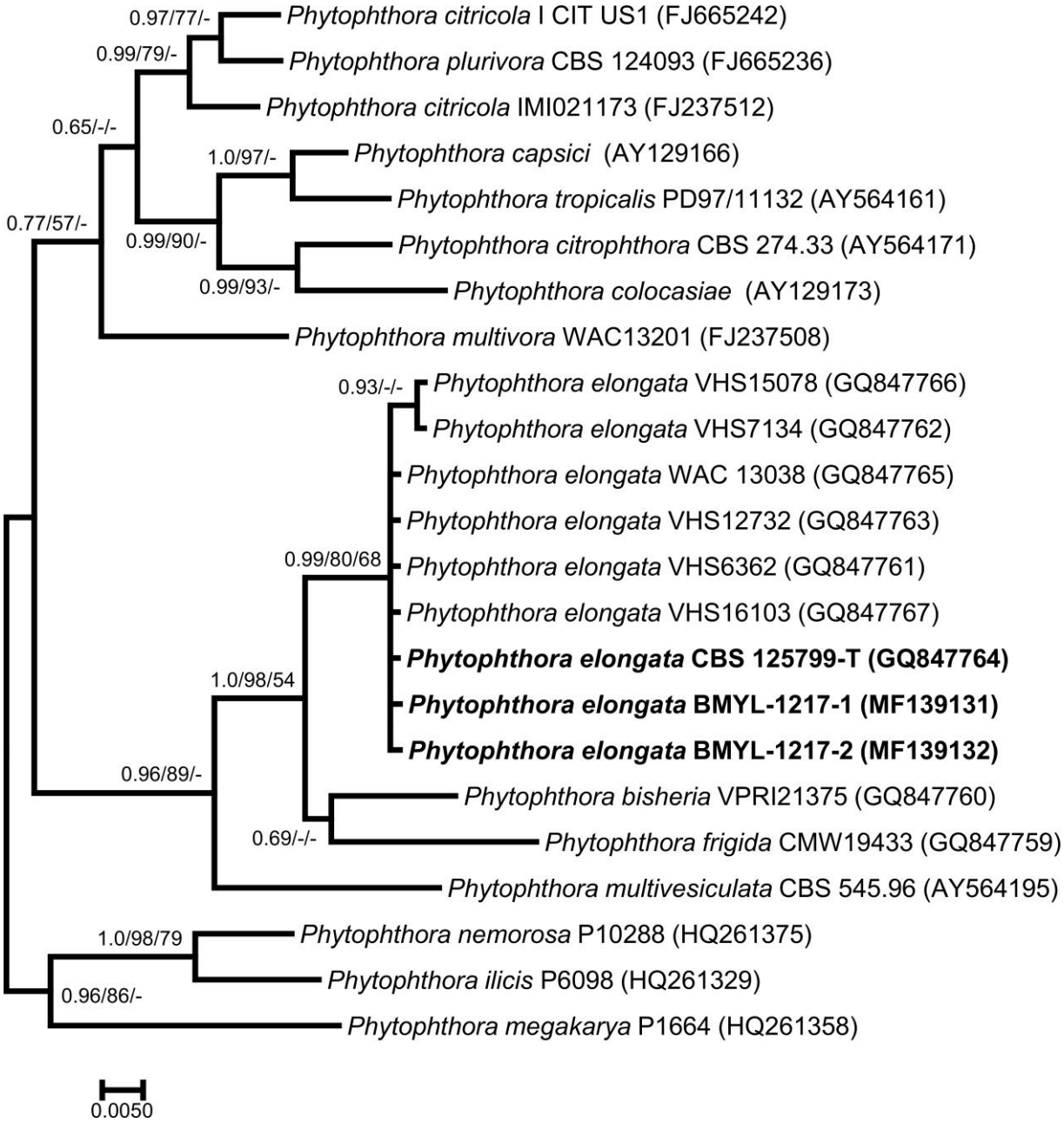

Figure 3 – Bayesian phylogenetic reconstruction of Clade 2 of *Phytophthora sensu lato* based on *cox*1 sequences with support values from Bayesian inference, Maximum Likelihood, and Minimum Evolution, in the respective order. CBS 125799 (= VHS13482) is the ex-type culture of *P. elongata*, while the strains BMYL-1217-1 and BMYL-1217-2 are from the Philippines. The scale bar indicates the number of substitutions per site. (-) denotes a lack of support in the respective method.

Of the 10 clades of *Phytophthora*, Clade 2 was described as one of the largest groups, composed of 21 taxa (Kroon et al. 2012), 17 of which are listed in the *Phytophthora* database (http://www.phytophthoradb.org/). Clade 2 *Phytophthora* spp. have been reported as pathogens of a variety of plants, e.g. *P. siskiyouensis* on *Umbellularia californica* and *Lithocarpus densiflorus* (Reeser et al. 2007), *P. colocasiae* on *Colocasia esculenta* (Raciborski 1990), *P. inflata* on *Ulmus americana* (Caroselli & Tucker 1949), and *P. meadii* on *Hevea brasiliensis* (McRae 1918). These species have been isolated either from the roots, foliage, or fruits of the plant host or from the rhizosphere.

Table 2 Estuarine *Phytophthora* spp. as recorded in literature

Species	Substrate	Country	Clade	Reference
P. elongata	Mangrove leaf litter	Philippines	2	This study
P. estuarina	*Laguncularia racemosa*	Brazil	9	Guo et al. 2016
	Rhizophora mangle			
P. insolita	Mangrove leaf litter	Philippines	9	Bennett and Thines 2017
	Rhizophora	China		Zeng et al. 2009
P. inundata	*Zostera marina*	Netherlands	6	Man In 'T Veld et al. 2011
P. gemini	*Zostera marina*	Netherlands	6	Man In 'T Veld et al. 2011
P. rhizophorae	*Rhizophora mangle*	Brazil	9	Guo et al. 2016

Phytophthora elongata has been reported as a pathogen of *Eucalyptus marginata* from Western Australia (Rea et al. 2010) and is reported in this study as an additional member of the estuarine oomycetes (Table 2) and the first species for the clade 2 group to be isolated from a marine environment. This species was previously considered as a member of the *P. citricola* species complex and referred to as *Phytophthora* sp. 2 (Burgess et al. 2009), *Phytophthora* sp. WA2 (Stukely et al. 2007), or *P. citricola* subgroup SG1 (Bunny 1996, Stukely et al. 2007). The nomenclatural description of *P. elongata* (Rea et al. 2010) stated that sporangia are often elongated, thus the species epithet, and varied in shape from ovoid, obpyriform, elongated obpyriform, ampuliform, limoniform, to various distorted shapes. The Philippine strains produced ovoid, obypriform, and limoniform sporangia, but no elongated sporangia or any sporangial extensions and shape variations were observed during sporulation under the conditions used in this study. These differences in shape could be a reflection of different sporulation conditions used or could be part of the natural variation in this pathogen. Since *P. elongata* is a pathogen of woody angiosperm plants, there is the possibility that this species could be a pathogen of mangrove tree species in the Philippines, which should probably be further investigated in the future. However, it is also conceivable that *P. elongata* has been introduced from Australia together with its hosts, as eucalypts were seen on the bay where the *P. elongata* strains were isolated.

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