Phylogeny of Mangrove Oomycetes

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Reuel Matignas Bennett
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Abstract

Taxa under scrutiny in this thesis are *Halophytophthora*-like oomycetes. The genus *Halophytophthora*, proposed in 1990, is an assemblage of unrelated species grouped together on the basis habitat preference, i.e. the mangrove or saltmarsh biome, and morphological similarity to *Phytophthora*. The premise "*Phytophthora*-like species from the mangrove environment" became the genus concept for *Halophytophthora* and lasted for almost 2 decades which resulted to the addition of several species (i.e. *H. elongata*, *H. exoprolifera*, *H. porrigovesica*, *H. kandeliae*, *H. masteri*, and *H. tartarea*). At the onset of molecular phylogenetics, *Halophytophthora* was inferred as a highly polyphyletic taxon and the genus concept was found to be unsuitable. This thesis adds to this, since six *Phytophthora* spp. were isolated from the mangrove environment, two of which were found in the Philippines (*Phytophthora elongata* and *Phytophthora insolita*). After a thorough assessment of the morphologic and phylogenetic data of taxa included in this thesis, several taxonomic novelties were introduced – a new family (Salispinaceae), a new genus (*Calycofera*), new species (*Calycofera cryptica*, *Phytopythium dogmae*, *Phytopythium leanoi*, *Salisapilia coffeyi*, and *Salispina hoi*), and new combinations (*Calycofera operculata*, *Salisapilia bahamensis*, *S. elongata*, *S. epistomia*, *S. masteri*, *S. mycoparasitica*). In addition, Salisapiliaceae and *Salisapilia* were emended.

Keywords: Estuary, *Calycofera*, Diversity, *Halophytophthora*, Peronosporaceae, Phylogeny, *Phytophthora*, *Phytopythium*, *Salisapilia*, Salisapiliaceae, *Salispina*, Salispinaceae, Systematics, Taxonomy

Kurzzusammenfassung

Die in dieser Dissertation untersuchten Arten sind *Halopyhtophthora*-ähnliche Oomyceten. Die Gattung *Halophythophthora*, welche 1990 beschrieben wurde, ist eine Ansammlung nicht-verwandter Arten, die aufgrund ihrer Habitatpräferenzen, Mangroven- bzw. Salzmarschbiome, und morphologischer Ähnlichkeit zu *Phytophthora* zusammengefasst wurden. Die Prämisse „*Phytophthora*-ähnliche Arten aus Mangroven" als Gattungskonzept für *Halophytophthora* besteht nun seit fast 2 Dekaden. Während dieser Zeit wurden eine ganze Reihe von Arten der Gattung hinzugefügt (z.B. *H. elongata*, *H. exoprolifera*, *H. porrigovesica*, *H. kandeliae*, *H. masteri* und *H. tartarea*). Mit dem Auftauchen molekularer Phylogenetik wurde jedoch klar, dass es sich bei *Halophytophthora* um ein höchst polyphyletisches Taxon handelt, dessen Gattungskonzept unbrauchbar war. Dieser Problematik nimmt sich diese Dissertation ausgehend von sechs *Phytophthora* Arten an, die aus Mangroven-Ökosystemen isoliert wurden, wovon zwei auf den Philippinen gefunden wurden (*Ph. elongata* und *Ph. insolita*). Nach detaillierter Untersuchung morphologischer und phylogenetischer Merkmale, der in dieser Thesis bearbeiteten Spezies, konnten mehrere taxonomische Neuerungen eingeführt werden – eine neue Familie (Salispinaceae), eine neue Gattung (*Calycofera*), fünf neue Arten (*Calycofera cryptica*, *Phytopythium dogmae*, *Phytopythium leanoi*, *Salisapilia coffeyi* und *Salispina hoi*) sowie einige Neukombinationen (*Calycofera operculata*, *Salisapilia bahamensis*, *S. elongata*, *S. epistomia*, *S. masteri*, *S. mycoparasitica*). Zudem wurden die Gruppen Salisapiliaceae und *Salisapilia* korrigiert.

Keywords: Mündungsgebiet, *Calycofera*, Diversität, *Halophytophthora*, Peronosporaceae, Phylogenie, *Phytophthora*, *Phytopythium*, *Salisapilia*, Salisapiliaceae, *Salispina*, Salispinaceae, Systematik, Taxonomie

Summary

This thesis presents a comprehensive investigation into the taxonomy and phylogeny of mangrove oomycetes, focusing on the Philippines. This particular group of oomycetes has been largely ignored for the past two or more decades, thereby creating a huge gap in the knowledge of these interesting fungal-like eukaryotes. Therefore, a need for an integrative taxonomic and phylogenetic analyses into these underexplored saprotrophic oomycetes deemed necessary – which is, primarily, the aim of this research. The research started with the isolation of different mangrove oomycetes from selected areas in the Philippines and acquisition of type strains from the Netherlands (Westerdijk Institute-KNAW, formerly Centralbureau voor Schimmelcultures-KNAW) and Japan (NITE Biological Resource Research Centre). Classical taxonomic and modern phylogenetic methods were integrated to establish new taxa (family, genera, new species), new combinations, and emendations to known taxonomic ranks (family, genera and species).

In the mangrove or estuarine environment, a total of six *Phytophthora* spp. (*Ph. elongata*, *Ph. estuarina*, *Ph. insolita*, *Ph. inundata*, and *Ph. gemini*) were recorded, two of which (*Ph. insolita* and *Ph. elongata*) are presented in this thesis as isolates from the Philippines, whereas the remaining *Phytophthora* spp. were reported elsewhere. *Phytophthora elongata*, a pathogen of *Eucalyptus marginata* in Australia, is the first species from the clade 2 *Phytophthora* to be reported as a mangrove oomycete; whereas *Ph. insolita*, an oospore producing *Phytophthora* devoid of antheridia and a pathogen of *Citrus*, is the third mangrove oomycete species for the clade 9 group (along with *Ph. estuarina* and *Ph. rhizophorae*). This is perplexing since *Ph. elongata* and *Ph. insolita* were thought to be as hemibiotrophic pathogens of land plants; and their tolerance to extreme salinity was unknown. However, these species are indeed capable of osmotic tolerance. The previously

accepted genus concept of *Halophytophthora* as "*Phytophthora*-like species from the mangrove environment" is thus rejected and the tolerance to the saline environment by some species of *Phytophthora* is possibly a reversal to its ancestral trait.

Another interesting group of mangrove oomycetes explored in this thesis are those now placed in the Salisapiliaceae. This taxon was proposed in 2010 to accommodate homothallic *Halophytophthora*-like species with a hyaline apical plug nested at or protruding through the discharge tube, an absence of vesicle prior to or during zoospore release, and hyphae with small diameter. The family was composed of three congeners, *S. sapeloensis* (type species), *S. nakagirii*, and *S. tartarea* (basionym, *H. tartarea*). However, of the species of *Salisapilia*, *S. nakagirii* was described based on strong phylogenetic placement and morphology of gametangia, and no sporangia were observed at the time the species was proposed. This then challenges the morphological circumscription of *Salisapilia*. In this thesis, a morphologic and phylogenetic study of Salisapiliaceae was conducted. Based on the presented data, sporangia of *S. nakagirii* deviated from the proposed diagnosis of *Salisapilia*, on the basis of the development of a vase-shaped semi-persistent or semi-evanescent vesicle; and the absence of a well-formed hyaline apical plug. It was proposed that *S. nakagirii* is papillate, however, the sporangia have an apapillate discharge tube filled with a non-sporogenous fluid mass of protoplasmic origin. Further, after maturity of zoospores, the sporogenic mass shrinks away from the discharge tube leaving an impression of a papilla. Based on the presented phylogenetic reconstruction, the genus *Salisapilia* includes five additional species, resulting from new combinations, (i.e. *S. bahamensis*, *S. elongata*, *S. epistomia*, and *S. mycoparasitica*), and a new species, *S. coffeyi*. Following an ancestral trait analysis, the character "discharge tube without apical hyaline plug" is potentially seen as an ancestral trait and that the character "discharge tube with apical

hyaline plug" is derived. Consequently, the description of the genus *Salisapilia* was emended to reflect this.

The recently described genus *Salispina*, which included the three species, *S. intermedia* (type species), *S. lobata*, and *S. spinosa*, is a taxon proposed in 2016 to accommodate *Halophytophthora*-like species with spiny or aculeolate sporangia. The genus includes species with aculeolate sporangia without a vesicle prior to or during zoospore release, a discharge tube and varying patterns of spine formation on the surface of the sporangia. Based on morphology, this genus is divergent from *Halophytophthora*, *Phytophthora*, *Phytopythium*, and *Salisapilia*. Based on phylogeny, it is the sister taxon to *Sapromyces elongatus* (Rhipidiaceae, Rhipidiales). The morphological differences between *Salispina* and *Sapromyces* are pronounced in terms of the development of sporangia and hyphal structure. *Sapromyces* forms a slender thallus with constrictions separating hyphal segments, a trait only weakly, if at all, observable among congeners of *Salispina*. When considering the morphological differences between *Salispina* and the other genera of Rhipidiaceae, few species of Rhipidiaceae have aculeolate sporangia (e.g. *Araiospora spinosa*, *Araispora coronata*, *Nellymyces megaceros*). At the time *Salispina* was proposed, the genus was considered as a taxon *incertae sedis*, however, a new family is proposed in this thesis, Salispinaceae, to accommodate this genus within Rhipidiales.

The genus *Phytopythium*, formerly referred to as the Clade K of *Pythium*, shares morphological characteristics with both *Phytophthora* and *Pythium*. The majority of *Phytopythium* spp. are pathogens of post-harvest fruits or plant species, albeit one species, *Pp. kandeliae*, which was formerly considered a member of *Halophytophthora*, was recorded as a saprotroph on *Kandelia candel* leaves, a mangrove plant commonly found in tropical countries. In this thesis, two new species of saprotrophic *Phytopythium* are

introduced, *Pp. leanoi* and *Pp. dogmae*. The former taxon is part of the *Pp. kandeliae* species-complex and is morphologically divergent from the *Pp. kandeliae* ex-type culture on the basis of formation of gametangia, the proliferation of sporangia, and the absence of a crescent-shaped apical translucent matrix. The second new congener, *Pp. dogmae* is a member of the clade 2 group of *Phytopythium* and is closely related to *Pp. chamaehyphon*, *Pp. helicoides*, and *Pp. palingenes*, but differs significantly based on combined morphological traits, i.e. the development of sporangia, discharge tubes, and the proliferation of sporangia. The new species of *Phytopythium* are both found in Philippine mangroves. The role of *Phytopythium* spp. in Philippine mangrove should be assessed to investigate the pathogenic potential of these supposedly saprotrophic mangrove oomycetes.

The morphological integrity of *Phytopythium* was recently challenged based on several published phylogenetic trees. *Halophytophthora operculata*, a mangrove oomycete and formerly classified in *Phytophthora*, groups with the *Phytopythium* clade, however with conflicting placements on several phylogenetic reconstructions using different genes. The phylogenetic position of *H. operculata* was resolved in this thesis and was inferred as basal to *Phytopythium*. *Halophytophthora operculata* is divergent from *Phytopythium* in terms of the development of sporangiogenic hyphae, wherein this structure is twisted for *H. operculata*, while it is simple with no projections for *Phytopythium*. The branching pattern of *H. operculata* is monochasial with externally proliferating sporangia, whereas branching pattern for *Phytopythium* is irregular and proliferation is nested-, extended-internal or external. The shape of sporangia for *H. operculata* is tulip-shaped, whereas various other forms, including globose, pyriform, to obpyriform, are known from *Phytopythium*. As a consequence, the genus *Calycofera* was proposed to accommodate *C. operculata* and its

sister taxon *C. cryptica* – a species described based on nucleotide sequence divergence from *C. operculata.* The proposal for *Calycofera* was a way to conserve the well-defined morphological diagnosis of *Phytopythium*.

As a concluding remark to this thesis, several proposals (new taxa, combinations, and emendation) were made to strengthen the natural grouping of mangrove oomycetes by using combined molecular and morphological approaches. The presented phylogeny of saprotrophic mangrove oomycetes is useful in understanding the evolutionary patterns of oomycetes in general; and that the intrinsic value of these organisms should not be neglected as these organisms are seen as key players in the ecosystem.

Zusammenfassung

Diese Dissertation beinhaltet eine umfassende Untersuchung der Taxonomie und Phylogenie von Oomyceten der Mangrovenwälder der Philippinen. Diese besondere Gruppe von Oomyceten wurde in den letzten zwei und mehr Dekaden größtenteils ignoriert, was eine Lücke im Wissen über diese interessante Gruppe pilzähnlicher Eukaryoten hinterließ. Daher gibt es einen großen Bedarf an integrativen taxonomisch-phylogenetischen Untersuchungen dieser schlecht untersuchten, saprotrophen Oomyceten. Dies ist das vorrangige Ziel dieser wissenschaftlichen Arbeit. Die Basis der Untersuchungen bildete die Isolierung von diversen Oomyceten der Mangrovenbiomen ausgewählter Gebiete der Philippinen und dem Erwerb von Stämmen aus den Kultursammlungen der Niederlande (Westerdijk Institute-KNAW, formerly Centralbureau voor Schimmelcultures-KNAW) und Japans (NITE Biological Resource Research Centre). Klassische taxonomische und moderne phylogenetische Methoden wurden kombiniert, um neue Taxa (Familien, Gattungen, Arten) zu beschreiben, sowie Neukombination und notwendige Korrekturen beschriebener taxonomischer Ränge (Familien, Gattungen und Arten) herauszuarbeiten.

Insgesamt sechs *Phytophthora*-Arten (*Ph. elongata*, *Ph. estuarina*, *Ph. insolita*, *Ph. inundata*, und *Ph. gemini*) aus Mangroven- bzw. Mündungsökosystemen wurden kultiviert, von denen in dieser Dissertation zwei Isolate von den Philippinen (*Ph. insolita* und *Ph. elongata*) näher charakterisiert werden. *Ph. elongata*, ein Pathogen von *Eucalyptus marginata* in Australien, ist die erste Art aus Klade 2 von *Phytophthora*, die einen Oomycete eines Mangrovenbioms darstellt. *Ph. insolita* wiederum ist eine Oosporen bildende, antheridienlose *Phytophthora*-Art und ein Pathogen von *Citrus*. Sie ist die dritte Oomyceten-Art aus Mangrovenökosystemen aus Klade 9 (neben *Ph. estuarina* und *Ph. rhizophorae*).

Überraschend ist, dass *Ph. elongata* und *Ph. insolita* als hemibiotrophe Pathogene von Landpflanzen bisher nicht für ihre Toleranz extremer Salinität bekannt waren. Da diese Arten, wie hier gezeigt, gleichwohl zu osmotischer Toleranz fähig sind, stellt dies das zuvor akzeptierte Gattungskonzept von *Halophytophthora* als „*Phytophthora*-ähnliche Arten der Mangroven“ in Frage. Die Fähigkeit einiger *Phytophthora*-Arten saline Umweltbedingungen zu tolerieren, stellt vermutlich eine Umkehr zu ursprünglichen Eigenschaften dar.

Eine weitere interessante Gruppe Mangroven bewohnender Oomyceten, sind Arten, die nun den Salisapiliaceae zugeordnet werden. Dieses Taxon wurde 2010 als eine Gruppe homothallischer *Halophytophthora*-ähnlicher Arten mit einem hyalinen, apikalen Pfropfen, welcher in den Entleerungsschlauch eingelassen oder über diesen hinausstehend ist. Weiterhin sind sie durch die Abwesenheit von Vesikeln vor oder während der Entleerung der Zoosporen und durch Hyphen mit kleinem Durchmesser charakterisiert. Die Familie setzt sich aus drei Artverwandten zusammen *S. sapeloensis* (die Typusart), *S. nakagirii* und *S. tartarea* (Basionym *H. tartarea*). Von diesen *Salisapilia*-Arten wurde *S. nakagirii* ausschließlich auf Basis phylogenetischer Analysen und der Morphologie der Gametangien beschrieben, jedoch wurden keine Sporangien beobachtet, was für eine vollumfängliche Beschreibung innerhalb der Gattung *Salisapilia* jedoch notwendig ist. Diese Dissertation untersucht die Familie Salisapiliaceae, anhand morphologischer und phylogenetischer Merkmale. Die hier dargestellten Daten legen nahe, dass die Sporangien von *S. nakagirii* von den beschriebenen Charakteristika der Gattung *Salisapilia* abweichen. Deutlich wird dies, in der Entwicklung von Vasen-förmigen semi-überdauernden bzw. semi-vergänglichen Vesikeln und der Abwesenheit eines ausgebildeten hyalinen, apikalen Pfropfens. Weiterhin wurde *S. nakagirii* als papillös beschrieben, doch haben die Sporangien einen warzenfreien

Entleerungsschlauch, der mit einer nicht-sporogenen, fluiden Masse protoplasmatischen Ursprungs gefüllt ist. Die die sporogene Masse zieht sich nach der Reife der Zoosporen aus dem Entleerungsschlauch zurück, was den Eindruck einer Warze zurücklässt. Basierend auf der dargestellten phylogenetischen Rekonstruktion beinhaltet die Gattung Salisapilia nun fünf zusätzliche Arten, die aus Neukombinationen hervorgegangen sind (z.B. *S. bahamensis*, *S. elongata*, *S. epistomia* und *S. mycoparasitica*) sowie die neu beschriebene Art *S. coffeyi*. Die anschließende Ancestral-Trait-Analyse zeigte zudem, dass das Merkmal „Entleerungschlauch ohne apikalem, hyalinen Pfropfen" potenziell ein eher ursprüngliches Merkmal ist und die Eigenschaft „Entleerungsschlauch mit apikalem, hyalinem Pfropfen" eher ein abgeleitetes Merkmal darstellt. Konsequenterweise wurde die Beschreibung der Gattung *Salisapilia* dahingehend korrigiert diese Merkmale widerzuspiegeln.

Die kürzlich beschriebene Gattung *Salispina*, die drei Arten (*S. intermedia* (Typusart), *S. lobata* und *S. spinosa*) beinhaltet, wurde 2016 als Taxon vorgeschlagen um *Halophytophthora*-ähnliche Arten mit stacheligen oder aculeolaten Sporangien zusammenzufassen. Die Gattung beinhaltet Arten mit Sporangien ohne Vesikel vor oder während der Entleerung der Zoosporen, einer Entleerungsschlauch sowie wechselnden Mustern von Stacheln auf der Oberfläche der Sporangien. Damit unterscheidet sich diese Gattung morphologisch von *Halophytophthora*, *Phytophthora* und *Salisapilia* und zeigt sich phylogenetisch als eine Schwestergattung zu *Sapromyces elongatus* (Rhipidiaceae, Rhipidiales). Die morphologischen Unterschiede zwischen *Salispina* und *Sapromyces* sind in der Entwicklung der Sporangien und in der Struktur der Hyphen zu finden. Hierbei zeigt *Sapromyces* durch einen schlanken Thallus mit Einschnürungen, wodurch hyphale Segmente abgegrenzt werden, eine Eigenschaft, die nur schwach bzw. gar nicht bei den Artverwandten

der Gattung *Salispina* beobachtet werden kann. Weitergehende Vergleiche der morphologischen Charakteristika von *Salispina* und anderen Gattungen der Rhipidiaceae zeigen zudem, dass nur wenige Arten der Rhipidiaceae aculeolate Sporangien haben (z.B. *Araiospora spinosa*, *Araispora coronata*, *Nellymyces megaceros*). Zur Zeit der Beschreibung von *Salispina* konnte die Gattung als *incertae sedis* keiner höheren Gruppe zugeordnet werden. In dieser Arbeit wird die neue Familie Salispinaceae erhoben, um der Stellung der Gattung innerhalb der Rhipidiales Rechnung zu tragen.

Die Gattung *Phytopythium*, die zuvor als Klade K von *Pythium* bezeichnet wurde, teilt morphologische Charakteristika sowohl mit *Phytophthora* als auch mit *Pythium*. Die Mehrzahl der *Phytopythium*-Arten sind Pathogene von geernteten Früchten und Pflanzen, von denen nur *Pp. kandeliae*, welche zuvor der Gattung *Halophytophthora* zugeordnet wurde, saprotroph auf Blättern von *Kandelia candel* lebt, einer in tropischen Ländern weit verbreiteten Mangrovenpflanze. Diese Dissertation beschreibt zwei neue Arten von saprotrophen *Phytopythium*-Arten, *Pp. leanoi* und *Pp. dogmae*. *Pp. leanoi* ist dabei Teil des *Pp. kandeliae*-Artkomplexes und unterscheidet sich von der Typusart *Pp. kandeliae* in der Bildung der Gametangien, der Reifung der Sporangien und der Abwesenheit der halbmondförmigen, apikalen, durchscheinenden Matrix. Der zweite Artverwandte *Pp. dogmae* ist ein Mitglied der Klade 2-Gruppe von *Phytopythium* und nah verwandt mit *Pp. chamaehyphon*, *Pp. helicoides* und *Pp. palingenes* unterscheidet sich jedoch signifikant in einigen, voneinander abhängigen, Merkmalen wie der Entwicklung der Sporangien, der Entleerungsschläuchen und der Reifung der Sporangien. Diese neuen *Phytopythium*-Arten wurden beide in den Mangroven der Philippinen gefunden. Eine weitere Untersuchung ihrer

Rolle in diesem Ökosystem scheint notwendig, um auch das Pathogenitätspotential dieser vermutlich saprotrophen Oomyceten der Mangroven zu verstehen.

Kürzlich veröffentlichte Phylogenien stellten das Gattungskonzept von *Phytopythium* in Frage. *Halophytophthora operculara*, ein Oomycet der Mangroven und zuvor der Gattung *Phytophthora* zugeordnet, gruppiert zusammen mit *Phytopythium*, jedoch widersprüchlich in den verschiedenen phylogenetischen Rekonstruktionen, wenn unterschiedliche Gene verwendet werden. Diese Widersprüche konnten im Rahmen dieser Arbeit geklärt und *H. operculata* als basale Art zu *Phytopythium* bestimmt werden. Morphologisch unterscheidet sie sich von *Phytopythium* in den sporogenen Hyphen, die bei *H. operculata* gedreht sind, während sie einfach und ohne Ausbuchtungen bei *Phytopythium* zu finden ist. Weiterhin ist das Verzweigungsmuster von *H. operculata* monochasial mit extern reifenden Sporangien, während *Phytopythium* ein irreguläres Verzweigungsmuster mit verschachteltem, erweitert-intern oder extern reifenden Sporangien zeigt. Die Form der Sporangien bei *H. operculata* ist tulpenförmig, bei *Phytopythium* sind jedoch verschiedene andere Formen wie globos, pyriform bis zu obpyriform beschrieben. Daher wurde die Gattung *Calycofera* mit den zwei Arten *C. operculata* und der genetisch abweichenden Schwesterart *C. cryptica* erhoben. Dies ermöglichte es, die morphologisch gut abgegrenzte Gattung *Phytopythium* zu erhalten.

Abschließend bleibt festzuhalten, dass diese Dissertation zahlreiche taxonomische Neuerungen (neue Arten, Neukombinationen, Korrekturen) gemacht hat, um die natürliche Gruppierung der Oomyceten der Mangrovenbiome unter Verwendung molekularer und morphologischer Merkmale zu verbessern. Die vorgestellten Phylogenien saprotropher Oomyceten der Mangroven der Philippinen leistet somit einen entscheidenden Beitrag zur Verbesserung des Verständnisses der Evolution der Oomyceten. Da diese zu den

Schlüsselorganismen dieser Ökosysteme zählen, darf ihr intrinsischer Wert nicht länger ignoriert werden.

Introduction

History of knowledge on estuarine oomycetes from saltmarsh and mangrove habitats

The Phylum Oomycota is a group of fungal-like eukaryotes of the Kingdom Straminipila and is composed of approximately 1,700 species grouped into 90 genera (Beakes and Thines 2017, Wijayawardene et al. 2020). Studies conducted on oomycetes are focused mainly on the pathogenic groups, which include white blister rusts (e.g. *Albugo* spp., *Pustula* spp.), downy mildews (e.g. *Bremia* spp., *Peronospora* spp., *Plasmopara* spp.), *Phytophthora* spp., *Pythium* spp., and fish pathogens (e.g. *Saprolegnia* spp.). An ecologically important group of oomycetes that is hardly investigated is the saprotrophic oomycetes from saltmarsh, estuarine, or mangrove environments (Figure 1). Nigrelli and Thines (2013) suggested that there are approximately 60 known species of marine oomycetes recorded in the literature, and to date, 30 species are known from mangrove and saltmarsh habitats (Hulvey et al. 2010, Nigrelli and Thines 2013, Bennett and Thines 2017, Bennett et al. 2017a, 2017b, 2017c, 2018, Bennett and Thines 2019).

Figure 1. A mangrove sampling site in the Philippines (Image by Gabrielle Beatrix B. Francsisco). Typical for mangroves is their distinct root system. (A–B) Pneumatophores. (C) Stilt root system. There are 40 known species of mangrove-forming plants in the Philippines, distributed over 16 families (Primavera 2000).

Formerly, an oomycete isolated from the rhizosphere, a leaf or any water sample having an ovoid, obovoid, obpyriform, pyriform, ellipsoid or fusiform sporangium, is cultivable, was often considered a member of the genus *Phytophthora* (Bennett and Thines 2017, Bennett et al. 2017b). *Phytophthora vesicula*, a saprotrophic oomycete, was the first *Phytophthora* species reported from the marine environment (Anastasiou and Churchland 1969). This was then followed by Fell and Master (1975), Pegg and Alcorn (1982), and Gerrettson-Cornell (1984) who reported additional mangrove oomycetes (*Ph. avicenniae*, *Ph. bahamensis*, *Ph. batemanensis*, *Ph. epistomium*, *Ph. mycoparasitica*, *Ph. operculata*, *Ph. polymorphica*, *Ph. spinosa* var. *spinosa*, and *Ph. spinosa* var. *lobata*). Ho and Jung (1990) proposed *Halophytophthora* as the generic concept, i.e. the *Phytophthora*-like species from the estuarine or mangrove ecosystem, to accommodate the abovementioned species. This resulted in their combination to *Halophytophthora*, despite the broad generic circumscription needed for this. Nonetheless, this concept was accepted for approximately two decades and resulted in the addition of several species, i.e. *Halophytophthora elongata*, *H. exoprolifera*, *H. kandeliae*, *H. masteri*, *H. tartarea*, and *H. porrigovesica* (Ho et al. 1991, Ho et al. 1992, Nakagiri et al. 1994, Nakagiri et al. 2001, Ho et al. 2003). However, a freshwater species, *H. fluviatilis* (Yang and Hong 2014), was isolated from a stream in the USA using *Rhododendron* as a bait, and, to date, is the only known freshwater congener. Nakagiri (2002), in a technical conference report, suggested that *H. spinosa* was not a member of *Halophytophthora* and was seen to be closely related to *Sapromyces* (Rhipidiaceae). This questioned the integrity of *Halophytophthora* as a monophyletic group and likewise with the generic concept of *Phytophthora*-like species from the estuarine or mangrove environment. In the studies of Huvley et al. (2010) and Lara and Belbahri (2011) the polyphyly of *Halophytophthora* was ascertained, setting the stage for further revision.

Hulvey et al. (2010) proposed the family Salisapiliaceae to accommodate species from the saltmarsh environment. This family is strongly supported phylogenetically and was monotypic, comprising only of *Salisapilia*. Three species were included in this genus, *Salisapilia sapeloensis* (type species), *S. nakagirii*, and *S. tartarea* (Basionym: *Halophytophthora tartarea*). The genus *Phytopythium* (Bala et al. 2010) was proposed to accommodate species having shared characteristics of *Pythium* and *Phytophthora*. This monophyletic group was previously referred to as the clade K based on the phylogenetic

study of the genus *Pythium* (Lévesque and de Cock 2004). However, *Halophytophthora kandeliae* is nested in this genus, and as a result, Thines (2014) proposed the combination *Phytopythium kandeliae*, which was supported by strong morphological and phylogenetic evidence. More recently, *Salispina* was proposed to accommodate the two varieties of *H. spinosa* in *Salispina* as *Salispina spinosa* and *Salispina lobata* together with the type species, *S. intermedia* (Li et al. 2016). However, *Salispina* was placed as the sister taxon to *Sapromyces* rather than Peronosporaceae or Salisapiliaceae (Li et al. 2016). But due to low support for this seemingly unusual grouping, no higher-level placement of the genus *Salispina* was proposed.

Overview of the estuarine oomycetes phylogeny

Formerly, mangrove oomycetes belong to either Peronosporaceae or Salisapiliaceae (Figure 2). The family Peronosporaceae contains complex oomycete species and includes the large group of downy mildews (e.g. *Bremia*, *Peronospora*, *Plasmopara*), the highly paraphyletic *Phytophthora*, *Halophytophthora* s.l., and *Phytopythium* (Beakes and Thines 2017).

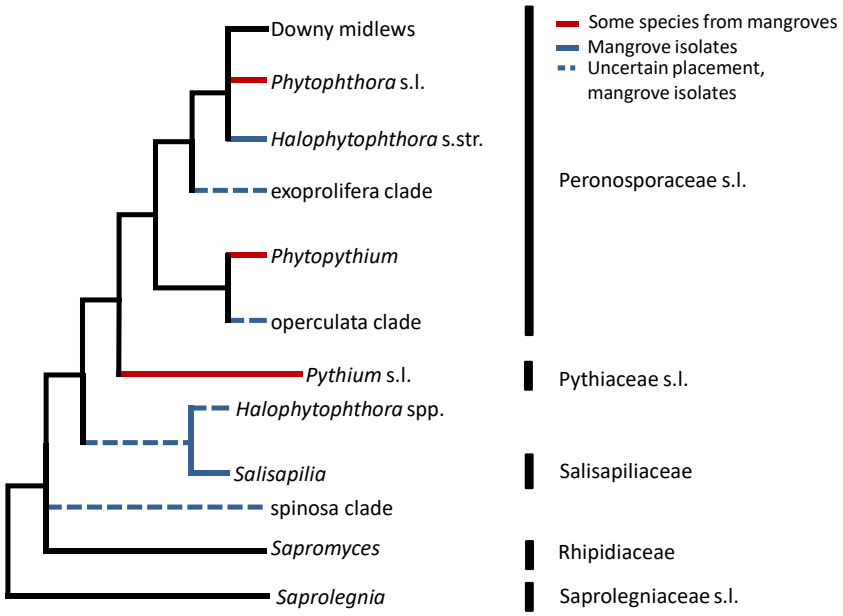

Figure 2. An overview of the phylogeny of estuarine and mangrove oomycetes (after Hulvey et al. 2010, Nigrelli and Thines 2013, Thines 2014, Li et al. 2016)

Apart from downy mildew genera, *Phytopythium* is the only monophyletic taxon. Recently, *Halophytophthora operculata* (basionym, *Phytophthora operculata*) was found to be the sister clade to *Phytopythium*. Another issue observed was that several species of *Halophytophthora* are grouped with *Salisapilia* (e.g. *H. elongata, H. epistomium*) or are forming a separate clade (e.g. *H. exoprolifera*). Salisapiliaceae is a well-established taxon based on the phylogeny of Hulvey et al. (2010) and is composed of three species, *Salisapilia sapeloensis* (type species), *S. nakagirii*, and *S. tartarea*.

Sporangia and Gametangia: Structures for survival and perpetuation

Oomycetes are thriving in different moist biomes due to their ability to colonize substrates by actively swimming towards them, which results in their wide evolutionary success (Thines 2014). Reproductive structures of saprotrophic oomycetes play a role in both colonization and survival (Figure 3).

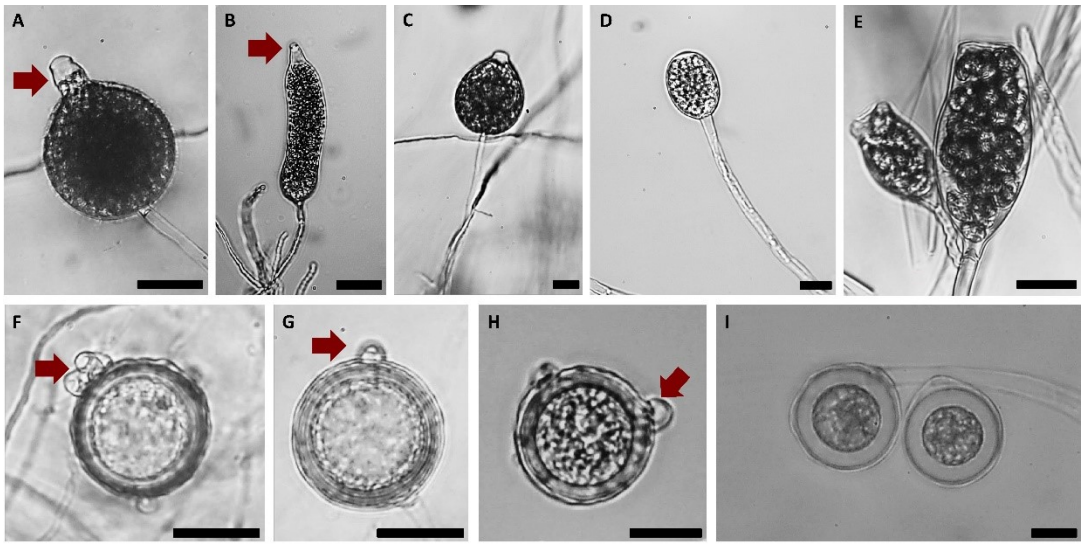

Figure 3. Sporangia of different species of mangrove oomycetes. **A** *Salisapilia nakagirii*. **B** *Halophytophthora elongata*. **C** *Halophytophthora batemanensis*. **D** *Halophytophthora porrigovesica*. **E** *Halophytophthora operculata*. **F–I** Gametangia. **F–H** *Phytopythium* sp. **I** *Phytophthora insolita*. Scale bars = 20 µm

Sporangia (or zoosporangia), which are formed through sporulation, are pouch-like structures of different shapes in which zoospores are formed (Figure 3A – E). The process of sporulation is defined as the formation and development of sporangia until the release of

zoospores. Zoospores are diploid, biflagellate, heterokont cells that are chemotactically attracted to different plant exudates (Leaño et al. 2000). They are formed after mitotic division of the protoplasmic component of sporangia. After complete maturity of zoospores, as triggered by external stimuli, they are released from the sporangia. Once zoospores locate a suitable substrate for colonization, they retract their flagella, encyst, and germinate, forming a germ-tube that will later develop into a dense mycelium (Leaño et al. 2000).

Oomycetes develop oogonia (female) and antheridia (male), which are collectively known as gametangia (Figure 3F – I). Antheridium attachment is a taxonomically informative feature in several genera and is described as either paragynous when the antheridium is on the side of the oogonium or amphigynous when the antheridium surrounds the base of the oogonium. Some species of saprotrophic or hemibiotrophic oomycetes have constricted to roundish antheridia (Bala et al. 2010, de Cock et al. 2015) (Figure 3F – H). The origin of antheridia from vegetative hyphae is a taxonomically valuable character in several groups and is classified as either monoclinous when the antheridium branches from the hypha bearing the oogonium or diclinous when the antheridial hypha is separate from the hypha bearing the oogonium. Oogonia are globose structures containing the oospore. Projections of the wall of oogonia are somehow varied among and within the groups and can be highly ornamented amongst *Pythium* spp. (van der Plaats-Niterink 1981). An oospore forms after fertilization of the oosphere by a nucleus transferred from the antheridium. Oospores are thick-walled resting zygotes and are capable of long-term survival in soil, water, or plant tissue. Once environmental conditions favor germination, the oospore develops a germ tube, and either a new mycelial network is formed or primary zoosporangium, which releases. In heterothallic species, where antheridia and oogonia are formed by distinct mycelia, the presence of both mating types is necessary for oospore formation, whereas in homothallic species sexual reproduction mostly takes place by selfing.

Ecology of mangrove oomycetes

Ecologically, *Halophytophthora* are ubiquitous decomposers living in mangrove leaf litter (Nakagiri 2000, Leaño 2001), driftwoods (Leong et al. 1998; Tan and Leong 1992),

marsh grass litter (Hulvey et al. 2010), and in *Avicennia marina*-trunk cankers and decayed rootlets (Pegg and Foresberg 1981). In Asian countries, especially mangrove leaves are common habitats of oomycetes and can be colonized from the initial to the final stage of leaf decay (Leaño et al. 2000). Of the different mangrove plant genera, *Halophytophthora* spp. were reported to colonize *Avicennia*, *Aegiceras*, *Bruguiera*, *Cerips*, *Rhizophora*, *Sonneratia*, and *Xylocarpus* (Leaño 2001, Nakagiri 2000, Pegg and Forsberg 1981, Tan and Pek 1997). Association between a mangrove species and an oomycetes observed were always saprotrophic, and in current literature no study dealt with the capability of mangrove oomycetes to act as pathogens of mangrove plants. However, *Halophytophthora mycoparasitica* is reported as a mycoparasite of *Pestalotia* sp. and *Penicillium* sp. (Fell and Master 1975).

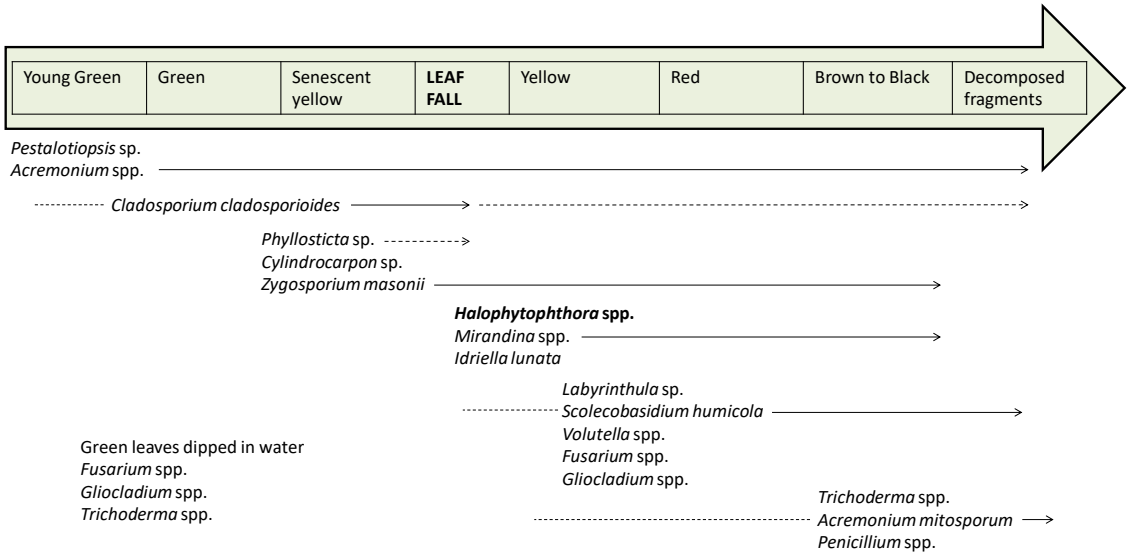

Figure 4. Succession of *Halophytophthora* spp. and other fungal species in a mangrove leaf (Presentation modified from Nakagiri et al. 1989)

Halophytophthora spp. were considered to be the first colonizers of fallen mangrove leaves (Newell et al. 1987, Nakagiri et al. 1989) (Figure 4). These species play an important role as decomposers. Once a senescent mangrove leaf falls into the water system, zoospores of *Halophytophthora* spp. are chemotactically attracted to the leachates and attach itself rapidly on the surface of the substrate for further colonization of the leaf through mycelial extension. For this, several enzymes (e.g. cellulase) are produced by saprotrophic oomycetes to degrade mangrove leaves (Raghukumar et al. 1994). Decomposition of leaf substrates occurs rapidly due to the quick infestation by zoospores

(Nakagiri et al. 1989). Through this process of decomposition as mediated by *Halophytophthora* spp. and other saprotrophic oomycetes, fallen leaves are transformed into a food source for small invertebrates (Fell and Master 1980).

Responses of mangrove oomycetes to abiotic factors (e.g. temperature, salinity, and pH) are comprehensively summarized by Leaño et al. (2000). It was inferred that *Halophytophthora* spp. are well-adapted to a wide range of pH (6.0 – 9.0), salinity (0 – 60 ‰) and temperature (10 – 35°C) and some were able to grow and sporulate throughout most of these ranges. This ability of oomycetes corresponds to the constant fluctuation of abiotic factors in a mangrove ecosystem and is a probable reason for their abundance in such habitats (Leaño et al. 2000).

In line with this, using *H. vesicula* as a model organism, Leaño (2001) attributed the ecological success of *Halophytophthora* spp. in colonizing mangrove leaves were to the abundant zoospore production, wide tolerance to pH, salinity, and temperature, ability to compete against higher fungi in colonizing mangrove leaves, and ability of zoospores to quickly attach firmly to a substrate.

Oomycetes in the Philippines

Oomycete research in the Philippines is relatively young as until recently, only a few species have been recorded in the scientific literature dealing with Philippine ecosystems. Several genera have been reported from the soil, and freshwater and marine environments as saprotrophs (e.g. *Halophytophthora* spp.) or pathogens (e.g. *Phytophthora* spp., *Pythium* spp., *Haliphthoros* spp., downy mildews and white blister rust), but these reports remained mostly anecdotal. After the 1980s, studies on Philippine oomycetes became stagnant with only a few studies carried during this period as compared to neighboring Asian territories (e.g. China, Japan, Taiwan, Thailand).

Increased interest in the study of *Halophytophthora* species started in the 1990s; however, the systematics, distribution, and ecology of *Halophytophthora* in the Philippines are very limited. Leaño (2001) reported the occurrence of *Halophytophthora* in Panay, Philippines, enlisting five species of *Halophytophthora*, namely *H. vesicula*, *Phytopythium*

kandeliae (≡ *H. kandeliae*), *Salisapilia bahamensis* (≡ *Ph. bahamensis*, *H. bahamensis*), *S. epistomium* (≡ *Ph. epistomium*, *H. epistomium*), and *Salispina lobata* (≡ *Ph. spinosa* var. *lobata*, *H. spinosa* var. lobata), and one unidentified *Halophytophthora* species. A decade later this was followed by Antazo et al. (2012) who isolated three unidentified *Halophytophthora* spp. from Marinduque. With this limited number of species in relation to the geographical position and climate of the Philippines, it is assumed that *Halophytophthora* and other marine oomycetes (e.g. *Phytopythium*, *Salisapilia*) are abundant and diverse from known species (in 16 families) of mangroves (Primavera 2000). Further, the pathogenic lifestyle of *Halophytophthora* is yet to be established since these organisms are thought to be facultative phytopathogens (e.g. *H. mycoparasitica* and *H. epistomium*). Table 1 summarizes a non-exclusive list of oomycetes recorded in Philippine literature.

Table 1. Philippine oomycetes recorded in the literature

Species	Isolation source / host / substrate	Family*	Reference
Myzocytium megastomum	Obligate endoparasite, *Cladophora*, *Pithophora*	Incertae sedis	Dogma 1986
	Closterium		Dogma 1975
Myzocytium proliferum	*Spirogyra*	Incertae sedis	Dogma 1975
Salispina lobate (≡ *Phytophthora spinosa* var. *lobata*)	Saprotroph, *X. moluccensis*	Incertae sedis	Leaño 2001
Albugo candida	Obligate pathogen, crucifers	Albuginaceae	Dogma 1986
Albugo ipomoeae-panduratae	Obligate pathogen, *Ipomoea*	Albuginaceae	Dogma 1986
Haliphthoros milfordensis	Pathogen, *Scylla serrata*	Haliphthoraceae	Leaño 2002
Haliphthoros philippinensis	Pathogen, *Penaeus monodon*	Haliphthoraceae	Hatai et al. 1980
	Scylla serrata		Leaño 2002
Lagenidium humanum	Saprotroph, soil	Lagenidiaceae	Dogma 1986

Lagenidium giganteum	Saprotroph, parasite of mosquito larvae	Lagenidiaceae	Dogma 1986
Lagenidium oophilum	Parasite, nematode eggs		
Lagenidium pygmaeum	Saprotroph, soil and freshwater	Lagenidiaceae	Dogma 1986
Leptolegniella keratinophilum	Soil	Leptolegniellaceae	Dogma 1986
	(baited using snake skin, hair)		
Apodachlya minima	soil, saprotroph	Leptomitaceae	Dogma 1986
Leptomitus lacteus	Saprotroph, foul and polluted waters	Leptomitaceae	Dogma 1986
Olpidiopsis karlingae	Parasite, *Karlingia rosea*	Olpidiopsidaceae	Dogma 1986
Olpidiopsis pythii	Parasite, *Pythium intermedium*	Olpidiopsidaceae	Dogma 1975
Olpidiopsis luxurians	Parasite, *Aphanomyces laevis*	Olpidiopsidaceae	Dogma 1986
Halophytophthora bahamensis (≡ *Phytophthora bahamensis*)	Saprotroph, *Avicennia lanata*	Peronosporaceae	Leaño 2001
Halophytophthora epistomium (≡ *Phytophthora epistomium*)	Saprotroph, *Rhizophora apiculata*, *Sonneratia* sp., *Xylocarpus granatum*	Peronosporaceae	Leaño 2001
Halophytophthora vesicula (≡ *Phytophthora vesicula*)	Saprotroph, *Avicennia lanata*, *A. officinalis*, *Ceriops decandra*, *R. apiculata, Sonneratia* sp., *X. granatum*, *X. moluccensis*	Peronosporaceae	Leaño 2001
Phytophthora cactorum	Pathogen, *Theobroma cacao*	Peronosporaceae	Mendiola and Espino 1916
Phytophthora capsica	Pathogen, *Piper nigrum*	Peronosporaceae	Tsao et al. 1994
Phytophthora citrophthora	Pathogen, *Citrus*	Peronosporaceae	Rosario 1968,
	Nephelium, *Sandoricum*		Tsao et al. 1994
Phytophthora colocassiae	Pathogen, *Colocasia esculenta*	Peronosporaceae	Mendiola and Espino 1916
Phytophthora haveae	Pathogen, *Sandoricum koetjape*	Peronosporaceae	Tsao et al. 1994
Phytophthora infestans	Pathogen, *Solanum tuberosum*	Peronosporaceae	Lee 1921
	tomato, potato		Dogma 1986

Phytophthora meadii	Pathogen, *Hevea brasiliensis*	Peronosporaceae	Teodoro 1926
Phytophthora nicotianae	Pathogen, *Piper nigrum*	Peronosporaceae	Tsao et al. 1994
	Citrus		Lee 1921
	Solanum melongena		Ocfemia 1925
	Carica papaya		Quimio and Quimio 1974
	Anonas comosus		Quebral et al. 1962
	Citrullus lunatus		Quimio and Quimio 1974
Phytophthora palmivora	Pathogen, ominivorous	Peronosporaceae	Reinking 1919, Teodoro 1926, Celino 1933
			Ela 1968, Rosario 1968
			Dogma 1986, Tsao et al. 1994, Borines et al. 2014
Phytophthora parasitica (synonym of *P. nicotianae*)	Pathogen, *Cocos*, *Theobroma*, *Gossypium*, *Lycopersicum*, *Solanum*, *Hibiscus*, *Musa*, *Citrus*, *Rheum*, *Lilium*, *Capsicum*, *Ananas*, *Nicotiana*, *Ricinus*, *Grammatophyllum*, *Pollia*, *Catharanthus*, *Manihot*, *Vigna*, *Bryophyllum*	Peronosporaceae	Dogma 1986
Phytophthora phaseoli	Pathogen, *Sandoricum koetjape*	Peronosporaceae	Clara 1928
Phytopythium kandeliae (≡ *Halophytophthora kandeliae*)	Saprotroph, *Avicennia lanata*, *R. apiculata*, *Sonneratia* sp.	Peronosporaceae	Leaño 2001
Bremia lactucae	Obligate pathogen, lettuce	Peronosporaceae	Dogma 1986
Plasmopara parasitica	Obligate pathogen, crucifers	Peronosporaceae	Dogma 1986
Plasmopara viticola	Obligate pathogen, grapes	Peronosporaceae	Dogma 1986

Pseudoperonospora cubensis	Obligate pathogen, cucurbits	Peronosporaceae	Dogma 1986
Peronosclerospora miscanthi (≡ *Sclerophthora miscanthi*)	Obligate pathogen, *Miscanthus*, *Saccharum*, *Sorghum*, corn	Peronosporaceae	Dogma 1986
Pe. philippinensis (≡ *S. philippinensis*)	Obligate pathogen, corn *Saccharum*, *Sorghum*, *Euchlaena*	Peronosporaceae	Weston 1920 Dogma 1986
Pe. sacchari (≡ *S. sacchari*)	Obligate pathogen, sugarcane, *Euchlaena*, *Tripsacum*, *Sorghum*	Peronosporaceae	Dogma 1986
Pe. sorghi (≡ *S. sorghi*)	Obligate pathogen, sorghum, *Euchlaena*, *Heteropogon*, *Panicum*, *Pennisetum*, *Saccharum*, *Dichantium*, *Panicum*	Peronosporaceae	Dogma 1986
Pe. spontanea (≡ *S. spontanea*)	Obligate pathogen, corn, *Saccharum*	Peronosporaceae	Dogma 1986
Pythium aphanidermatum	Pathogen, sugarcane, corn, sorghum, radish, tobacco, *Carica*, cucumber, *Solanum*	Pythiaceae	Dogma 1986
Pythium arrhenomanes	Pathogen, corn, wheat, sugarcane	Pythiaceae	Dogma 1986
Pythium debaryanum	Pathogen, omnivorous species	Pythiaceae	Dogma 1986
Pythium echinulatum	Saprotroph, normally can be pathogenic similar to *Py. debaryanum*, *Py. arrhenomanes*, and *Py. Aphanidermatum*	Pythiaceae	Dogma 1986
Pythium monospermum	Saprotroph, soil and freshwater	Pythiaceae	Dogma 1986
Pythium proliferum	Saprotroph, or pathogen of strawberry	Pythiaceae	Dogma 1986
Pythium torulosum	Saprotroph, freshwater and very moist soils	Pythiaceae	Dogma 1986
Achlya ambisexualis	Freshwater, saprotroph	Saprolegniaceae	Dogma 1986
Achyla Americana	Pathogen, Rice grain rot	Saprolegniaceae	Dogma 1986
Achyla bisexualis	Freshwater and moist soils	Saprolegniaceae	Dogma 1986
Achyla flagellate	Pathogen, Rice grain rot	Saprolegniaceae	Dogma 1986
Achlya proliferoides	Freshwater, saprotroph	Saprolegniaceae	Dogma 1986

Dictyuchus anomalus	Freshwater, soil, saprotroph	Saprolegniaceae	Dogma 1986
Saprolegnia diclina	Pathogen of fishes, extremely rare in the Philippines	Saprolegniaceae	Dogma 1986
Thraustotheca clavate	Saprotroph, highland soils	Saprolegniaceae	Dogma 1986
Aphanomyces cladogamus	Parasite, tomato, spinach, eggplant, lettuce, sugar beets	Verrucalvaceae	Dogma 1986
Aphanomyces helicoides	Freshwater, saprotroph	Verrucalvaceae	Dogma 1986
Aphanomyces keratinophilus	Freshwater, saprotroph	Verrucalvaceae	Dogma 1986
Aphanomyces laevis	Freshwater, saprotroph; or as parasite of some desmids, crayfish, and Philippine milkfish (*Chanos* sp.), catfish and carps	Verrucalvaceae	Dogma 1986
Plectospira gemmifera	Pathogen, sugarcane, moist soils	Verrucalvaceae	Dogma 1986

*based on Beakes and Thines 2017; Entries highlighted with red were reported in the mangrove environment.

Hypothesis

This thesis presents the following research hypotheses:

1) An oomycete isolated from the mangrove environment is a member of the *Halophytophthora* group following the *Phytophthora*-like species concept;
2) *Salisapilia* is not a species-rich group and requires no phylogenetic and morphologic revisions;
3) *Salispina* is a member of the family Rhipidiaceae, Rhipidiales;
4) *Phytopythium* spp. are not thriving in Philippine mangrove leaves; and
5) *Halophytophthora operculata* is a congener of the monophyletic *Phytopythium*.

Objectives of the Research

The central aim of this study is to revise the systematics of saprotrophic oomycetes using classical and molecular phylogenetic approaches and to explore the diversity of mangrove oomycetes in the Philippines. Specifically, this research aims to:

1) Challenge the genus concept of *Phytophthora*-like species in the mangrove environment;
2) Revise the systematics of *Salisapilia*;
3) Determine the phylogenetic placement of *Salispina* in Peronosporomycetes;
4) Identify *Phytopythium* spp. from the Philippine mangrove environment; and
5) Resolve the *Phytopythium – Halophytophora operculata* clade.

Significance of the study

The existing knowledge on this group of oomycetes has been insufficient for the past three or more decades. Few researchers are interested in this group despite the economic and ecological potential of saprotrophic oomycetes. Thus, by bridging the gap from the 80's up to the present time, this study will present the foundation of the newly revised systematics of saprotrophic oomycetes that will serve as a guide for future research – be it in the field of Phylogeny, Taxonomy, and Biotechnology.

Taxonomy. This research is a major step in updating the classification, description, and nomenclature of mangrove oomycetes.

Phylogeny. This research will aid in the establishment of natural groupings for some enigmatic taxa, i.e. *Phytophthora* and *Halophytophthora.* Further, findings generated from this thesis will help in understanding ecological shifts, lifestyle transitions, and thallus organization in oomycetes as key aspects in the phylogeny of the Peronosporalean galaxy.

Biotechnology. There have been several studies that explore the fatty acid components (e.g. Polyunsaturated fatty acids and monounsaturated fatty acids) of saprotrophic oomycetes. Mangrove oomycetes reported in this thesis are potential organisms that can be tested for fatty acid mass production.

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Thesis Publications

Publications 1 and 2: Challenging the genus concept of *Phytophthora*-like species in the mangrove environment

This chapter presents two isolated *Phytophthora* spp. from the Philippines, *Ph. insolita,* and *Ph. elongata*. The data highlights that the previous genus-concept of *Halophytophthora*, as *Phytophthora*-like species from the mangrove environment, is no longer valid as mangroves are seen as a cryptic habitat of different *Phytophthora* species. Manuscripts presented in this chapter are:

Bennett RM, Thines M (2017) Confirmation that *Phytophthora insolita* (Peronosporaceae) is present as a marine saprotroph on mangrove leaves and the first report of the species in the Philippines. Nova Hedwigia, 105:185–196. DOI: https://doi.org/10.1127/nova_hedwigia/2017/0404

Bennett RM, Dedeles GR, Thines M (2017) *Phytophthora elongata* (Peronosporaceae) is present as an estuarine species in Philippine mangroves. Mycosphere, 8:959–967. DOI: 10.5943/mycosphere/8/7/11

Publication 3: The revision of *Salisapilia* (Salisapiliaceae)

This chapter presents the phylogenetic analyses of *Salisapilia*. An emendation of the genus *Salisapilia* and *S. nakagirii* are proposed together with five new combinations and one new species. Further, this chapter presents the taxonomic analyses for Salisapiliaceae.

Bennett RM, Thines M (2019) Revisiting Salisapiliaceae. Fungal Systematics and Evolution, 3:171–184. DOI: doi.org/10.3114/fuse.2019.03.10

Publication 4: A revision of *Salispina*

A new congener for *Salispina, S. hoi,* is herewith presented and the family Salispinaceae is proposed to accommodate *Salispina*. The physiological responses (i.e. sporulation and colony radial growth) of *S. spinosa*, *S. lobata*, and *S. hoi* to reduced oxygen concentration are presented complementary to the morphological and phylogenetic delineation of the proposed taxa.

Bennett RM, Devanadera MK, Dedeles GR, Thines M (2018) A revision of *Salispina*, with its placement in a new family, Salispinaceae (Rhipidiales), and the description of its fourth species, *S. hoi* sp. nov. IMA Fungus, 9(2):259–269.

Publication 5: *Phytopythium* spp. from Philippine mangroves leaves

This chapter presents two new members of *Phytopythium* spp. isolated from Philippine mangrove leaves. The proposed taxa, *Pp. leanoi* and *Pp. dogma,* are the second and third *Phytopythium* spp. reported for the Philippines. The *Pp. kandeliae* species complex is likewise resolved using combined morphological and phylogenetic analyses.

Bennett RM, Nam B, Dedeles GR, Thines M (2017) *Phytopythium leanoi* sp. nov. and *Phytopythium dogmae* sp. nov., *Phytopythium* species associated with mangrove leaf litter from the Philippines. Acta Mycologia. 52(2):1103. DOI: https://doi.org/10.5586/am.1103

Publication 6: The *Phytopythium – Halophytophthora operculata* clade

The phylogenetic and morphological analyses of *H. operculata* and its sister taxon, *Phytopythium* is presented in this chapter. The deep morphological divergence between *H. operculata* and *Phytopythium* strongly supported *Calycofera*, to accommodate *H. operculata,* thus *C. operculata*; and its cryptic species, *C. cryptica* – which was described based on diagnostic nucleotide bases.

Bennett RM, de Cock AWAM, Lévesque CA, Thines M (2017) *Calycofera* gen. nov., an estuarine sister taxon to *Phytopythium*, Peronosporaceae. Mycological Progress. 16:947–945. DOI: 10.1007/s11557-017-1326-9

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Confirmation that *Phytophthora insolita* (Peronosporaceae) is present as a marine saprotroph on mangrove leaves and first report of the species for the Philippines

Reuel M. Bennett[1,2] and Marco Thines[1,2,3,*]

[1] Biodiversity and Climate Research Centre (BiK-F), Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, 60325 Frankfurt am Main, Germany

[2] Department of Biosciences, Institute of Ecology, Evolution and Diversity, Goethe University Frankfurt am Main, Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany

[3] Integrative Fungal Research Cluster (IPF), Georg-Voigt-Str. 14-16, 60325 Frankfurt am Main, Germany

With 3 figures and 3 tables

Abstract: Over the past 10 years evidence has increased that *Phytophthora* species are common in estuarine and coastal environments. These findings made it seem possible that *Phytophthora* spp. might also be present in mangrove habitats of the Philippines. In this manuscript *P. insolita,* an oospore-producing *Phytophthora* species devoid of antheridia, is reported as an additional member of estuarine oomycetes and reported from the Philippines for the first time. The Philippine strain, *P. insolita* H3YB, was isolated from a fallen senescent mangrove leaf in Hinigaran, Negros Occidental, Philippines. The morphology of H3YB and its phylogenetic placement agreed with its identification as *P. insolita*. Further targeted studies into the ecology of *Phytophthora* species in mangroves might shed light on the ecological role of these species in the mangrove ecosystem.

Key words: estuarine ecosystem; *Halophytophthora*, Oomycetes, phylogeny, *Phytophthora*.

Introduction

The genus *Phytophthora* harbours several of the economically most important hemibiotrophic oomycetes. This genus contains more than 100 species and nearly 20 species were isolated from aquatic environments including some unidentified strains (Brasier et al. 2003, Hong et al. 2008, Hüberli et al. 2013, Hulvey et al. 2010, Man In 'T Veld et al. 2011, Reeser et al. 2011, Scibetta et al. 2012). These were either

*****corresponding author: marco.thines@senckenberg.de

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isolated from leaf baits (Hüberli et al. 2013), irrigated agricultural land (Brasier et al. 2003), decayed leaves and seeds of *Zostera marina* (Man In 'T Veld et al. 2011), or mangrove leaf samples (Guo et al. 2016). Pathogenic and saprophytic species of *Phytophthora* require fluid water for reproducing asexually (Hüberli et al. 2013). In the Philippines, *Phytophthora* species were commonly reported on post-harvest fruits and agricultural plantations (Borines et al. 2014). Important species present in the Philippines are *Phytophthora palmivora* on *Artocarpus heterophyllus* (Langka, Tagalog) (Borines et al. 2014), *P. cactorum* on *Theobroma cacao* (Kakaw, Tagalog) (Mendiola & Espino 1916), *P. capsici* on *Piper nigrum* (Paminta, Tagalog) (Tsao et al. 1994), *P. phaseoli* on *Sandoricum koetjape* (Santol, Tagalog) (Clara 1928) and *P. infestans* on *Solanum tuberosum* (Patatas, Tagalog) (Lee 1921).

Another notable group of *Phytophthora*-like organisms are those from the estuarine environment. Initially, some species were classified as *Phytophthora* but most were later classified in the genus *Halophytophthora* (e.g. *H. avicenniae, H. bahamensis, H. batemanensis, H. epistomium, H. mycoparasitica, H. polymorphica, H. vesicula*). These species were separated from the genus *Phytophthora* because of their ecological preference and morphological differences (Ho & Jong 1990). However, with increasing efforts in isolating and describing *Phytophthora* spp. in estuarine and mangrove systems, it becomes apparent that the ecological preference is insufficient to delineate *Halophytophthora* and *Phytophthora*. Recently, isolated strains in an estuarine environment from Brazil were described as *P. estuarina* and *P. rhizophorae* (Guo et al. 2016). Another marine species was described from *Zostera marina* (*P. gemini*) in The Netherlands (Man In'T Veld et al. 2011). *Phytophthora ramorum,* a pathogen of several ornamental plants, was isolated from a brackish creek in Washington in 2010 (Chastagner, unpublished) as mentioned in the report of Preuett et al. (2016). These recent findings highlight the fragmentary knowledge on *Phytophthora* species from saline environments, and it was the aim of the current study to investigate the presence of *Phytophthora* in Philippine mangroves.

Materials and methods

Isolation: Mangrove leaf litter from selected areas in the Philippines was collected and placed in sealed plastic bags. Strips of mangrove litters were blot-dried and laid-over in an agarized vegetable juice (VJ) with Nystatin (500 mg/ml) and Rifampicin (30 mg/ml) or Streptomycin (0.5 mg/ml). Coenocytic hyphae growing at the edge of the leaf strips were cut and placed on new VJ media with antibiotics until axenic. Cultures were maintained at 20°C in VJ with or without antibiotics. Colony patterns were examined on Potato Carrot Agar (PCA, Medium No. 2, NBRC, Japan), Vegetable Juice Agar (VJA, 200 ml clarified Gemüsesaft, Alnatura, Germany in 1 L deionized water with 1.5% Agar) and Potato Dextrose Agar (PDA, Carl Roth GmbH, Karlsruhe, Germany).

Production of sporangia and chlamydospores: Sporangia were induced using 50% soil extract (sterile or non-sterile), distilled water and saline solution at NaCl concentrations of 0, 10, 20, and 30 ppt from 3- to 7-day-old cultures in 60 mm Petri plates incubated in a dark compartment or with continuous light. Alternatively, sesame or hemp seeds were placed on the periphery of a 3- to 7-day-old culture and after hyphal colonization on the seed surface; these were then transferred to a solution of either water, soil extract and saline solution (0–30 ppt) or a combination of each solution. Structures were observed and photos were taken using Canon Digital Camera EOS 500D attached to a Motic® AE31 trinocular microscope (Motic, Wetzlar Germany).

Table 1. Primer combinations used for PCR amplification.

Markers	Primer	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*2	*cox*2-F	GGC AAA TGG GTT TTC AAG ATC C	Hudspeth et al. 2000
	*cox*2-RC4	TGA TTW AYN CCA CAA ATT TCR CTA CAT TG	Choi et al. 2015

DNA extraction, PCR amplification, and phylogenetic inference: The H3YB strain was grown on a clarified VJ agar incubated at room temperature in the dark for 3 to 7 days. Mycelia from the culture media were harvested and genomic DNA extraction was performed following the instruction manuals for the BioSprint 96 Kingfisher flex (Thermo Fisher Scientific, Waltham, Massachusetts, USA) robot and Qiagen DNA (Qiagen GmbH, Hilden, Düsseldorf, Germany) plant tissue extraction kit. PCR amplification of the internal transcribed spacers (ITS), and cytochrome oxidase 2 (*cox*2) were done using the primers listed in Table 1.

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 product. Cycling conditions for ITS were as follows: initial denaturation at 94°C for 4 min, it was then followed by 36 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 20 s, elongation at 72°C for 1 min and final elongation at 72°C for 4 min. Whereas cycling conditions for the *cox2* region were: initial denaturation at 94°C for 4 min, followed by 36 cycles of denaturation at 94 °C for 40 s, annealing at 51°C for 40 s, elongation at 72°C for 40 s, and final elongation at 72°C for 4 min. PCR reactions were carried out on an Eppendorf Mastercycler pro (Eppendorf AG, Hamburg, Germany). Amplified PCR products were sequenced at the Biodiversity and Climate Research Centre (BiK-F) sequencing laboratory using *cox*2-RC4 for *cox*2 and LR0 for the ITS region. Sequences were analyzed, assembled to contigs and edited using Geneious software version 5.0.4 (Biomatters Ltd., USA). Sequences of *P. insolita* were deposited to NCBI as KY076608 for the ITS and KY076609 for *cox*2. Edited contigs in FASTA format were uploaded to TrEase database website (http://www.thines-lab.senckenberg.de/trease/) (Mishra et al., unpublished) for the phylogenetic tree construction following these protocols: BLAST search (Altschul et al. 1990) using the megablast algorithm for automated sequence-fetching and multiple sequence alignment using MAFFT with G-INS-i model (Katoh et al. 2002). In addition, type sequences were manually searched and selected from GenBank (www.ncbi.nlm.nih.gov/genbank/) (Table 3) prior to MAFFT alignment. GenBank accession numbers of obtained sequences are listed in Table 3. Phylogenetic trees were computed using FastTree (Price et al. 2010), RAxML (Stamatakis 2014) and MrBayes (Ronquist et al. 2012), for Minimum Evolution, Maximum Likelihood, and Bayesian inference, respectively.. Specifications for the FastTree program include Generalized Time-Reversible (GTR) as the algorithm for 1,000 bootstrap replicates. The GTR-GAMMA algorithm was used for 1,000 bootstrap replicates in RAxML. For MrBayes, the model 6 (GTR) was chosen to generate 1,000,000 generations, sampling every 10,000th tree, with discarding the first 30% of the sampled trees. Phylogenetic trees, in Newick format were merged using the TrEase webserver. Downloaded files were viewed using MEGA ver. 6 or 7 (Tamura et al. 2013).

Results

Phytophthora insolita H3YB: Growth, Sporulation, and Morphology: The strain H3YB was isolated from mangrove leaf-litter in Hinigaran, Negros Occidental, Philippines. The strain was morphologically identified as *P. insolita* on the basis of production of oogonia and oospores in the absence of antheridia, as well as the morphology of sporangia. The isolate grew well on clarified VJ agar and PCA when incubated at room temperature in a dark compartment. The colony pattern on VJ and

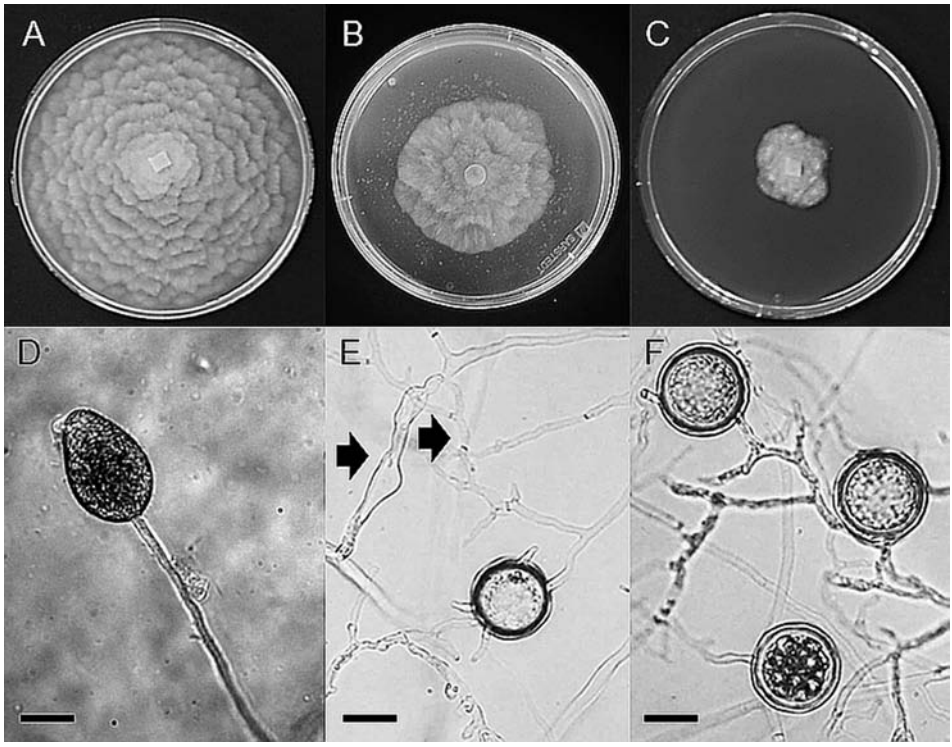

Fig. 1. *Phytophthora insolita* H3YB: colony pattern on (A) Potato Carrot Agar (B) Vegetable Juice Agar, and (C) Potato Dextrose Agar after 7 days of incubation in the dark at room temperature. (D) Sporangium. (E) Oogonium, note the presence of several hyphal outgrowths on the periphery of the oogonium. (F) Developing oogonia. Arrows point to coralloid hyphae and the presence of a septum. Bars = 20 µm.

PCA media was chrysanthemum to petaloid-like (Fig. 1). It grew well within 20–30°C and colony growth stalled at ≥35 °C. Development of zoosporangia and the release of zoospores was only observed when mycelia grown on VJ-agar blocks were incubated in non-sterile soil solution at 20–23°C with continuous light. Sporulation of *P. insolita* H3YB was induced in neither sterile distilled water nor saline solutions at 10 to 30 ppt in either dark or light incubations. The strain H3YB was observed to have a non-caducous, apapillate sporangia and an oogonia with oospore devoid of antheridia. The morphological comparison (sporangia, oogonia and oospores) of different *P. insolita* isolates from recorded literature and the isolate H3YB is presented in Table 2.

Phylogenetic placement of Phytophthora insolita H3YB: *Phytophthora insolita* H3YB, an oospore producing *Phytophthora* devoid of antheridia, is a member of the clade 9 group. Phylogenetically, the *P. insolita* H3YB strain slightly differs from the ex-type strain of *P. insolita* (IMI288805) ITS sequence (Fig. 2). The *cox*2 phylogeny presented a similar topology, with two phylogenetically distinct lineages for different *P. insolita* strains. However, there are no available *cox*2 sequences for the type specimen

Table 2. Summary of the morphological differences of *Phytophthora insolita* isolates based on recorded literature.

Structure	This study	Ann & Ko 1980	Ho et al. 2002	Testa et al. 2005
Hyphae	aseptate, septa forms in older cultures; coralloid or catenulate	aseptate, septa forms at maturity	terminal or intercalary hyphal swellings	*
(Zoo-)Sporangia				
Attachment	terminal	terminal	terminal	*
Shape	ovoid, obpyriform	ovoid	ovoid, obpyriform	*
Papilla	apapillate	apapillate	apapillate	*
Size (µm)	(25–)27–32–37(–40) × (23–)24–26–28(–30) (n = 100)	29–39 × 36–68	(27–)36–51(–62) × (22–)27–39(–51)	34–56 × 22–38
Dehiscence	non-dehiscence or non-caducous	non-caducous	non-caducous	*
Type of Proliferation	nested and external proliferation	nested proliferation	Nested – internal proliferation	*
Zoospores				
Shape	reniform, elliptical or elongated	*	*	*
Development	develops inside the sporangium and release directly through an opening at the apex of the sporangia	develops inside the sporangium	develops inside the sporangium	*
Vesicle	absent	*	*	*
Oogonia				
Shape	globose, smooth walled	spherical, smooth walled to slightly undulating	spherical	*
Attachment	terminal or lateral	terminal	*	*
Diameter (µm)	(30–)32–34–36(–40) (n = 100)	29–36	(27–)29–33(–37)	26–36
Oospore	plerotic, nearly plerotic	nearly plerotic	plerotic	*
Shape	spherical	spherical, thick walled	spherical, thick walled	*
Diameter (µm)	(25–)26–28–30(–32) (n = 100)	27–31	26–33	22–30
Antheridia	absent, not observed	absent, not observed	not observed	*
Chlamydospores	present, globose	present	*	*

* not mentioned in the corresponding study

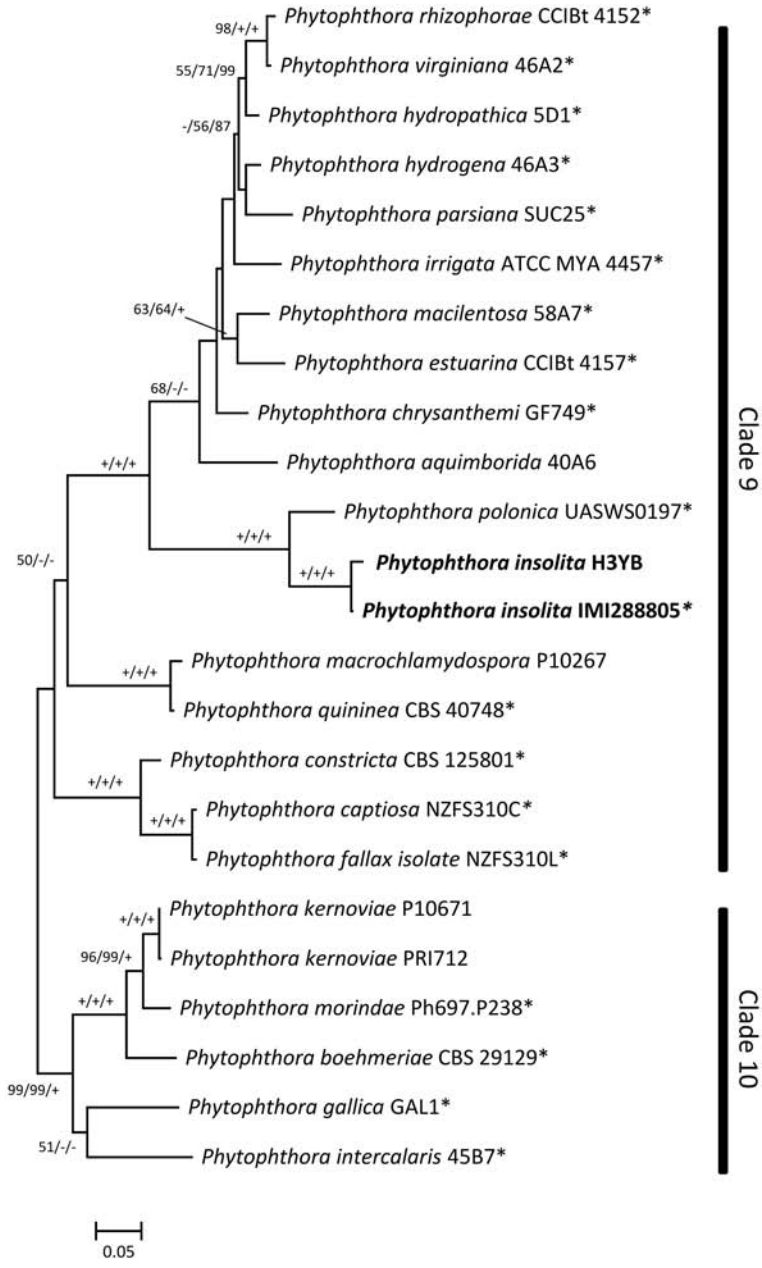

Fig. 2. Phylogenetic tree from Maximum Likelihood inference based on ITS sequences of published *Phytophthora* spp. and *P. insolita* H3YB with support from Maximum Likelihood (RAxML) and Minimum Evolution (FastTree) bootstrap replicates, as well as Bayesian posterior probabilities (MrBayes), in the respective order. Scale bar indicates the number of substitutions per site; (-) indicates an alternate, unsupported or weakly supported topology; (+) maximum bootstrap support; (*) ex-type specimen.

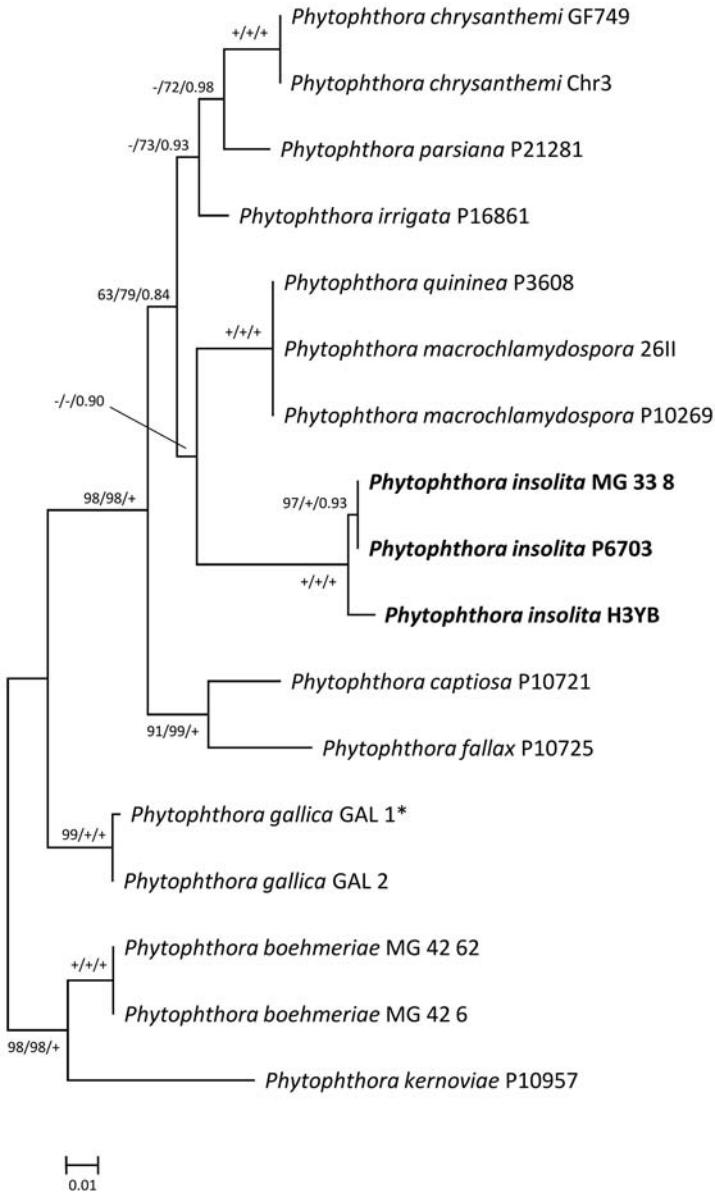

Fig. 3. Phylogenetic tree from Maximum Likelihood (RAxML) based on *cox*2 sequences with support from Maximum Likelihood (RAxML) and Minimum Evolution (FastTree) bootstrap replicates, as well as Bayesian posterior probabilities (MrBayes), in the respective order. Scale bar indicates the number of substitutions per site; (-) indicates an alternate, unsupported or weakly supported topology; (+) maximum bootstrap support; (*) ex-type specimen.

Table 3. GenBank accession numbers of different *Phytophthora* spp. used in the phylogenetic analyses.

Species name	ITS	*cox*2
P. aquimborida	FJ666127	
P. boehmeriae	HQ643149	DQ365736, DQ365736
P. captiosa	DQ297402	JF771334
P. chrysanthemi	AB437135	AB465510, AB465509
P. constricta	HQ013225	
P. estuarina	KT886034	
P. fallax	DQ297391	HM534966
P. gallica	DQ286726	EF192239, EF192238
P. hydrogena	KC249959	
P. hydropathica	EU583793	
P. insolita	GU993897	DQ365744, HM534993
P. intercalaris	KT163268	
P. irrigata	FJ196758	HM534963
P. kernoviae	HQ261604, HQ643261	JF771502
P. macilentosa	KF192700	
P. macrochlamydospora	HQ261606	JF771508, KP070629
P. morindae	FJ469147	
P. parsiana	AY659739	HM535007
P. polonica	DQ396409	
P. quininea	HQ643338	JF771559
P. rhizophorae	KT886031	
P. virginiana	KC295544	

P. insolita IMI288805, so it cannot be inferred, if the group distinct from H3YB would also contain the ex-type strain. The strain H3YB grouped with *P. insolita* strains MG338 and P6703 with strong bootstrap support for all analyses (Fig. 3).

Discussion

The morphology of *P. insolita* H3YB is similar to all reported *P. insolita* isolates, including the type specimen (Ann & Ko 1980). Similarly, when the ITS and *cox2* barcodes of clade 9 species were subjected to barcode gap analyses, using the Automated Barcoding Gap Discovery program (Puillandre et al. 2012), H3YB was suggested to be *P. insolita.* Lastly, blast search of H3YB strain using *cox*2 barcode resulted in 99% similarity to *P. insolita*.

Phytophthora insolita is unique among *Phytophthora* spp. since it is capable of producing oospores in the absence of antheridia. There have been reports on the occurrence of parthenogenic oospores, as described by Ho et al. (2002) among strains of *P. infestans* and *P. fragariae* (Pethybridge & Murphy 1913, Savage et al. 1968). However, this seems to happen only rarely in culture. Oospores of *P. insolita* were

observed in cultures after 1 month when incubated at room temperature in the dark. This was similar to the findings of Ho et al. (2002) where it was reported that oogonia developed after 1 to 5 months of incubation at 25°C in the dark. Further, Ann & Ko (1980) reported that 20% vegetable juice with a final pH of 7.4 was best for oospore production and that no oospores were produced using 40% vegetable juice with a low pH (pH 4.4).

Production of sporangia and release of zoospores was more difficult to induce for the H3YB strain compared to literature on other *P. insolita* isolates. Ann & Ko (1980) achieved sporulation after 24 h incubation at 25°C in light by simply placing an agar block with mycelia in a 20 ml sterile distilled water. Ho et al. (2002) achieved similar results when mycelial agar blocks were placed in sterile distilled water incubated at room temperature under indoor lighting. The H3YB strain did not sporulate in different saline solutions and sterile distilled water, but only in a non-sterile soil extract solution. This probably gave a more natural environment for the strain to induce the development of sporangia and eventual release of zoospores.

Phylogenetically, the genus *Phytophthora* is composed of 10 clades based on several gene analyses (Cooke et al. 2000, Martin et al. 2014). Of the 10 clades of *Phytophthora* s.l., clade 9 is interesting since this group is composed of species with apapillate, non-caducous and internally-nested proliferating sporangia. Further Yang et al. (2014) inferred that members of this group were able to tolerate up until 40°C. Members of the clade 9 group were either isolated from soil samples, plant substrates and irrigation areas, and most were considered as pathogens of several plant species. However, *P. estuarina* and *P. rhizophorae* were isolated from a mangrove site in Brazil which then expands the ecological preference for this clade.

In a survey of *Phytophthora* spp. in Hainan Island, Zeng et al. (2009) were able to isolate a *Phytophthora* sp. that showed similarity to asexual strains of *P. insolita* on *Rhizophora* sp. leaves submerged in seawater. However, the identity of the isolate could not be validated due to the absence of oogonia and oospores. Based on the current study it could be confirmed that *P. insolita* is present in mangrove ecosystems, it seems likely that the assumption of Zeng et al. (2009) regarding the identity of their *Phytophthora* sp. strain was correct. Based on recorded literature, *P. insolita* was found in Taiwan (Ann & Ko 1980, 1994), Hainan Island, China (Ho et al. 2002), Nagpur, India (Bawage et al. 2013, Das et al. 2012), Ohio, USA (Testa et al. 2005), and Hinigaran, Negros Occidental, Philippines (this study). *Phytophthora insolita* was recorded to infect Citrus (Ann & Ko 1980, Bawage et al. 2013, Das et al. 2012), *Rhododendron* (Testa et al. 2005), *Euphorbia pulcherrima* (Ann & Ko 1994), *Nymphaea tetragona*, *Solanum melongena* and *Vatica mangachapoi* (Zeng et al. 2009), but was collected as a saprotroph in decaying mangrove leaf litter in the Philippines in this study.

In the report of Preuett et al. (2016) on the effect of salinity on the survival, growth and sporulation of *P. ramorum*, it was construed that *P. ramorum* can survive in a wide range of salinity and could infect a variety of plant species (e.g. *Ilex vomitoria*, *Parthenocissus quinquefolia*, *Magnolia virginiana*, and *Magnolia grandiflora*) that have some tolerance to salt. Ecologically, it is early to infer if there is a shift of preference among freshwater or soil-dwelling *Phytophthora* species (e. g. *P. insolita*, *P. inundata*,

and *P. ramorum*) to marine or estuarine environments or if this is a plesiomorphic trait. However, Duniway (1979) inferred that several *Phytophthora* spp. have a tolerance to salinity which could possibly explain the occurrence of *P. insolita*, *P. inundata* and *P. ramorum* on the estuarine or marine environment. The current accounts regarding the occurrence of *Phytophthora* spp. in saline environments suggest a greater diversity of *Phytophthora* spp. in such habitats currently known. This is in line with Man In 'T Veld et al. (2011) who noted that the occurrence of *Phytophthora* spp. in marine environments received little attention and the low number of species known from the marine environment is probably due to a low sampling frequency in marine habitats. In the light of the finding that *P. insolita* was found as the 5th species of *Phytophthora* in the marine environment, it seems warranted to direct more attention to brackish and coastal environments to investigate the ecology and distribution of *Phytophthora* species. Based on the findings of the present study and previous findings (Man In't Veld et al. 2011; Guo et al. 2016) it seems possible that many *Phytophthora* species have additional reservoirs in these environments or are specialised to these, with their ecological roles awaiting exploration.

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

Bennett RM[1,2,*], Dedeles GR[4], and Thines M[1,2,3]

[1]*Senckenberg Biodiversity and Climate Research Centre, Senckenberg Gesellschaft für Naturforschung, Senclenberganlage 25, 60325 Frankfurt am Main*
[2]*Department of Biosciences, Institute of Ecology, Evolution and Diverstiy, Goethe University Frankfurt am Main, Max-von-Laue-Straße 9, 60438 Frankfurt am Main, Germany*
[3]*Integrative Fungal Research Cluster (IPF), Georg-Voigt-Straße 14-16, 60325 Frankfurt am Main, Germany*
[4]*UST Collection of Microbial Strains, Research Centre for the Natural and Applied Sciences, University of Santo Tomas, Manila, Philippines*

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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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Corresponding Author: Reuel M. Bennett – Reuel.Bennett@senckenberg.de, reuelmbennett@gmail.com

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.

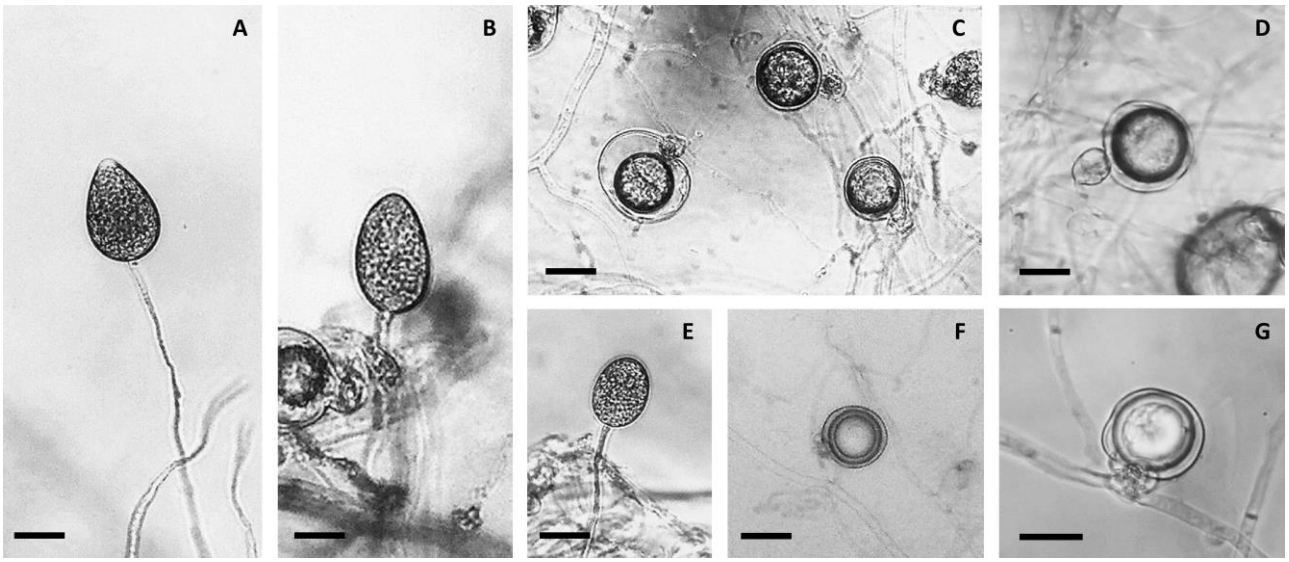

Figure 1 – *Phytophthora elongata.* BMYL-1217. A, B, E Sporangia. C, D, F, G Gametangia. Note the presence of the paragynous antheridia; Oospores, aplerotic. Scale bars = 20 µm.

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.

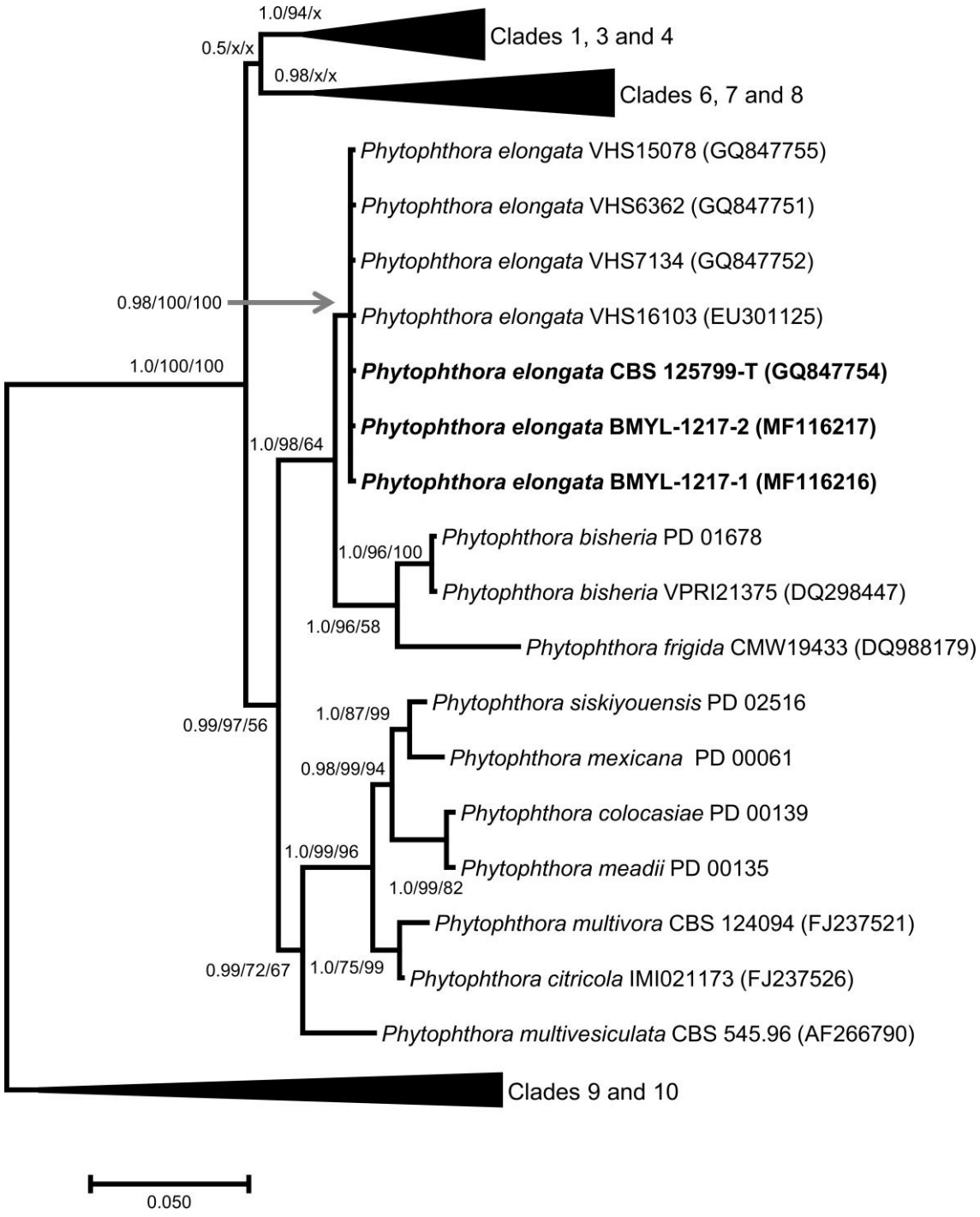

Figure 2 – ITS-based Bayesian phylogenetic inference of the genus *Phytophthora*. Support values from Bayesian inference, Maximum Likelihood, and Minimum Evolution, in the respective order. CBS 125799 (= VHS13482) is the ex-type culture of *P. elongata*, while the strains BMYL-1217-1 and BMYL-1217-2 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.

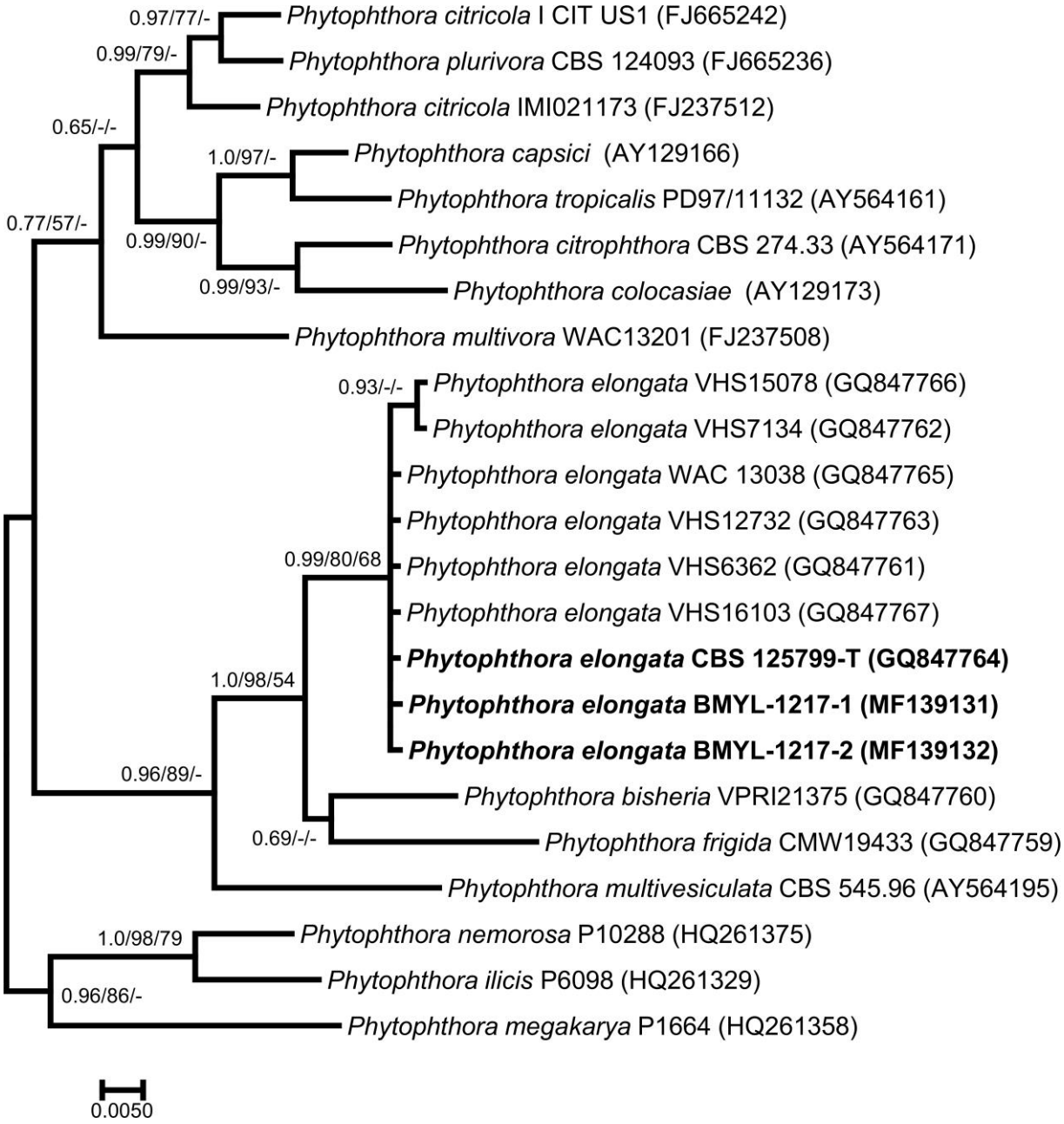

Figure 3 – Bayesian phylogenetic reconstruction of Clade 2 of *Phytophthora sensu lato* based on *cox*1 sequences with support values from Bayesian inference, Maximum Likelihood, and Minimum Evolution, in the respective order. CBS 125799 (= VHS13482) is the ex-type culture of *P. elongata*, while the 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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Revisiting *Salisapiliaceae*

R.M. Bennett[1,2], M. Thines[1,2,3*]

[1]Senckenberg Biodiversity and Climate Research Centre (SBik-F), Senckenberganlage 25, 60325 Frankfurt am Main, Germany
[2]Department of Biological Sciences, Institute of Ecology, Evolution and Diversity, Goethe University Frankfurt am Main, Max-von-Laue-Str. 9, 60438, Frankfurt am Main, Germany
[3]Integrative Fungal Research Cluster (IPF), Georg-Voight-Str. 14-16, 60325 Frankfurt am Main, Germany

*Corresponding author: m.thines@thines-lab.eu

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Abstract: Of the diverse lineages of the Phylum *Oomycota*, saprotrophic oomycetes from the salt marsh and mangrove habitats are still understudied, despite their ecological importance. *Salisapiliaceae*, a monophyletic and monogeneric taxon of the marine and estuarine oomycetes, was introduced to accommodate species with a protruding hyaline apical plug, small hyphal diameter and lack of vesicle formation during zoospore release. At the time of description of *Salisapilia*, only few species of *Halophytophthora*, an ecologically similar, phylogenetically heterogeneous genus from which *Salisapilia* was segregated, were included. In this study, a revision of the genus *Salisapilia* is presented, and five new combinations (*S. bahamensis*, *S. elongata*, *S. epistomia*, *S. masteri*, and *S. mycoparasitica*) and one new species (*S. coffeyi*) are proposed. Further, the species description of *S. nakagirii* is emended for some exceptional morphological and developmental characteristics. A key to the genus *Salisapilia* is provided and its generic circumscription and character evolution in cultivable *Peronosporales* are discussed.

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INTRODUCTION

The Phylum *Oomycota* is a monophyletic group of fungal-like eukaryotes of the Kingdom *Straminipila* (Beakes & Thines 2017). Members of this group are saprotrophs, pathogens, or parasites of various plant and animal species in both aquatic and terrestrial environments. Habitats in which oomycetes seem to play a major role are the mangrove and salt marshes (Marano *et al.* 2016). Fallen senescent leaves of mangrove and salt marsh plants have proven to be rich in oomycete decomposers, which were originally subsumed as estuarine or marine *Phytophthora* (Fell & Master 1975, Pegg & Alcorn 1982, Nakagiri *et al.* 1989). Based on their environmental preference, they were later assigned to a morphologically diverse genus of their own, *Halophytophthora* (Ho & Jong 1990).

Halophytophthora was found to be polyphyletic on the basis of phylogenetic studies (Hulvey *et al.* 2010, Lara & Belbahri 2011). Based on recent phylogenetic analyses, there are only five known species of the *Halophytophthora s. str.*, namely *H. vesicula* (the type species of the genus), *H. avicenniae*, *H. batemanensis*, *H. polymorphica* (Hulvey *et al.* 2010, Lara & Belbahri 2011, Nigrelli & Thines 2013, Marano *et al.* 2014, Thines 2014), and the freshwater isolate, *H. fluviatilis* (Yang & Hong 2014) – the only known congener to date which was isolated from a freshwater biome. A few species of *Halophytophthora* were transferred to *Phytopythium* (*Phytopythium kandeliae*, basionym: *H. kandeliae*) (Thines 2014), and *Salispina* (*Salispina lobata*, basionym: *Phytophthora spinosa* var. *lobata*, and *Salispina spinosa*, basionym: *Phytophthora spinosa* var. *spinosa*) (Li *et al.* 2016); whereas some species were either associated with *Phytophthora* or *Salisapilia*, or are forming separate lineages (Li *et al.* 2016, Marano *et al.* 2014, Jung *et al*. 2017).

The genus *Salisapilia*, which type species, *Salisapilia sapeloensis*, was isolated from *Spartina alterniflora*, was described based on the following features contrasting to *Halophytophthora*: a small hyphal diameter, the formation of an apical or protruding hyaline plug, the absence of an evanescent or persistent vesicle during zoospore release, and homothallism. However, *Salisapilia nakagirii*, a homothallic species described by Hulvey *et al.* (2010), did not develop sporangia under the cultivation conditions applied, so the description of this species was based only on the morphology of gametangia and its phylogenetic placement within *Salisapiliaceae*. However, Hulvey *et al*. (2010) included only a small fraction of the species described in *Halophytophthora* in their dataset. Thus, it cannot be ruled out that several lineages not strongly supported as nested within *Halophytophthora* (Lara & Belbahri 2011) represent members of the genus *Salisapilia*. It was the aim of this study to close this knowledge gap by detailed phylogenetic and morphological analyses.

MATERIALS AND METHODS

Acquisition of strains and sporulation

Ex-type strains of *Halophytophthora* and *Salisapilia* were either acquired from NBRC in Japan or the Westerdijk Fungal Biodiversity Institute (formerly CBS-KNAW) in the Netherlands. Strains were

cultivated and maintained on clarified-vegetable juice agar (VJA) (Medium No. 15 NBRC, using Alnatura Gemüsesaft or Campbell V8 Juice) (http://www.nite.go.jp/en/nbrc/cultures/media/culture-list-e.html) with or without antibiotics: Nystatin (500 mg/mL), as well as Rifampicin (30 mg/mL) or Streptomycin (0.5 mg/mL).

All strains used in this study were tested for sporulation in saline solution at 0, 10, 20 and 30 promille (w/v) from 3–7-d-old cultures in 60 mm Petri plates. Plates were incubated in the dark at room temperature for 18–24 h or until sporangia were formed. Morphological characteristics were observed using a Motic AE31 trinocular inverted microscope (Motic, Wetzlar, Germany) and photos were taken using a Canon Digital Camera EOS 500D (Canon, Tokyo, Japan). Isolates were also grown on agarised media: Potato Carrot Agar (PCA), Peptone Yeast Glucose Agar (PYGA) and Potato Dextrose Agar (PDA) at room temperature (~20–25 °C) (Crous *et al.* 2009).

DNA extraction, PCR, and phylogenetic reconstruction

Cultures were grown on VJA plates at room temperature in a dark compartment. After 7–10 d, mycelia were harvested and subjected to DNA extraction following the method outlined in Bennett *et al*. (2017a). Extracted genomic DNA for all samples was amplified by PCR for the internal transcribed spacers (ITS), and the large nuclear ribosomal subunit (LSU). The primers ITS1-O (Bachofer 2004) and LR0 (Moncalvo *et al.* 1995) were used for the ITS region, while LR0R (Moncalvo *et al*. 1995) and LR6-O (Riethmüller *et al*. 2002) were used for the LSU region.

The 25 µL PCR reaction mixes contained 1× PCR Buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.8 µg bovine serum albumin, 0.4 µM of each primer, 0.5 U *Taq* polymerase and 10–50 ng of DNA. Cycling conditions for the ITS included an initial denaturation at 94 °C for 4 min, followed by 36 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 60 s; and a final elongation at 72 °C for 4 min. For the LSU region, initial denaturation was set at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 53 °C for 20 s, and elongation at 72 °C for 2 min; and a final elongation at 72 °C for 7 min. All amplification reactions were carried out in an Eppendorf Mastercycler Pro equipped with a vapoprotect lid (Eppendorf AG, Hamburg, Germany).

PCR amplicons were sequenced by the laboratory centre of the Senckenberg Biodiversity and Climate Research Centre (SBiK-F, Frankfurt am Main, Germany) using the primer used in PCR. Sequences were analysed, assembled into contigs, and edited using Geneious v. 5.0.4 (Biomatters Ltd., USA). Edited contigs in FASTA format and ex-type sequences from the NCBI (https://www.ncbi.nlm.nih.gov/nuccore) and the *Phytophthora* database (http://www.phytophthoradb.org/) (Table S1) were uploaded to the TrEase webserver (http://www.thines-lab.senckenberg.de/trease/) for multiple sequence alignment using MAFFT, version 7 (Katoh *et al.* 2002). A primary phylogenetic tree computation using Minimum Evolution (ME) was generated using FastTree, version 1 (Price *et al.* 2009) as implemented on the TrEase webserver following the Generalized Time-Reversible (GTR) algorithm and 1 000 bootstrap replicates. Maximum Likelihood (ML) inference was done as the secondary tree using the FastTree, version 2 (Price *et al.* 2010) with the GTR algorithm model and 1 000 bootstrap replicates. A third phylogenetic reconstruction was done using Bayesian Inference (BI) as implemented in the TrEase webserver using MrBayes, version 3.2 (Ronquist *et al.* 2012). For Bayesian analysis the 6-GTR substitution model was used and 1 M generations were run, with trees sampled at every 10 000th generation, discarding the first 30 % of the sampled trees to ensure sampling always reached the stationary phase. After checking that there were no supported conflicts between the datasets, alignments of each locus were concatenated into a single alignment file using SequenceMatrix (Vaidya *et al.* 2010) and phylogenetic trees of concatenated alignments were generated following the above-mentioned protocols. Phylogenetic trees were viewed using MEGA v. 6 or 7 (Tamura *et al*. 2013).

Ancestral state reconstruction for papilla and hyaline apical plug

The ancestral state reconstruction of the papilla and the hyaline apical plug was done using observed or recorded characteristics for *Halophytophthora* (Anastasiou & Churchland 1969, Gerrettson-Cornel & Simpson 1984), *Salisapilia* (Table 1), and other members of *Peronosporaceae* (e.g. *Phytophthora*, *Phytopythium*, and *Pythium*) (van der Plaats-Niterink 1981, de Cock *et al.* 1987, Paul 1987, Erwin & Ribeiro 1996, Paul *et al*. 1999, Paul 2000, Nechwatal & Oßwald 2003, Uzuhashi *et al*. 2010, Kroon *et al.* 2012, de Cock *et al.* 2015). The traits were mapped on the Bayesian phylogeny of the concatenated dataset using Mesquite v. 3.2, and the likelihood ancestral reconstruction algorithm (Maddison & Maddison 2018) was run using the following the character data: (0) Papilla (P) forming an apical plug (AP); (1) P not forming an AP; (2) Semi-papilla (SP) forming an AP; (3) SP not forming an AP; (4) Non-papillate; and, "?" when sporangial germination was neither reported nor observed.

RESULTS

Phylogenetic reconstructions

According to the phylogeny based on concatenated sequences of ITS and LSU in this study (Fig. 1), strains *Halophytophthora bahamensis* NBRC 32556 (Fig. 2), the strain NBRC 32557 (Fig. 4), which was named as *H. bahamensis*, but is not conspecific with the ex-type strain, *H. elongata* NBRC 100786 (Fig. 3), *H. epistomia* NBRC 32617 (Fig. 5), *H. masteri* NBRC 32604 (Fig. 6), and *H. mycoparasitica* NBRC 32966 (= NBRC 32967) (Fig. 7) clustered with other members of the *Salisapiliaceae* with strong to maximum support (Fig. 1). Further, these strains were distinct from *S. nakagirii* LT6456 (= CBS 127947) (Fig. 8), *S. sapeloensis* LT6440 (= CBS 127946) (Fig. 9), and *S. tartarea* CBS 208.95 (Fig. 10).

Morphology

Halophytophthora bahamensis NBRC 32556 (Fig. 2E–F), *H. elongata* NBRC 100786 (Fig. 4C–D), *H. epistomia* NBRC 32617 (Fig. 5E–F), *H. masteri* NBRC 32604 (Fig. 6C–D), and *H. mycoparasitica* NBRC 32966 (= NBRC 32967) (Fig. 7E–F), were all forming a distinct hyaline apical plug at the apex of the discharge tube similar to *S. sapeloensis* CBS 127946 (Fig. 9E) and *S. tartarea* CBS 208.95 (Fig. 10E–F). The apical hyaline plug was indistinct in *S. nakagirii* CBS 127947 (Fig. 8F–G). The shape of sporangia varied among species. The mode of zoospore release was either directly through a discharge tube or by the formation

Table 1. Morphological comparison of *Salisapilia* spp. Measurements for sporangia are given as (min.–)average_minus_SD–SD–average_plus_SD(–max.).

Structure	***S. sapeloensis*** (Hulvey *et al*. 2010)	***S. coffeyi*** (This study)	***S. bahamensis*** (Fell & Master 1975)	***S. elongata*** (Ho *et al*. 2003)	***S. epistomia*** (Fell & Master 1975, Ho *et al*. 1990[a])	***S. nakagirii*** (Hulvey *et al*. 2010, This study[b])	***S. masteri*** (Nakagiri *et al*. 1994)	***S. mycoparasitica*** (Fell & Master 1975)	***S. tartarea*** (Nakagiri *et al*. 1994)
Hyphal diam (µm)	1–2	1–3	1–3	3–9	2–4	1–2	2–10	2–9	1–3(–9)
Septa	Occurs at maturity	Occurs at maturity	Occurs at maturity	Occurs at maturity	Occurs at maturity	Occurs at maturity	Occurs at maturity	Develop numerous septa with age	Non-septate, or septate with age
Branching pattern	Branched or unbranched	Branched or unbranched	Branching, rare	Unbranched	Branching, rare	Branched or unbranched	Branched or unbranched	Branched or unbranched	Unbranched or branched
Sporangiogenic hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae	Undifferentiated to vegetative hyphae
Sporangia									
Size (µm)	34–97 (av. 59)	44.05–107.33 × 6.51–16.92 (av. 74.01 × 10.32)	26–119 × 19–43 (av. 61 × 28) 39–97 × 14–31 (av. 68 × 23)	115–530 × 32–64	43–184 × 56–107 (av. 127.6 × 63.3)	81.5–205.25 × 32.25–113 (av. 136.88 × 66.43) [b]	26–92 × 18–91 (av. 64 × 62.6)	26–131 × 14–111 (av. 82 × 61)	20–104 × 18–96 (av. 55.6 × 47.6)
Discharge tube size (µm)	6–18	4.81–13 × 2.58–3.94 (av. 9.07 × 3.12)	3–7	-	10–51 × 9–10	6.18–18.02 × 4.3–8.7 (av. 11.95 × 6.63) [b]	5–28 × 6–10	Av. 22, tapering	10–22 × 4–8
Apical plug size (µm)	3–8, protruding	~1–3	1–2 (width)	10 × 5.6	14–90 × 9–10	Indistinct [b]	5–24 × 5–14	5–15 × 3–10	11–29 × 5–8
Surface	Smooth, partly rough	Smooth	Smooth	Smooth	Smooth	Non-smooth [b]	Smooth	Denticulate, few spines	Smooth
Vacuole	Absent	Present	Present	Absent	Absent	Absent [b]	Absent	Absent	Absent
Basal plug	Present in some, hyaline	Present, hyaline	Present, hyaline	Present, hyaline	Present, hyaline	Present, hyaline [b]	Present, hyaline	Present, hyaline	Present, hyaline
Detachment	Non-caducous	Non-caducous	Non-caducous	Non-caducous	Non-caducous	Non-caducous [b]	Non-caducous	Non-caducous	Non-caducous
Shape	Ovoid, obpyriform	Bursiform to often narrowly bursiform, obpyriform to narrowly-elongate and obclavate; Setiform appendages, absent	Highly variable, bursiform, multi-lobed, obclavate, obpyriform; Setiform appendages, present, aseptate to septate	Obovoid, obclavate, bursiform, cylindrical, elongated	Lageniform, obpyriform	Ovoid, globose, obpyriform [b]	Spherical, ovoid, obpyriform	Obnapiform	Spherical, ovoid to obpyriform

Table 1. (Continued).

Structure	***S. sapeloensis*** (Hulvey *et al*. 2010)	***S. coffeyi*** (This study)	***S. bahamensis*** (Fell & Master 1975)	***S. elongata*** (Ho *et al*. 2003)	***S. epistomia*** (Fell & Master 1975, Ho *et al*. 1990[a])	***S. nakagirii*** (Hulvey *et al*. 2010, This study[b])	***S. masteri*** (Nakagiri *et al*. 1994)	***S. mycoparasitica*** (Fell & Master 1975)	***S. tartarea*** (Nakagiri *et al*. 1994)
Zoospore release	Zoospores exit through the discharge tube after ejection of the apical plug	Zoospores exit through the discharge tube after ejection of the apical plug	Zoospores exit through the discharge tube after ejection of the apical plug	Zoospores exit through the discharge tube after ejection of the apical plug	Zoospores exit through the discharge tube after ejection of the apical plug	Zoospores are released in a vase-like discharge vesicle [b]	The apical plug is extruded, and a tubular vesicle is ejected. Zoospores exit through the opening	Zoospores exit through the discharge tube after ejection of the apical plug. Plug evanesces rapidly.	Zoospores exit through the discharge tube after ejection of the apical plug
Vesicle	Absent	Absent	Absent	Present, tubular	Absent	Present, vase-like [b]	Present, tubular	Absent	Absent
Oogonia									
Size (µm)	35–60, 49	Not observed	Not observed	Not observed	34–40, 37 [a]	33–48, 39	Not observed	Not observed	33–66
Surface	Smooth				Smooth [a]	Smooth			Smooth
Shape	Spherical, ovoid				Spherical [a]	Spherical			Spherical, tapered base
Oospore	Plerotic	Not observed	Not observed	Not observed	Plerotic [a]	Plerotic	Not observed	Not observed	Aplerotic
Size (µm)	28–56, 48				- [a]	28–44			24–62
Wall (µm)	2–9				4–5 [a]	1–7			3–10
Antheridia	Paragynous	Not observed	Not observed	Not observed	Paragynous [a]	Paragynous	Not observed	Not observed	Diclinous, paragynous
Size (µm)	2–9				6–24 × 2–8, 12–6 [a]	3–10			4–10
Shape	Simple, lobed or branched				- [a]	Club-shaped, lobed			Partly enwraps oogonia

- no data provided.

[a] Data from Ho *et al.* (1990).

[b] Characteristics of *S. nakagirii* observed in this study.

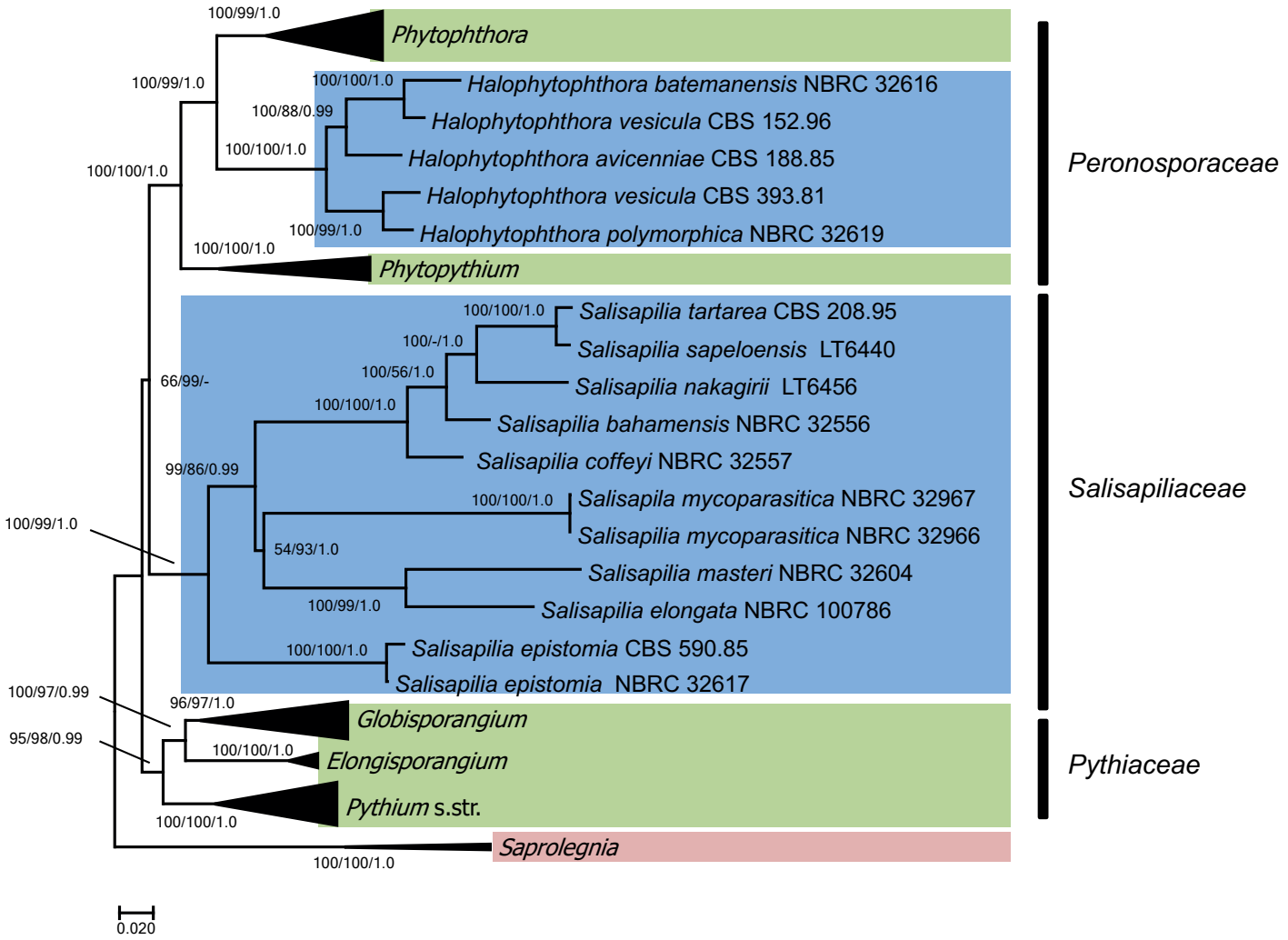

Fig. 1. Phylogenetic tree based on concatenated ITS and LSU alignments based on Minimum Evolution (ME) inference, with bootstrap support values from ME and Maximum Likelihood, as well as posterior probabilities from Bayesian Inference, in the respective order. (-) indicates support below 50 % (bootstrap) or 0.8 (posterior probability), or alternating but not strongly supported topology (support below 70 % bootstrap or 0.9 posterior probability). The scale bar indicates the number of nucleotide substitutions per site.

of an evanescent vesicle. A summary of the morphology of *Salisapilia* spp. is presented in Table 1.

The shape of sporangia of NBRC 32557 (Fig. 3E–H) was different from the ex-type culture of *H. bahamensis* (NBRC 32556) to which it had been assigned. The sporangia of the ex-type culture were bursiform, obclavate, obpyriform to highly variable and multi-lobed; whereas strain NBRC 32557 has narrowly bursiform, obpyriform to narrowly-elongated and obclavate sporangia. Variation of the shape of the sporangium was pronounced for *H. bahamensis* NBRC 32556, and some sporangia bore two discharge tubes. In contrast, NBRC 32557 always formed a single discharge tube and its sporangial shape was more stable. The strain NBRC 32557 releases its zoospore after extrusion of the small hyaline apical plug from the discharge tube. Zoospores are released directly out from the discharge pore and a vesicle was absent. After the sporangia had released zoospores, an umbonate or elevated basal plug was observed. Gametangia and chlamydospores were not observed for the strain NBRC 32557.

The ancestral trait reconstruction (Fig. 11) of the papilla and hyaline apical plug suggested that papillate and semi-papillate sporangia were putatively derived from non-papillate sporangia. Further, a sporangium with papilla forming a discharge tube and a hyaline apical plug appeared to be a synapomorphic trait for *Salisapilia*.

Taxonomy

Based on the presented phylogenetic and morphological analyses of the different taxa included in this study, the genus *Salisapilia* contains several additional species previously treated as members of *Halophytophthora*. As a consequence, five new combinations (i.e. *S. bahamensis*, *S. elongata*, *S. epistomia*, *S. masteri*, and *S. mycoparasitica*) and a new species (*S. coffeyi*) for the genus *Salisapilia* are introduced here. Measurements for sporangia are given as (min.–)average_minus_SD–SD–average_plus_SD(–max.).

Salisapilia Hulvey *et al.*, *Persoonia* **25**: 112 (2010), ***emend.*** MycoBank MB517465.

Colonies on VJ agar stellate, indistinct, petalloid; *aerial hyphae* limited; *vegetative hyphae* with regular branching, septae occur at maturity; *hyphal swellings* present, shape variable; *sporangia* produced in saline water, shape obpyriform, ovate, obovate, elongate to irregular; *proliferation* often external; *dehiscence or*

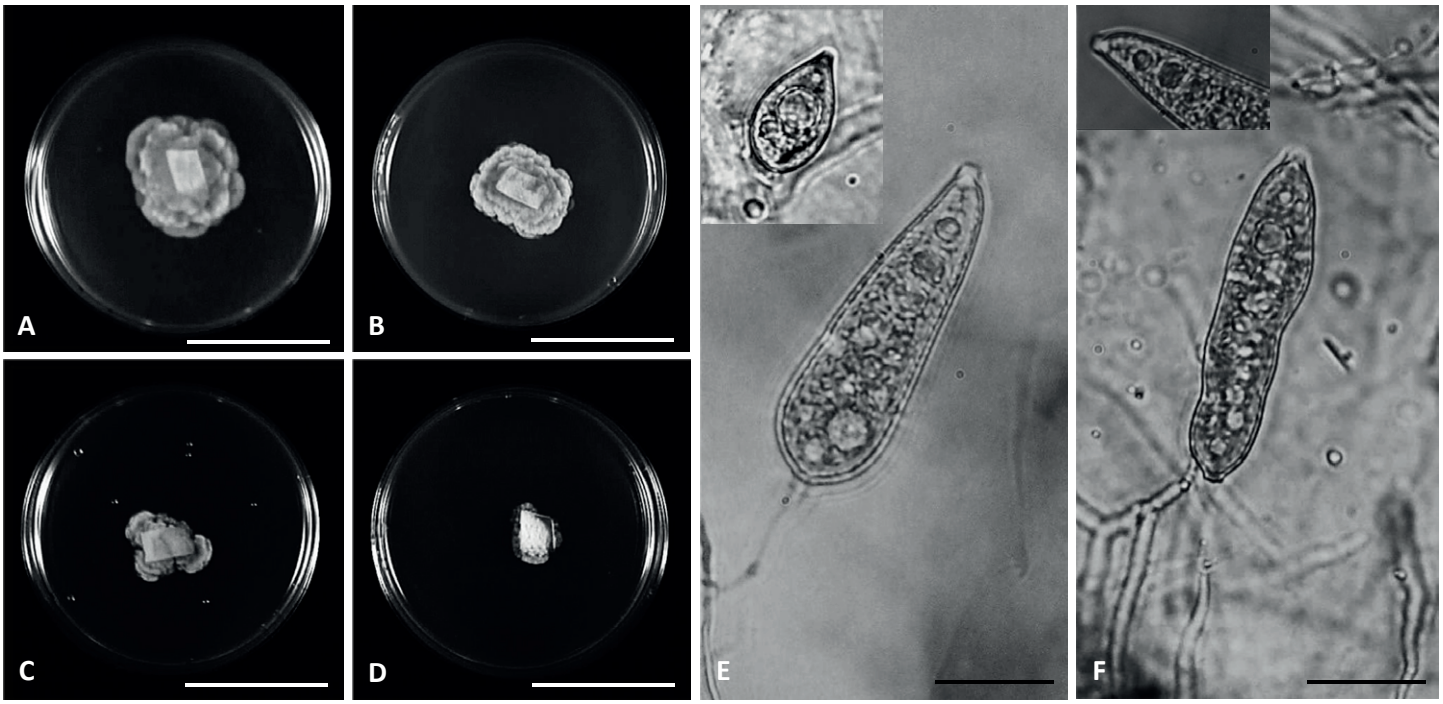

Fig. 2. *Salisapilia bahamensis* NBRC 32256. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Peptone yeast glucose agar. **D.** Potato dextrose agar. **E, F.** Mature, vacuolated sporangia, (inset figure, sporangium showing hyaline apical plug). Scale bars: A–D. = 30 mm, E, F. = 20 µm.

discharge tube present, usually with a hyaline plug at the apex; *zoospore release* occurs after dehiscence of the hyaline plug at the apex of the discharge tube, or zoospores exit directly from the discharge pore or through an evanescent tubular to vase-like vesicle; *gametangia* observed for some species; *antheridial attachment* paragynous, diclinous; *oogonia* smooth-walled; *oospores* spherical to ovoid, terminal or intercalary.

Type species: *Salisapilia sapeloenesis* Hulvey *et al*.

Synopsis of species included in *Salisapilia*

Salisapilia bahamensis (Fell & Master) R. Bennett & Thines, ***comb. nov*.** MycoBank MB823448. Fig. 2.

Basionym: *Phytophthora bahamensis* Fell & Master, *Canad. J. Bot*. **53**: 2913. 1975. MB320472.
Synonym: *Halophytophthora bahamensis* (Fell & Master) Ho & Jong, *Mycotaxon* **36**: 381. 1990. MB126014.

Typus: **Holotype** ATCC 28296, cultures ex-type = CBS 586.85 = IMI 330182 = NBRC 32556 , voucher ex ex-type strain NBRC3256 = USTH 014147, University of Santo Tomas Herbarium, Manila, Philippines.

Distribution: Bahamas, Philippines.

Salisapilia coffeyi R. Bennett & Thines, ***sp. nov*.** MycoBank MB823342. Fig. 3.

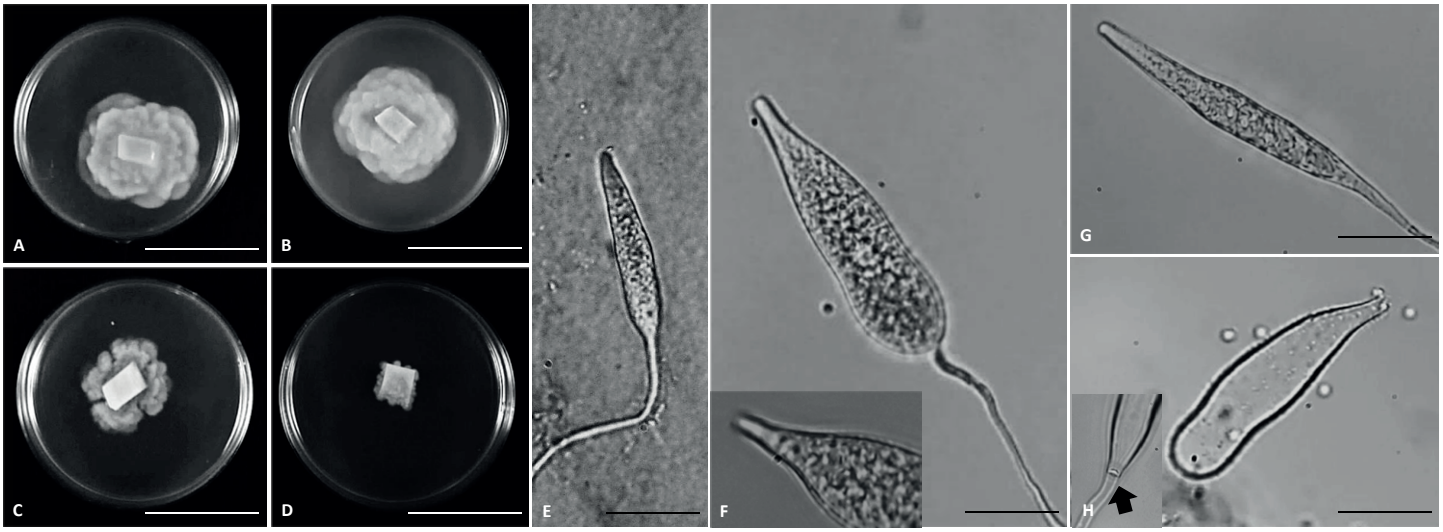

Fig. 3. *Salisapilia coffeyi* NBRC 32557. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Peptone yeast glucose agar. **D.** Potato dextrose agar. **E.** Immature sporangium. **F, G.** Mature sporangia, (inset figure) sporangium showing hyaline apical plug. **H.** Empty sporangium; inset, elevated or umbonate basal plug. Scale bars: A–D = 30 mm, E–H = 20 µm.

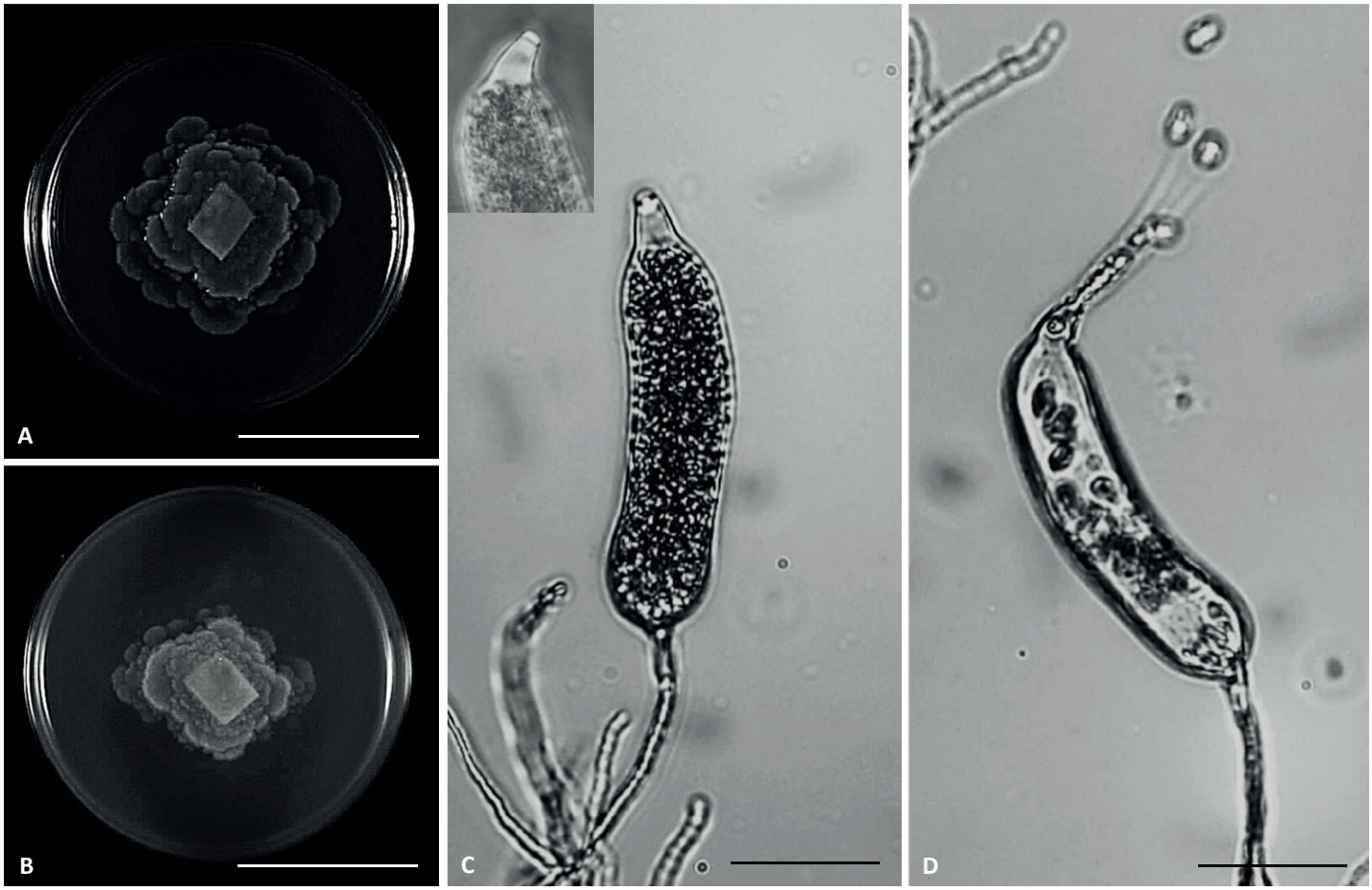

Fig. 4. *Salisapilia elongata* NBRC 100786. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Mature sporangium; hyaline apical plug (inset). **D.** Mature sporangium releasing zoospores through a tubular vesicle. Scale bars: A, B = 30 mm, C, D. = 20 µm.

Etymology: Dedicated to Michael Coffey for his contributions to the study of cultivable oomycetes.

Colony pattern on vegetable juice agar and potato carrot agar petaloid to rosette-like; *vegetative hyphae* highly branched, with septae at maturity; *sporangiogenic hyphae* undifferentiated from vegetative hyphae, bearing a single sporangium; *sporangia* smooth and thin-walled, with vacuoles, non-deciduous, (25.5–) 44–74–107(–126) × (4–)6.5–10.5–17(–19.5) µm, bursiform, narrowly bursiform, obpyriform to narrowly-elongate and obclavate, mostly with a tapering apex; *dehiscence tube* present; 5–13 × 2.5–4 µm; *dehiscence plug* present, hyaline, 1–3 µm in diameter; *basal plug* present, hyaline, raised to umbonate; *proliferation* external; *zoospore release* directly through the dehiscence tube after ejection of the dehiscence plug; *vesicle* not observed; *chlamydospores* not observed; *gametangia* not observed.

Typus: **Bahamas**, Conception Island, isolated from decaying leaf of *Rhizophora mangle*, Oct. 1972, *J.W. Fell & I.M. Master* (**holotype** USTH 014149, ex-type culture NBRC 32557, GenBank: ITS, MF979510; LSU, MF979503).

Salisapilia elongata (Ho & Chang) R. Bennett & Thines, ***comb. nov.*** MycoBank MB823450. Fig. 4.
Basionym: *Halophytophthora elongata* Ho & Chang, *Mycotaxon* **85**: 417. 2003. MB372647.

Typus: **Holotype** 17II2001, Y.M. Ju, Institute of Botany, Academica Sinica, Taipei, Taiwan, cultures ex-type BCRC 33983 = NBRC 100786.

Distribution: Taiwan, Philippines.

Salisapilia epistomia (Fell & Master) R. Bennett & Thines, ***comb. nov.*** MycoBank MB823449. Fig. 5.
Basionym: *Phytophthora epistomium* Fell & Master, *Canad. J. Bot*. **53**: 2913. 1975. MB320475.
Synonym: *Halophytophthora epistomia* (Fell & Master) Ho & Jong, *Abstracts IMC-4*, Regensburg, 1990. MB126016.

Typus: **Holotype** ATCC 28293, cultures ex-type IMI 330183 = CBS 590.85 = NBRC 32617, voucher ex ex-type strain NBRC32617 = USTH 014147, University of Santo Tomas Herbarium, Manila, Philippines.

Distribution: USA.

Salisapilia masteri (Nakagiri & Newell) R. Bennett & Thines, ***comb. nov.*** MycoBank MB823447. Fig. 6.
Basionym: *Halophytophthora masteri* Nakagiri & Newell, *Mycoscience* **35**: 227. 1994. MB363473.

Typus: **Holotype** NBRC H-12169, NITE Biological Resource Center, Japan, cultures ex-type IFO 32604 = ATCC 96906 = CBS 207.95 = NBRC 32604.

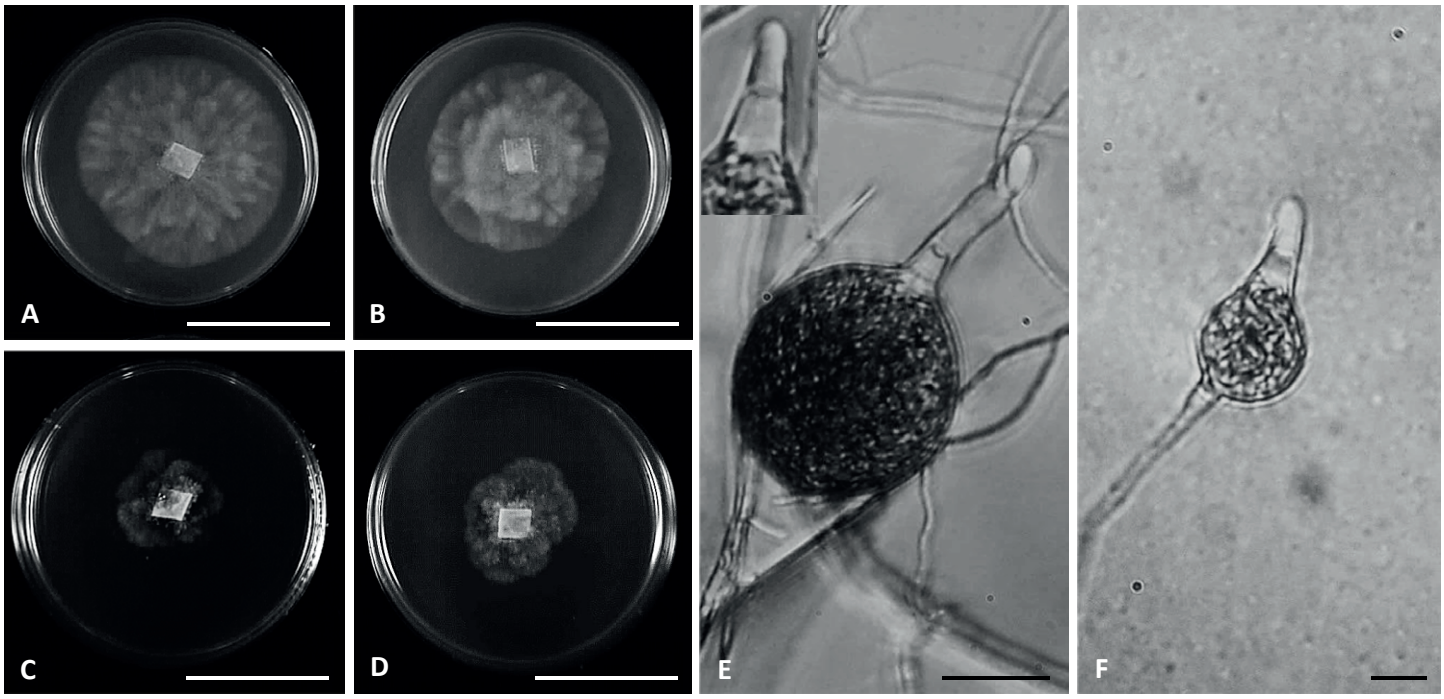

Fig. 5. *Salisapilia epistomia* NBRC 32617. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Peptone yeast glucose agar. **D.** Potato dextrose agar. **E–F.** Mature sporangia; hyaline apical plug (inset, Fig. 4E). Scale bars: A–D. = 30 mm, E, F. = 20 µm.

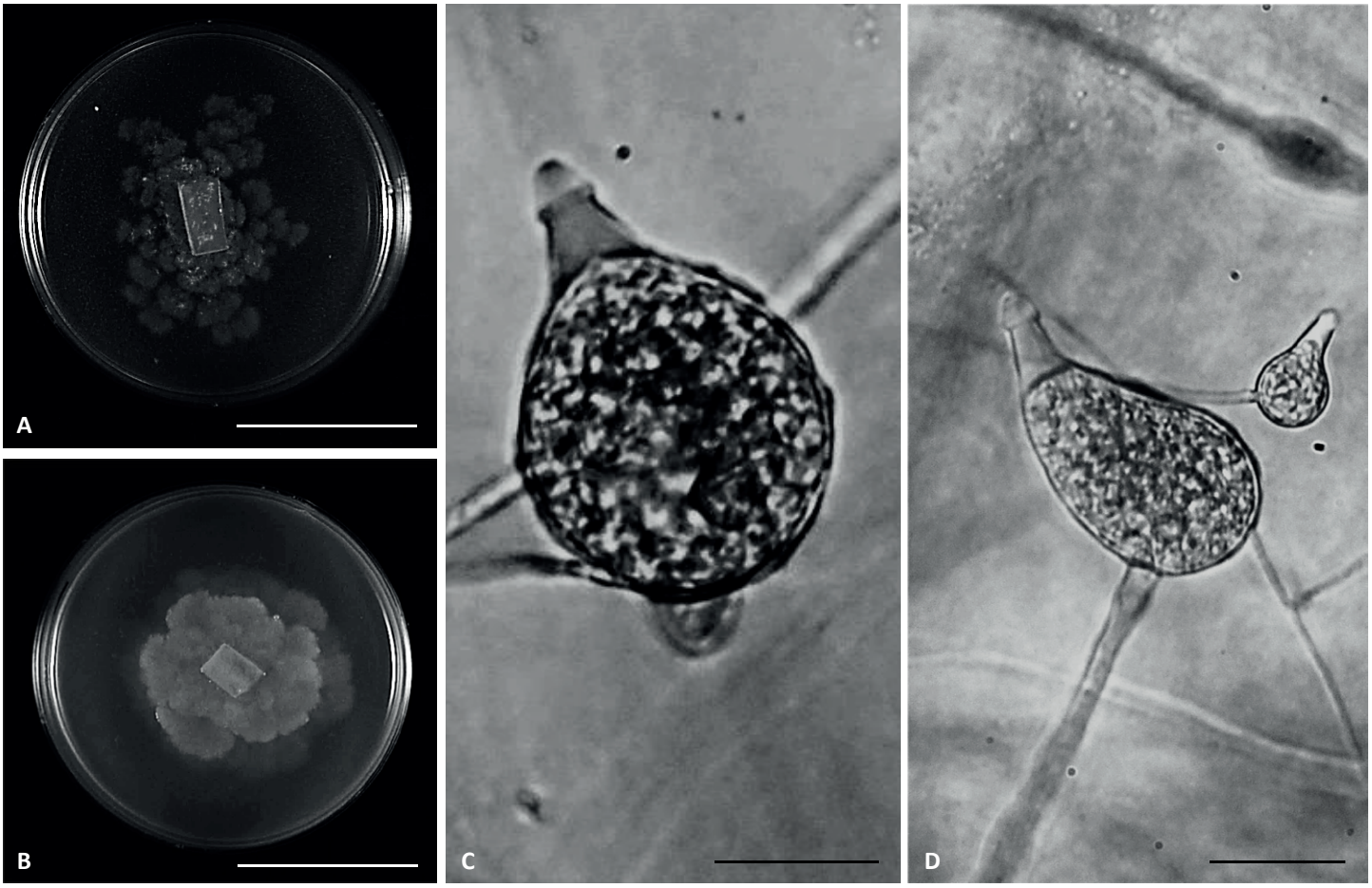

Fig. 6. *Salisapilia masteri* NBRC 32604. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C, D.** Mature sporangia. Scale bars: A, B = 30 mm, C, D = 20 µm.

Distribution: Bahamas.

Salisapilia mycoparasitica (Fell & Master) R. Bennett & Thines, ***comb. nov.*** MycoBank MB824539. Fig. 7.

Basionym: *Phytophthora mycoparasitica* Fell & Master, *Canad. J. Bot.* **53**: 2916. 1975. MB320485.
Synonym: *Halophytophthora mycoparasitica* (Fell & Master) Ho & Jong, *Mycotaxon* **36**: 381. 1990. MB126017.

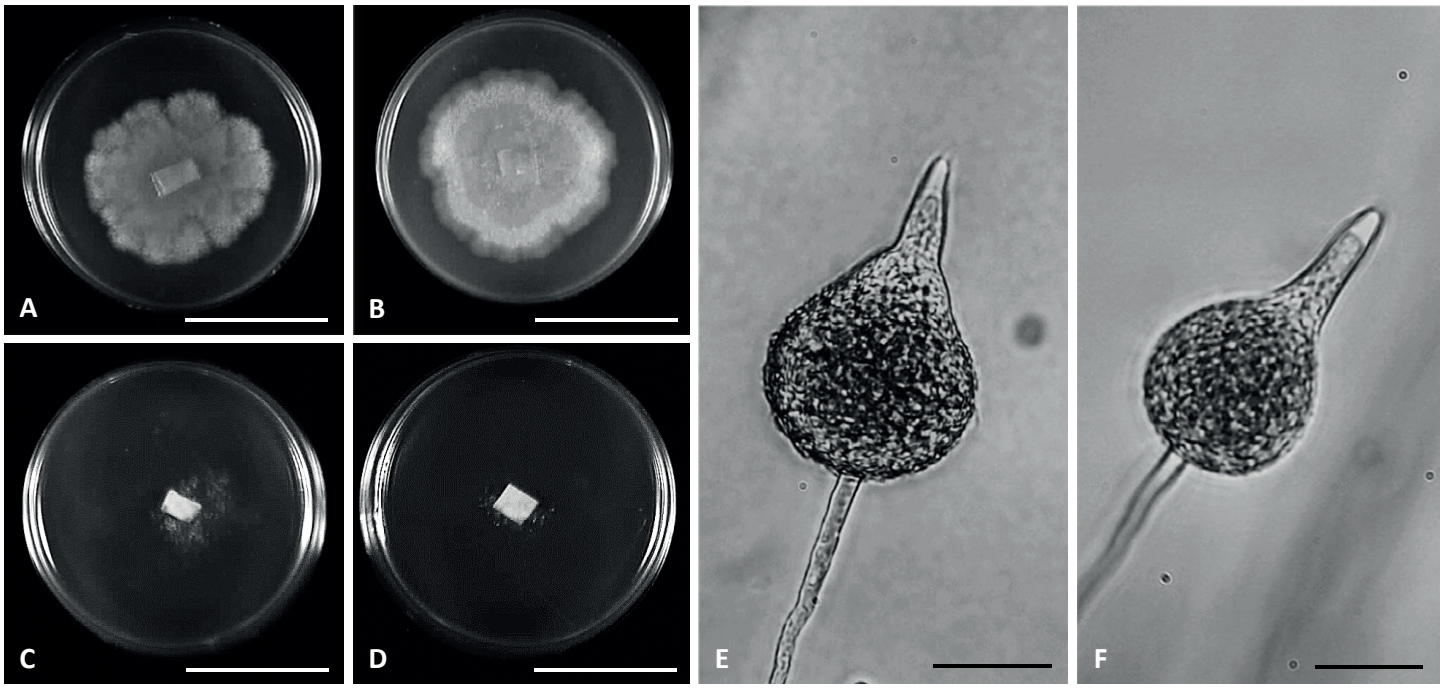

Fig. 7. *Salisapilia mycoparasitica* NBRC 32966. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Peptone yeast glucose agar. **D.** Potato dextrose agar. **E, F.** Mature sporangia. Scale bars: A–D = 30 mm, E–F = 20 µm.

Typus: **Holotype** ATCC 28292 (discarded), (**lectotype** designated here fig. 16, *Canad. J. Bot.* **53**: 2918 (1975), MBT386266; **epitype** designated here NBRC H-12221, MBT386249, ex-epitype culture NBRC 32966, NITE Bioresource Centre, Japan).

Other materials examined: NBRC 32967, NITE Bioresource Centre, Tokyo Japan.

Distribution: Malaysia, Japan.

Notes: The designated type, ATCC 28292, is no longer available, and no additional specimen was deposited in any recognised fungarium at the time *Phytophthora mycoparasitica* was proposed. Since neither inactive nor living material appears to remain from the collection of Fell & Master (1975), fig. 16 from that publication is designated as the **lectotype**, the specimen NBRC H-12221 is designated as the **epitype** and NBRC 32996 (NBRC, Japan) as the **ex-epitype culture**.

Salisapilia nakagirii Hulvey *et al.*, *Persoonia* **25**: 113. 2010, ***emend.*** MycoBank MB517466. Fig. 8.

Typus: **Holotype** CBS H-20478, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands, ex-type cultures CBS 127947 = NBRC 108757 = LT6456.

Distribution: USA.

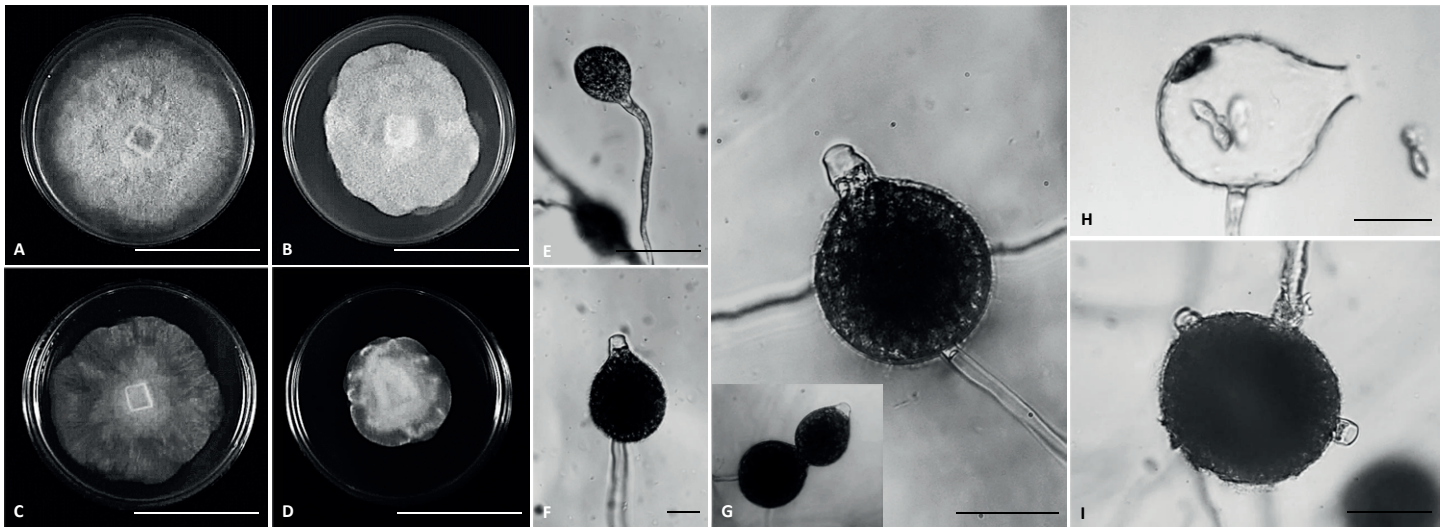

Fig. 8. *Salisapilia nakagirii* CBS 127947. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Peptone yeast glucose agar. **D.** Potato dextrose agar. **E.** Immature sporangium. **F–I.** Mature sporangia, (inset figure) modified shape of a sporangium. **H.** Empty sporangium. **I.** Mature sporangium with two discharge tubes. Scale bars: A–D = 30 mm, E–I = 20 µm.

Colony pattern on Vegetable juice agar and potato carrot agar indistinct; stellate to rosette-like on peptone yeast agar; *hyphae* branched with *septae* at maturity; *sporangia* ovoid, globose to obpyriform, (26–)81.5–137–205(–231) × (11.5–)32–66.5–113(–133.5) µm; *dehiscence tube* present, filled with non-sporogenous protoplasmic mass, size 6–18.0 × 4.5–8.5 µm; *hyaline apical plug* indistinct; *sporangial wall* wrinkled in some sporangia; *basal plug* present in few sporangia; *proliferation* external; *zoospore release* through an evanescent vesicle; *vesicle* vase-shaped; *gametangia* present; *antheridia* diclinous, paragynous, club-shaped or lobed, 3–10 µm in length; *oogonia* hyaline, spherical, 33–48 µm; *oospores* 28–44 µm, hyaline, with a uniformly refractile ooplast vacuole; wall 1–7 µm.

Salisapilia sapeloenesis Hulvey *et al*., *Persoonia* **25**: 113. 2010. MycoBank MB517467. Fig. 9.

Typus: **Holotype** CBS H-20477, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands, ex-type cultures NBRC 108756 = LT6440 = CBS 127946.

Distribution: USA.

Salisapilia tartarea (Nakagiri & Newell) Hulvey, Nigrelli, Telle, Lamour & Thines, ***comb. nov.*** MycoBank MB517468. Fig. 10.
Basionym: *Halophytophthora tartarea* Nakagiri & Newell, *Mycoscience* **35**: 224. 1994. MB363474.
Synonym: Salisapilia tartarea (Nakagiri & S.Y. Newell) Hulvey *et al*., *Persoonia* **25**: 114. 2010. Nom. inval., Art. 41.5 (Melbourne).

Typus: **Holotype** NBRC H-12168, NITE Biological Resource Center, Japan, ex-type cultures NBRC 32606 = ATCC 96905 = CBS 208.95.

Distribution: USA.

Note: Invalidly proposed in *Persoonia* **25**: 114 (2010), as the date of publication of the basionym was omitted.

DISCUSSION

Estuarine and saltmarsh oomycetes are a diverse group of heterokonts that recently received much attention. Members of this ecological group are in the genera *Halophytophthora* (Ho & Jong 1990), *Phytopythium* (Bala *et al.* 2010), *Salisapilia* (Hulvey *et al*. 2010), *Salispina* (Li *et al*. 2016), and *Calycofera* (Bennett *et al.* 2017b). Of these taxa, *Halophytophthora* and *Salisapilia* were regarded to be in need of taxonomic revision (Nigrelli & Thines 2013, Marano *et al.* 2014, Beakes & Thines 2017), and the latter genus was resolved in this study.

Members of the monogeneric *Salisapiliaceae* are characterised by a small hyphal diameter, a protruding hyaline apical plug, and the absence of a vesicle during zoospore release (Hulvey *et al*. 2010). However, the sporangia of *S. nakagirii* CBS 127947 were reported to release zoospores into a semi-persistent vesicle and that the typical hyaline apical plug was absent (Marano *et al.* 2014). These observations are largely confirmed in this study, demonstrating that *S. nakagirii* has an exceptional mode of sporulation, even though we classify the vesicle as evanescent, as the structure is not readily observable sometime after zoospore release. Marano *et al.* (2014) reached the conclusion that *S. nakagirii* is papillate; however, it appears that the discharge tube is rather filled with some non-sporogenous mass, which is protoplasmic of origin, and its distalmost part is probably homologous to the apical plug observed in other species of *Salisapilia*, giving the impression of a papilla (Gerrettson-Cornell & Simpson 1984).

Hulvey *et al*. (2010) suggested that the intricacies of zoospore release might be of phylogenetic relevance and, thus, useful for resolving some systematic complexities of saprotrophic oomycetes. However, the example of *S. nakagirii*,

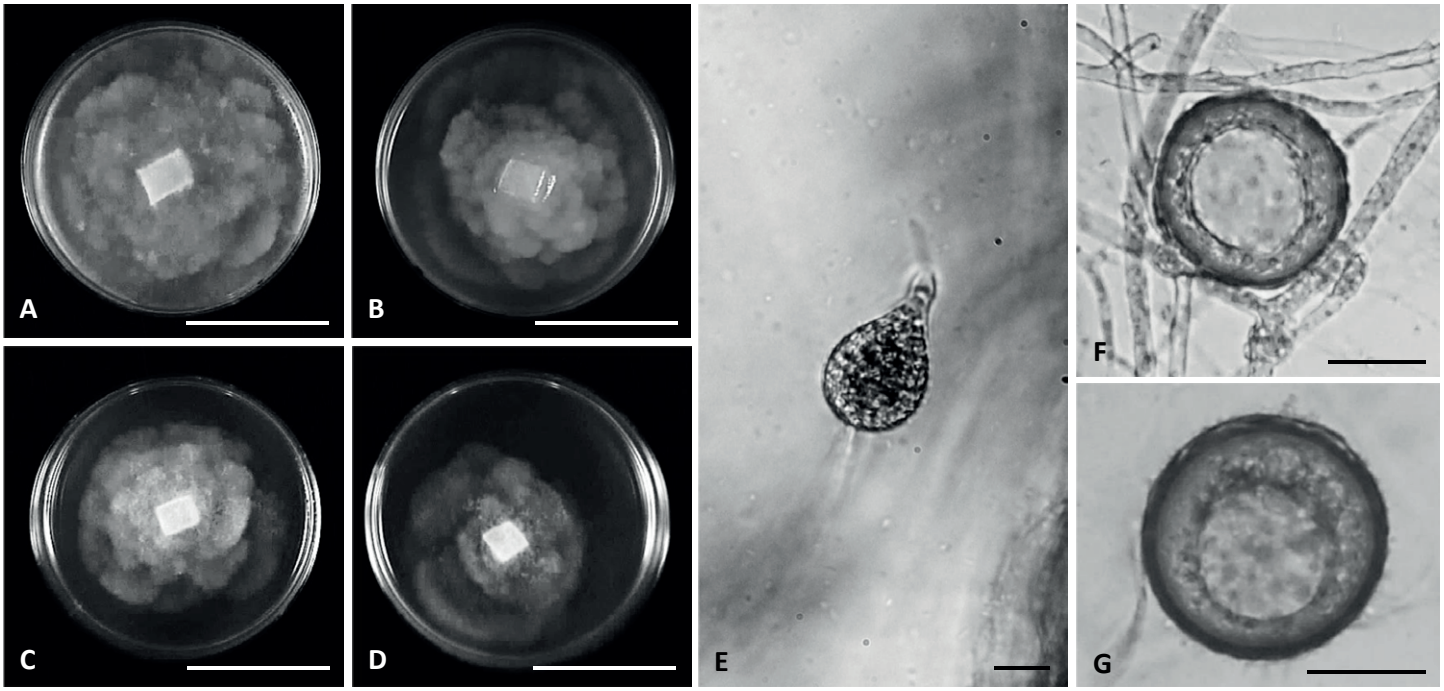

Fig. 9. *Salisapilia sapeloensis* CBS 127946. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Peptone yeast glucose agar. **D.** Potato dextrose agar. **E.** Mature sporangium. **F, G.** Oogonia. Scale bars: A–D = 30 mm, E–G = 20 µm.

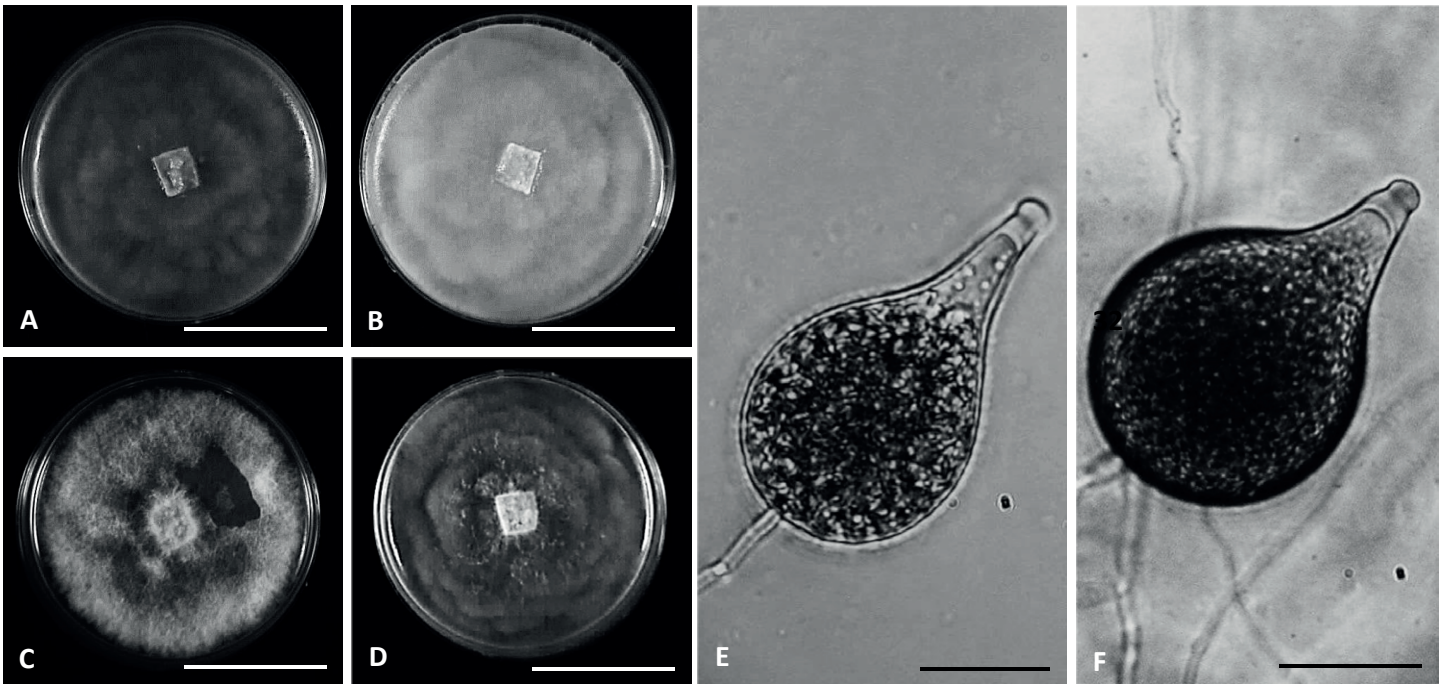

Fig. 10. *Salisapilia tartarea* CBS 208.95. Colony patterns on **A.** Vegetable juice agar. **B.** Potato carrot agar. **C.** Peptone yeast glucose agar. **D.** Potato dextrose agar. **E, F.** Mature sporangia. Scale bars: A–D = 30 mm, E, F = 20 µm.

in line with observations on other species of saprotrophic or hemibiotrophic *Peronosporales*, demonstrates the necessity to combine morphological and ontogenetic data with molecular phylogenetics, as the process of zoospore release might be variable within genera (Gisi *et al*. 1979, Gerrettson-Cornell & Simpson 1984, Bala *et al.* 2010, de Cock *et al*. 2015). Difficulty in finding clade specific-synapomorphies is common in saprotrophic and hemibiotrophic oomycetes. A good example of this is the paraphyletic genus *Phytophthora*, where the classification by Waterhouse (1963) or Stamps *et al*. (1990) does not reflect natural groupings resolved by multigene-phylogenies (Cooke *et al.* 2000, Kroon *et al*. 2004, Blair *et al.* 2008, Runge *et al*. 2011). *Halophytophthora elongata* (Ho *et al.* 2003) and *H. masteri* (Nakagiri *et al*. 1994) formed elongated to tubular-shaped, discharge-tube-like vesicles, similar to the vase-like vesicle of *S. nakagirii* prior to zoospore release.

While the absence of a vesicle does not seem to be a characteristic useful for delineating *Salisapilia*, the hyaline apical plug is a feature that seems to be of more diagnostic value. It is a usually readily observable cone-like structure nested at and eventually protruding from the apex of the discharge tube. Prior to zoospore release, the hyaline plug is ejected or detached from the discharge tube (Nakagiri *et al.* 1994, Ho *et al*. 2003) giving way for the release of zoospores. The only species in which this feature does not manifest is *S. nakagirii*. Haowever, variation in size of the hyaline apical plug is present among members of *Salisapilia* (Table 1). Based on the ancestral trait reconstruction analysis, it was observed that non-papillate sporangia appear as an ancestral trait to papillate and semi-papillate sporangia (Yang *et al*. 2017). In the present ancestral state reconstruction (Fig. 11), the absence of the hyaline apical plug seems to be a derived feature in *S. nakagirii,* and otherwise appears to be an exclusive synapomorphy for the genus *Salisapilia*.

Phylogenetically, *Salisapiliaceae* is a well-supported clade that appears to be a sister group to *Peronosporaceae* and *Pythiaceae* (Hulvey *et al*. 2010, this study). Hulvey *et al*. (2010) suggested that *H. bahamensis*, *H. epistomia*, *H. exoprolfiera,* and *H. operculata* might belong to the genus *Salisapilia*, but as no sequence data were available at that time to support this, Hulvey *et al*. (2010) refrained from proposing new combinations for any of these taxa. Of these species, *Halophytophthora operculata* was recently transferred to the genus *Calycofera* (Bennett *et. al*. 2017b), which was inferred to be the sister taxon to *Phytopythium*. Jung *et al*. (2017) suggested that *H. epistomia* might need to be accommodated in a genus of its own, but in the present study, it could be demonstrated that the morphology of *H. epistomia* fits well to the emended diagnosis of *Salisapilia*. Thus, it was combined into that genus instead of erecting a new one.

Key to the species of *Salisapilia*

1. Sporangia non-papillate; hyaline plug absent ..... ***S. nakagirii***
1. Sporangia papillate; hyaline plug present 2

2. Zoospore release through an evanescent vesicle 3
2. Zoospore release directly through the discharge tube 4

3. Dehiscence tube ragged appearance, with collar-like folds;
   sporangium shape ovoid, obpyriform, spherical ..... ***S. masteri***
3. Dehiscence tube smooth with cone-like plug; sporangium shape, mainly elongated, bursiform,
   cylindrical-elongated ..... ***S. elongata***

4. Sporangia vacuolated 5
4. Sporangia non-vacuolated 6

5. Sporangium shape bursiform; multi-lobed with aseptate or septate
   setiform appendages ..... ***S. bahamensis***
5. Sporangium shape narrowly bursiform, obpyriform, elongate to obclavate;
   single-lobed, setiform appendages absent ..... ***S. coffeyi***

6. Sexual structures absent; sporangium surface denticulate with few spines ..... ***S. mycoparasitica***
6. Sexual structures present, homothallic; sporangium surface smooth, spines absent 7

7. Oospores aplerotic ..... ***S. tartarea***
7. Oospores plerotic 8

8. Hyaline apical plug protruding through the discharge tube, 3–8 µm long;
   sporangium shape ovoid to obpyriform ..... ***S. sapeloensis***
8. Hyaline apical plug nested at the discharge tube, 14–90 µm long;
   sporangium shape langeniform to obpyriform ..... ***S. epistomia***

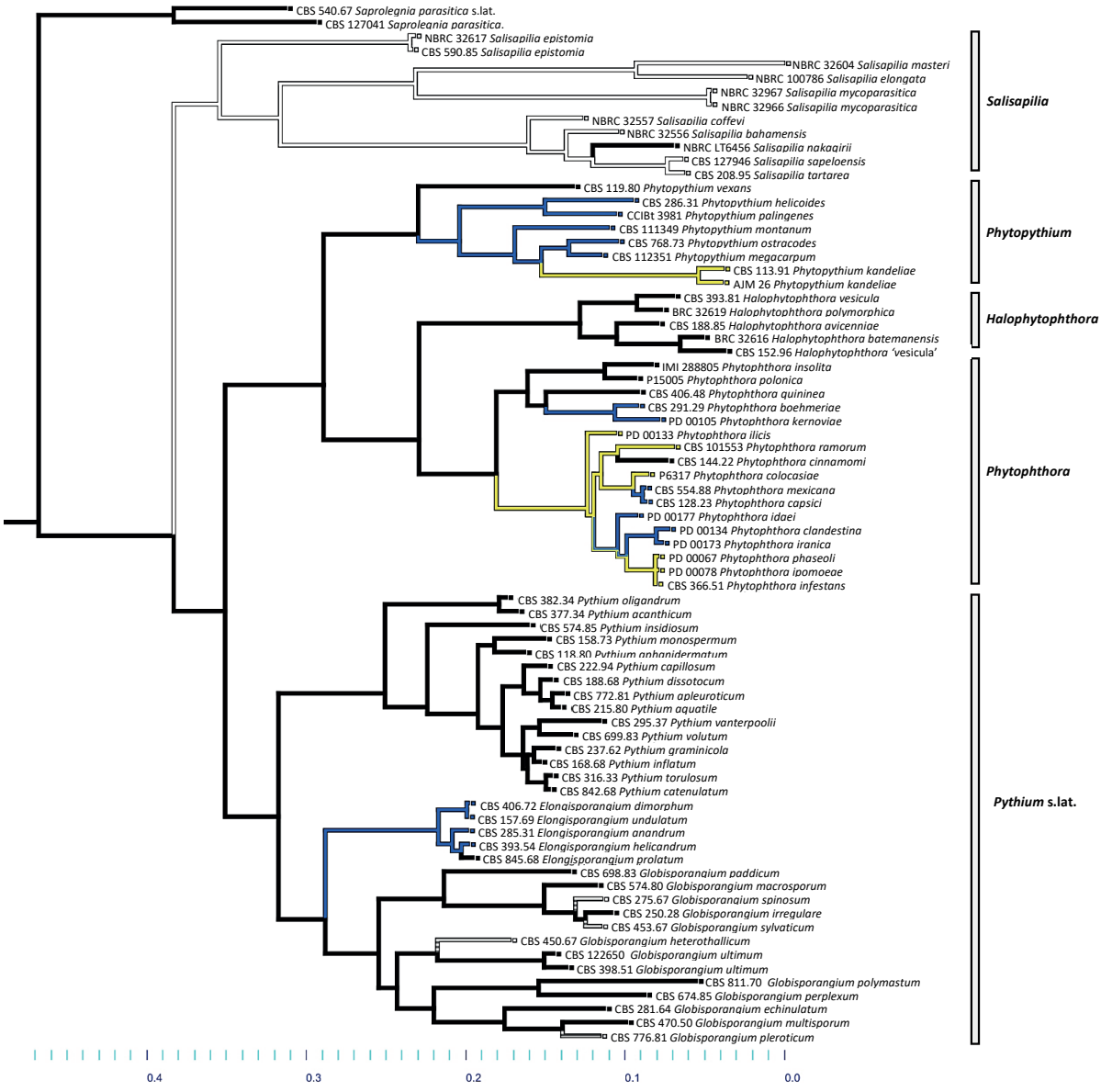

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Fig. 11. Ancestral trait reconstruction of the papilla and hyaline apical plug for *Elongisporangium*, *Globisporangium*, *Halophytophthora*, *Phytophthora*, *Phytopythium*, *Pythium*, and *Salisapilia*. White-coloured branches represent lineages with papillate sporangia bearing a hyaline apical plug; blue – papillate sporangia with no hyaline apical plug; yellow – semi-papillate sporangia with no hyaline apical plug; black – non-papillate sporangia. The scale corresponds to species divergence relative to nucleotide substitution rates based on the Bayesian phylogenetic inference.

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Supplementary Material: http://fuse-journal.org/

Table S1. GenBank numbers of sequences used in this study.

Fig. S1. Phylogenetic tree based on ITS sequences. The primary phylogenetic tree was inferred using Minimum Evolution (ME), with bootstrap support values from ME and Maximum Likelihood, and posterior probabilities from Bayesian Inference, in the respective order. (-) indicates unsupported alternating topology or bootstrap value and posterior probability of ≤ 50 / 0.8, respectively. The scale bar indicates the number of nucleotide substitutions per site.

Fig. S2. Phylogenetic tree based on LSU sequences. The primary phylogenetic tree was inferred using Minimum Evolution (ME), with bootstrap support values from ME and Maximum Likelihood, and posterior probabilities from Bayesian Inference, in the respective order. (-) indicates unsupported alternating topology or bootstrap value and posterior probability of ≤ 50 / 0.8, respectively. The scale bar indicates the number of nucleotide substitutions per site.

Table S1. GenBank numbers of sequences used in this study.

Species	Strain information	Other strain no.	ITS	LSU
Halophytophthora				
H. bahamensis	NBRC 32557	IFO 32557 ATCC28297 P3931*	MF979510	MF979503
H. bahamensis T	NBRC 32556	IFO 32556 ATCC 28296 CBS 586.85 IMI 330182 P3930*	MF979511	MF979504
H. batemanensis T	NBRC 32616	CBS 679.84 NBRC 32616 MG 25-3 MG 33-5 DAR 41559 IMI 327602 ATCC 56965	AF271223	DQ361227
H. elongata T	NBRC 100786	BCRC 33983	MF979512	MF979505
H. epistomia	CBS 590.85	NBRC 32617	HQ643220	HQ665279
H. epistomia T	NBRC 32617	IFO 32617 ATCC 28293 IMI 330183 CBS 590.85	MF979513	MF979506
H. masteri T	NBRC 32604	IFO 32604 ATCC 96906 CBS 207.95	MF979514	MF979507
H. mycoparasitica	NBRC 32967	IFO 32967	MF979515	MF979508
H. mycoparasitica	NBRC 32966	IFO 32966	MF979516	MF979509
H. polymorphica T	CBS 680.84	DAR 41562 IFO 32619 ATCC 56966 NBRC 32619	HQ643313	HQ665288
H. vesicula T	NBRC 32216	IFO 32216 CBS 393.81	JF750389	KT455418
H. vesicula	CBS 152.96		HQ232472	HQ232463
Phytopythium				
P. helicoides	CBS 286.31		HQ643383	HQ665186
P. kandeliae	AJM26		KJ399962	KJ399965
P. kandeliae	CBS 113.91		KJ399961	HQ665079
P. megacarpum	CBS 112351		AB725881	HQ665067
P. montanum	CBS 111349		AB725883	HQ665064
P. ostracodes	CBS 768.73		HQ643395	HQ665295
P. palingenes	CCIBt 3981		KR092139	KR092143
P. vexans	CBS 119.80		AY598713	HQ665090
Salisapilia				
S. nakagirii T	LT6456	CBS 127947 NBRC 108757	HQ232467	HQ232458
S. sapeloensis T	LT6440	CBS 127946 NBRC 108756	HQ232466	HQ232457
S. tartarea T	CBS 208.95	IFO 32606 NBRC 32606 ATCC 96905	HQ232473	HQ232464
Saprolegnia				
S. parasitica	CBS 540.67		AY310504	HQ665256
S. parasitica	CBS 127041		HQ111458	HQ395663
Phytophthora				
P. boehmeriae	CBS 291.29	PD 00181 P6950	NR147884	HQ665190
P. capsici	CBS 128.23		DQ464056	HQ665120
P. cinnamomi	CBS 144.22		KC478663	HQ665126
P. clandestina	CBS 349.86	P3942	PD_00134	PD_00134
P. colocasiae	P6317		PD_00139	PD_00139
P. idaei	CBS 971.95	P6767 IMI 313728	PD_00177	PD_00177
P. ilicis	P3939		PD_00133	PD_00133
P. infestans	CBS 366.51		HQ643247	HQ665217
P. insolita	IMI 288805	PD 00175 P6195	NR147858	EU080180

P. ipomoeae	P10225		PD_00078	PD_00078
P. iranica	CBS 374.72	P3882	PD_00173	PD_00173
P. kernoviae	P10958		PD_00105	PD_00105
P. Mexicana	CBS 554.88	P0646	PD_00061	PD_00061
P. phaseoli	P10145		PD_00067	PD_00067
P. polonica	P15005		PD_01107	PD_01107
P. quininea	CBS 406.48	P3247	PD_00126	PD_00126
P. ramorum	CBS 101553	PD 00065 P10103	NR147877	HQ665053
Pythium				
P. acanthicum	CBS 377.34		HQ643409	HQ665222
P. aphanidermatum	CBS 118.80		AY598622	HQ665084
P. apleroticum	CBS 772.81		AY598631	HQ665296
P. aquatile	CBS 215.80		AY598632	HQ665153
P. capillosum	CBS 222.94		AY598635	HQ665164
P. catenulatum	CBS 842.68		AY598675	HQ665302
P. dissotocum	CBS 166.68		AY598634	HQ665139
P. graminicola	CBS 327.62		HQ643545	HQ665211
P. inflatum	CBS 168.68		AY598626	HQ665140
P. insidiosum	CBS 574.85		AY598637	HQ665273
P. monospermum	CBS 158.73		AY598621	HQ665137
P. oligandrum	CBS 382.34		AY598618	HQ665223
P. torulosum	CBS 316.33		AY598624	HQ665206
P. vanterpoolii	CBS 295.37		AY598685	HQ665193
P. volutum	CBS 699.83		AY598686	HQ665291
Globisporangium				
G. echinulatum	CBS 281.64		AY598639	HQ665183
G. heterothallicum	CBS 450.67		AY598654	AY598654
G. irregulare	CBS 250.28		AY598702	HQ665172
G. macrosporum	CBS 574.80		AY598646	HQ665272
G. multisporum	CBS 470.50		AY598641	HQ665239
G. paddicum	CBS 698.83		AY598707	HQ665290
G. perplexum	CBS 674.85		AY598658	HQ665283
G. pleroticum	CBS 776.81		AY598642	HQ665298
G. polymastum	CBS 811.70		AY598660	HQ665301
G. spinosum	CBS 275.67		AY598701	HQ665181
G. sylvaticum	CBS 453.67		AY598645	HQ665236
G. ultimum	CBS 122650		HQ643864	HQ665103
G. ultimum	CBS 398.51		AY598657	HQ665227
Elongisporangium				
E. anandrum	CBS 285.31		AY598650	HQ665185
E. dimorphum	CBS 406.72		AY598651	HQ665229
E. helicandrum	CBS 393.54		AY598653	HQ665225
E. prolatum	CBS 845.68		AY598652	HQ665303
E. undulatum	CBS 157.69		AY598708	HQ665134

Strain information and abbreviation

T – ex-Type specimen
ATCC – American Type Culture Collection, USA
BCRC – Bioresource Collection and Research Center, Taiwan
CBS – Westerdijk Fungal Biodiversity (formerly Centraalbureau voor Schimmelcultures), The Netherlands
NBRC – NITE Biological Resource Centre, Japan
IMI – CABI Bioscience, part of the United Kingdom National Culture Collection
IFO – Institute for Fermentation Osaka, Japan
PD – sequence strains obtained from the *Phytophthora* database (http://www.phytophthoradb.org/)
*information obtained from the *Phytophthora* WOC Database, World *Phytophthora* Genetic Resource Collection (http://phytophthora.ucr.edu/)

Fig. S1

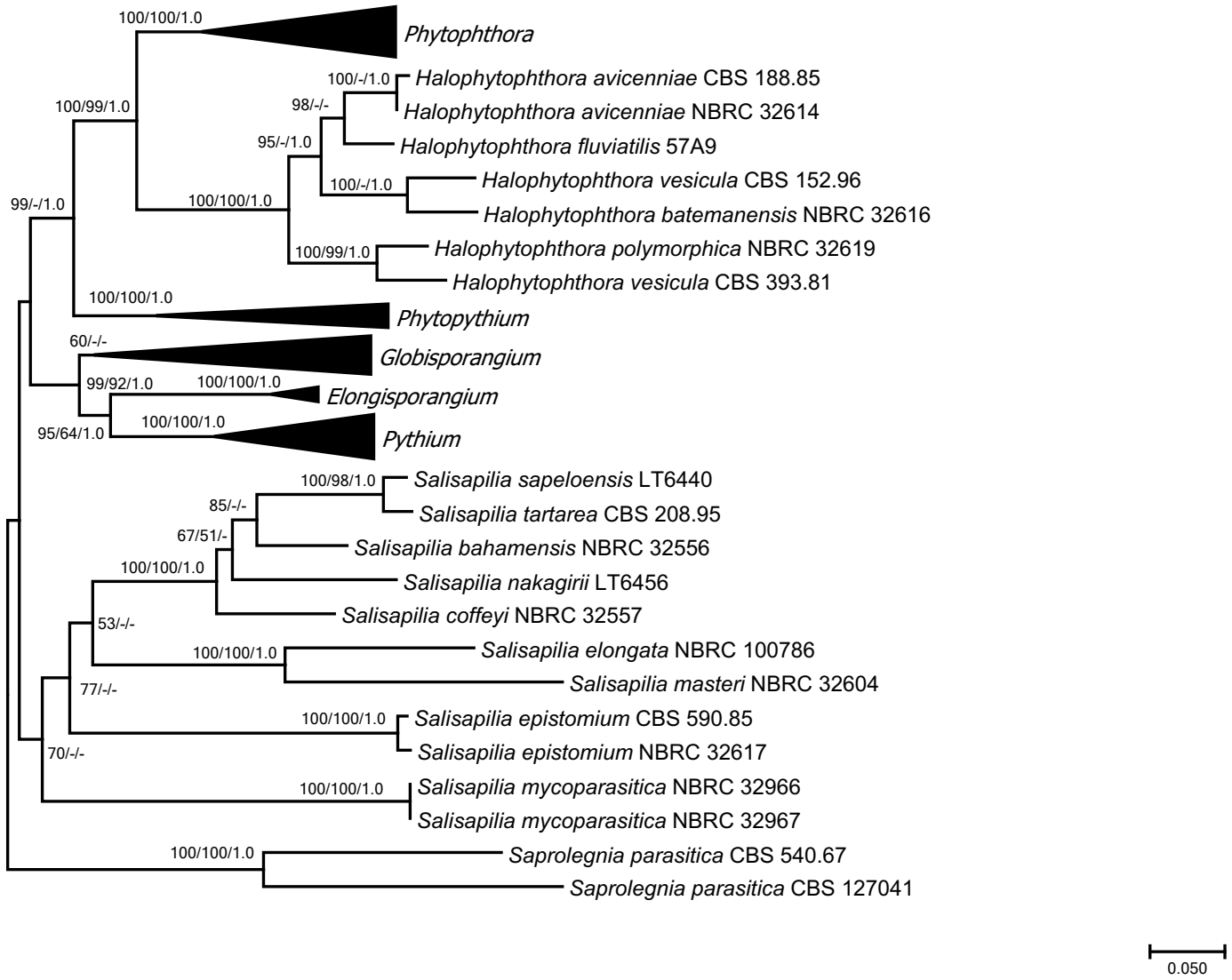
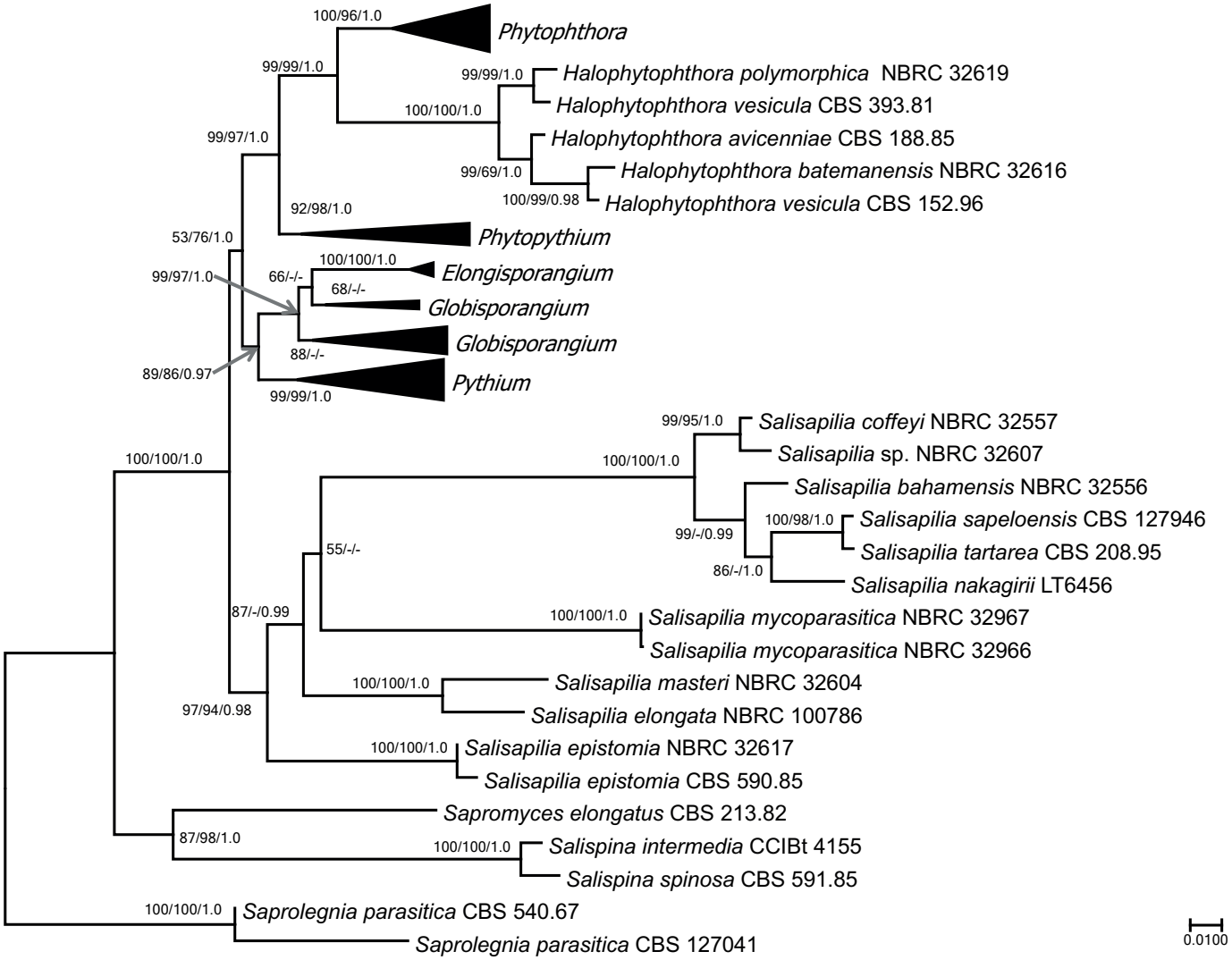

100/100/1.0
Phytophthora
100/-/1.0
Halophytophthora avicenniae CBS 188.85
100/99/1.0
98/-/-
Halophytophthora avicenniae NBRC 32614
95/-/1.0
Halophytophthora fluviatilis 57A9
100/-/1.0
Halophytophthora vesicula CBS 152.96
99/-/1.0
100/100/1.0
Halophytophthora batemanensis NBRC 32616
100/99/1.0
Halophytophthora polymorphica NBRC 32619
Halophytophthora vesicula CBS 393.81
100/100/1.0
Phytopythium
60/-/-
Globisporangium
99/92/1.0
100/100/1.0
Elongisporangium
95/64/1.0
100/100/1.0
Pythium
100/98/1.0
Salisapilia sapeloensis LT6440
85/-/-
Salisapilia tartarea CBS 208.95
67/51/-
Salisapilia bahamensis NBRC 32556
100/100/1.0
Salisapilia nakagirii LT6456
Salisapilia coffeyi NBRC 32557
53/-/-
100/100/1.0
Salisapilia elongata NBRC 100786
Salisapilia masteri NBRC 32604
77/-/-
100/100/1.0
Salisapilia epistomium CBS 590.85
Salisapilia epistomium NBRC 32617
70/-/-
100/100/1.0
Salisapilia mycoparasitica NBRC 32966
Salisapilia mycoparasitica NBRC 32967
100/100/1.0
Saprolegnia parasitica CBS 540.67
Saprolegnia parasitica CBS 127041
0.050

Fig. S2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Halophytophthora avicenniae CBS 188.85
99/97/1.0
Halophytophthora batemanensis NBRC 32616
99/69/1.0
Halophytophthora vesicula CBS 152.96
100/99/0.98
92/98/1.0
Phytopythium
53/76/1.0
100/100/1.0
Elongisporangium
99/97/1.0
66/-/-
68/-/-
Globisporangium
Globisporangium
88/-/-
89/86/0.97
Pythium
99/99/1.0
99/95/1.0
Salisapilia coffeyi NBRC 32557
Salisapilia sp. NBRC 32607
100/100/1.0
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Salisapilia bahamensis NBRC 32556
100/98/1.0
Salisapilia sapeloensis CBS 127946
99/-/0.99
Salisapilia tartarea CBS 208.95
86/-/1.0
Salisapilia nakagirii LT6456
55/-/-
87/-/0.99
100/100/1.0
Salisapilia mycoparasitica NBRC 32967
Salisapilia mycoparasitica NBRC 32966
100/100/1.0
Salisapilia masteri NBRC 32604
97/94/0.98
Salisapilia elongata NBRC 100786
100/100/1.0
Salisapilia epistomia NBRC 32617
Salisapilia epistomia CBS 590.85
Sapromyces elongatus CBS 213.82
87/98/1.0
100/100/1.0
Salispina intermedia CCIBt 4155
Salispina spinosa CBS 591.85
100/100/1.0
Saprolegnia parasitica CBS 540.67
Saprolegnia parasitica CBS 127041
0.0100

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ARTICLE

A revision of *Salispina*, its placement in a new family, *Salispinaceae* (*Rhipidiales*), and description of a fourth species, *S. hoi* sp. nov.

Reuel M. Bennett[1,2,3], Mark Kevin Devanadera[4], Gina R. Dedeles[4], and Marco Thines[1,2,3]

[1]Senckenberg Biodiversity and Climate Research Centre (SBiK-F), Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, D-60325 Frankfurt am Main, Germany; corresponding author e-mail: m.thines@thines-lab.eu
[2]Department of Biological Sciences, Institute of Ecology, Evolution and Diversity, Goethe University Frankfurt am Main, Max-von-Laue-Str. 9, D-60438 Frankfurt am Main, Germany
[3]Integrative Fungal Research Cluster (IPF), Georg-Voigt-Str. 14-16, D-60325 Frankfurt am Main, Germany
[4]Department of Biological Sciences-College of Science, Department of Biochemistry-Faculty of Pharmacy, and UST Collection of Microbial Strains (USTCMS), Research Center for the Natural and Applied Sciences, University of Santo Tomas, Manila 1015, Philippines

Abstract: The genus *Salispina* was recently described for saprotrophic estuarine oomycetes with aculeolate or spiny sporangia. The genus currently contains three species, *S. intermedia*, *S. lobata*, and *S. spinosa*, the latter two previously included in *Halophytophthora*. During a survey of mangrove-inhabiting oomycetes in the Philippines, an isolate of *Salispina* (USTCMS 1611), was obtained from a decaying mangrove leaf. This isolate differed from other species in the genus in a unique combination of morphological and biological characters. Phylogenetic analysis revealed it to be the sister lineage of *S. lobata.* Consequently, the new species name *S. hoi* is introduced for the isolate. In addition, *Salispina* spp. grouped with *Sapromyces* of *Rhipidiales* with strong support, but differs from all other known genera of the order in the weak formation of hyphal constrictions, and absence of basal thalli and a holdfast network. The new family *Salispinaceae* is, therefore, described to accommodate *Salispina* in the order *Rhipidiales*.

Key words:
Mangrove
new taxa
Oomycota
phylogenetics
Sapromyces
taxonomy

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INTRODUCTION

Mangroves are inhabited by saprotrophic oomycetes, fungal-like eukaryotes in the kingdom *Straminipila* (Fell & Master 1975, Leaño *et al.* 2000, Leaño 2001, Thines 2014, Marano *et al*. 2016, Bennett *et al*. 2017a). These organisms are the first colonisers of fallen senescent mangrove leaves and, thus, have an important role in the nutrient cycling in estuarine ecosystems (Newell *et al.* 1987, Nakagiri *et al.* 1989, Leaño *et al*. 2000). Of the diverse mangrove oomycetes, *Salispina* is a genus currently comprising three described species (Li *et al*. 2016): *S. intermedia* (type species), *S. spinosa* (syn. *Phytophthora spinosa* var. *spinosa*, *Halophytophthora spinosa* var. *spinosa*), and *S. lobata* (syn. *Phytophthora spinosa* var. *lobata*, *Halophytophthora spinosa* var. *lobata*). This genus was erected to accommodate saprotrophic mangrove oomycetes with aculeolate or spiny, variously shaped sporangia, and direct zoospore release through an apical discharge tube. However, the higher taxonomic affinity of *Salispina* remained uncertain, and the genus was not assigned to a family or order (Li *et al.* 2016).

In the Philippines, Leaño (2001) recognized *S. lobata* (as *H. spinosa* var. *lobata*) as the first record of *Salispina* for the Philippines, and we did not find any other report of these organisms in the Philippines. It was the aim of this study to investigate the presence of additional species of *Salispina* in Philippine mangroves, and to resolve the family and order classification.

MATERIALS AND METHODS

Isolation, morphological investigation, and sporulation

The isolation and purification of the isolate used in this study, which came from decaying leaves collected from mangroves at Davao del Sur, Philippines, followed the method of Bennett & Thines (2017). For morphological investigations, samples were processed as described in Bennett & Thines (2017), but values were rounded to the nearest half micron, except for mean values. For sporulation, the development of sporangia from agarised media plugs was observed in saline concentrations of 0–3 % incubated at room temperature (~20–25 °C) in a dark compartment. Zoospore release was induced by placing mycelia with mature sporangia in a saline solution (≥ 3.5 %) and at 35 °C without light. Colony radial growth at 20, 25, 30, and 35 °C was tested in vegetable juice agar (VJA, commercial V8 Juice, Campbell or Alnatura Gemüsesaft, Alnatura; NBRC, medium no. 15), with or without seawater (http://www.nite.go.jp/en/nbrc/cultures/media/culture-list-e.html); and potato carrot agar (PCA; Crous *et al.* 2009), based on Alnatura Demeter Karotten mit Kartoffeln,

Alnatura). Mean colony radial growth was measured for five days and expressed as mm/day following the method of Solis *et al*. (2010).

Salispina sp. USTCMS 1611, *S. spinosa* CBS 591.85, and *S. lobata* CBS 588.85 were tested for growth in VJA at room temperature (~20–25 °C) for 5 d using a candle jar incubation method as described below and mean colony radial growth was measured according to Solis *et al.* (2010). For sporangium development under depleted oxygen conditions, mycelium in VJA from a 7 d-old culture plate (three per strain) was cut and the resulting pieces of ~1-2 cm² were placed in 60 mm Petri dishes containing 3 % saline solution. The Petri dishes were placed in a desiccator with a burning candle instead of silica gel. Then the desiccator was closed, allowing the candle to consume the oxygen until the flame could not be supported anymore. Subsequently, the desiccator was incubated at room temperature (~20–25°C). Another set-up was incubated in ambient air on a work-bench at room temperature (~20–25 °C). For zoospore release, the same settings were used, except for incubation at 35 °C and a saline solution of 3.5 %.

DNA Extraction and PCR amplification

For DNA extraction, a phenol-isoamyl-chloroform method was used (Bennett *et al.* 2017b). Subsequently, PCR amplification of *cytochrome oxidase* 1 (*cox*1), *cytochrome oxidase* 2 (*cox*2), and large nuclear ribosomal subunit (nrLSU) was done using the PCR primers listed in Table 1. The PCR reaction mix contained 1× PCR buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.8 µg BSA, 0.4 µM of each primer, 0.5 U *Taq* polymerase and 10–50 ng DNA. PCR amplification of the *cox*1 region was done with an initial denaturation at 95 °C for 4 min, followed by 36 cycles of denaturation at 95 °C for 40 s, annealing at 51 °C for 40 s, and elongation at 72 °C for 60 s. A final elongation was done at 72 °C for 5 min. The cycling conditions for the *cox*2 region included an initial denaturation at 94 °C for 4 min, followed by 36 cycles of denaturation at 94 °C for 40 s, annealing at 51 °C for 40 s, and elongation at 72 °C for 40 s. A final elongation was carried out at 72 °C for 4 min.

For the LSU region, the cycling conditions were as follows: – initial denaturation 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 53 °C for 20 s, and elongation at 72 °C for 120 s. Subsequently, a final extension was carried out at 72 °C for 7 min.

PCR amplicons were sent to the SBiK-F Central Laboratory for sequencing with the primers used for PCR amplification. Sequences were assembled, converted into contigs and edited using Geneious version 5.0.4 (Biomatters, New Zealand). The resulting contigs were exported in fasta file format along with reference sequences selected from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide) (Table 2). The resulting sequences were uploaded to the TrEase phylogeny webserver (http://www.thines-lab.senckenberg.de/trease/) for sequence alignment and phylogenetic tree reconstruction. The program MAFFT (Katoh *et al.* 2002) was used for multiple sequence alignment of *cox*1, *cox*2, and nrLSU sequences. Specifically, the FFT-NS-1 (fast) model was the chosen algorithm for *cox*1 and *cox*2 due to the absence of gaps and because taxa used in the multiple sequence alignments were closely related species. The G-INS-i was the algorithm used for nrLSU sequences. The primary phylogenetic tree, Minimum Evolution (ME), was generated using FastTree (Price *et al.* 2009), with 1000 bootstrap replicates and following the Generalized Time-Reversible (GTR) algorithm. Maximum Likelihood (ML) was generated using RAxML (Stamatakis 2014) where GTR-GAMMA was the chosen algorithm supported by 1000 bootstrap replications. Bayesian Inference was done using MrBayes (Ronquist *et al.* 2012) with the GTR model of substitutions and running four incrementally heated chains for 1 000 000 generations, discarding the first 30 % of the resulting trees to ensure sampling of trees and posterior probability calculations from the stationary phase. After making sure no supported incongruences were present for the different loci, alignments of *cox*1, *cox*2, and nrLSU sequences were concatenated using SequenceMatrix (Vaidya *et al*. 2010) and phylogenetic trees were computed as outlined above. Phylogenetic trees were viewed and annotated using MEGA, version 6 and 7 (Tamura *et al.* 2013).

RESULTS

Morphology

Salispina sp. USTCMS 1611 was isolated from decaying leaves collected from mangroves at Davao del Sur. Colony morphology of the isolate was appressed on both VJA and PCA (Fig. 1A–B). The strain developed aculeolate sporangia similar to known taxa of *Salispina* (Fig. 1) (Table 3). Hyphae were 2–9 µm wide, with retraction septae forming in some hyphae in old cultures submerged in 2–3 % saline solution incubated at room temperature (~20–25 °C). The branching pattern was irregular. Sporulation was achieved when plugs with mycelia were placed in 2–3 % saline solution and incubated at room temperature (~20–25 °C) in the dark. Sporangiogenic hyphae are not differentiated

Table 1. PCR Primers used in this study.

Loci	Primer pair	Sequence (5' – 3')	Reference
*cox*1	Oom*cox*1_Levup	GCT TAA GTT CAG CGG GT	Robideau *et al.* (2011)
	Oom*cox*1_Levlo	CYT CHG GRT GWC CRA AAA ACC AAA	Robideau *et al.* (2011)
*cox*2	*cox*2-F	GGC AAA TGG GTT TTC AAG ATC C	Hudspeth *et al.* (2000)
	*cox*2-RC4	TGA TTW AYN CCA CAA ATT TCR CTA CAT TG	Choi *et al.* (2015)
nrLSU	LR0R	ACC CGC TGA ACT TAA GC	Moncalvo *et al.* (1995)
	LR6-O	CGC CAG ACG AGC TTA CC	Riethmüller *et al.* (2002)

Table 2. GenBank* sequences (accession numbers) used in this study.

Species	Strain information	*cox*1	*cox*2	LSU
Halophytophthora				
H. vesicula ex-type	NBRC 32216• (= CBS 393.81 = IFO 32216)	MG019397	MF991427	KT455418
Phytopythium				
P. helicoides	CBS 286.31•	MF397921	MF397926	HQ665186
P. kandeliae	CBS 111.91•	HQ708207	MF397928	HQ665065
P. megacarpum	CBS 112351•	HQ708435	AB690665	HQ665067
P. montanum	CBS 111349•	HQ708436	AB690667	HQ665064
P. ostracodes	CBS 768.73•	EF408874	AB690668	HQ665295
P. vexans	CBS 119.80•	EF426548	EF426547	HQ665090
Phytophthora				
Ph. boehmeriae	CBS 291.29• (= PD_00181 = P6950)	HQ261251	PD_00181	HQ665190
Ph. insolita	IMI 288805• (= PD_00175 = P6195)	PD_00175	PD_00175	EU080180
Ph. kernoviae	P10958•	PD_00105	PD_00105	PD_00105
Ph. quininea	CBS 406.48 (= P3247)•	PD_00126	PD_00126	PD_00126
Ph. ramorum	CBS 101553• (= PD_00065 = P10103)	HQ708387	PD_00065	HQ665053
Pythium				
Py. aquatile	CBS 215.80•	HQ708492	KJ595355	HQ665153
Py. capillosum	CBS 222.94•	HQ708529	KJ595360	HQ665164
Py. torulosum	CBS 316.33•	HQ708900	KJ595374	HQ665206
Py. inflatum	CBS 168.68•	HQ708610	KJ595352	HQ665140
Salisapilia				
S. sapeloensis ex-type	LT6440• (= CBS 127946 = NBRC 108756)	KT897704	KJ654178	HQ232457
Salispina				
S. intermedia ex-type	CCIBt 4155	KT886053	NS	KT920432
S. intermedia	CCIBt 4153	KT886052	NS	KT920431
S. intermedia	CCIBt 4156	KT886054	NS	KT920433
S. intermedia	CCIBt 4115	KT886055	NS	NS
S. hoi ex-type	USTCMS 1611•	MG019399	MF991430	MG385863
S. lobata ex-type	CBS 588.85 (= NBRC 32592 = IFO 32592 = ATCC 28291)	KT886056	MF991429	NS
S. spinosa ex-type	CBS 591.85• (= NBRC 32593 = IFO 32593 = ATCC 28294)	KT886057	MF991428	KT920434
Sapromyces				
S. elongatus	CBS 213.82•	MG019398	KT257452	AF235950
Saprolegnia				
S. parasitica	CBS 223.65•	NW012157837	NW012157837	HQ665165
S. ferax	P1.5.14	KP965743	KP965749	NS

NS: No sequence was used for the respective loci.

*Some sequences of *Phytophthora* spp. were downloaded from the *Phytophthora* database (http://www.phytophthoradb.org/).

•Strains used in multigene analyses.

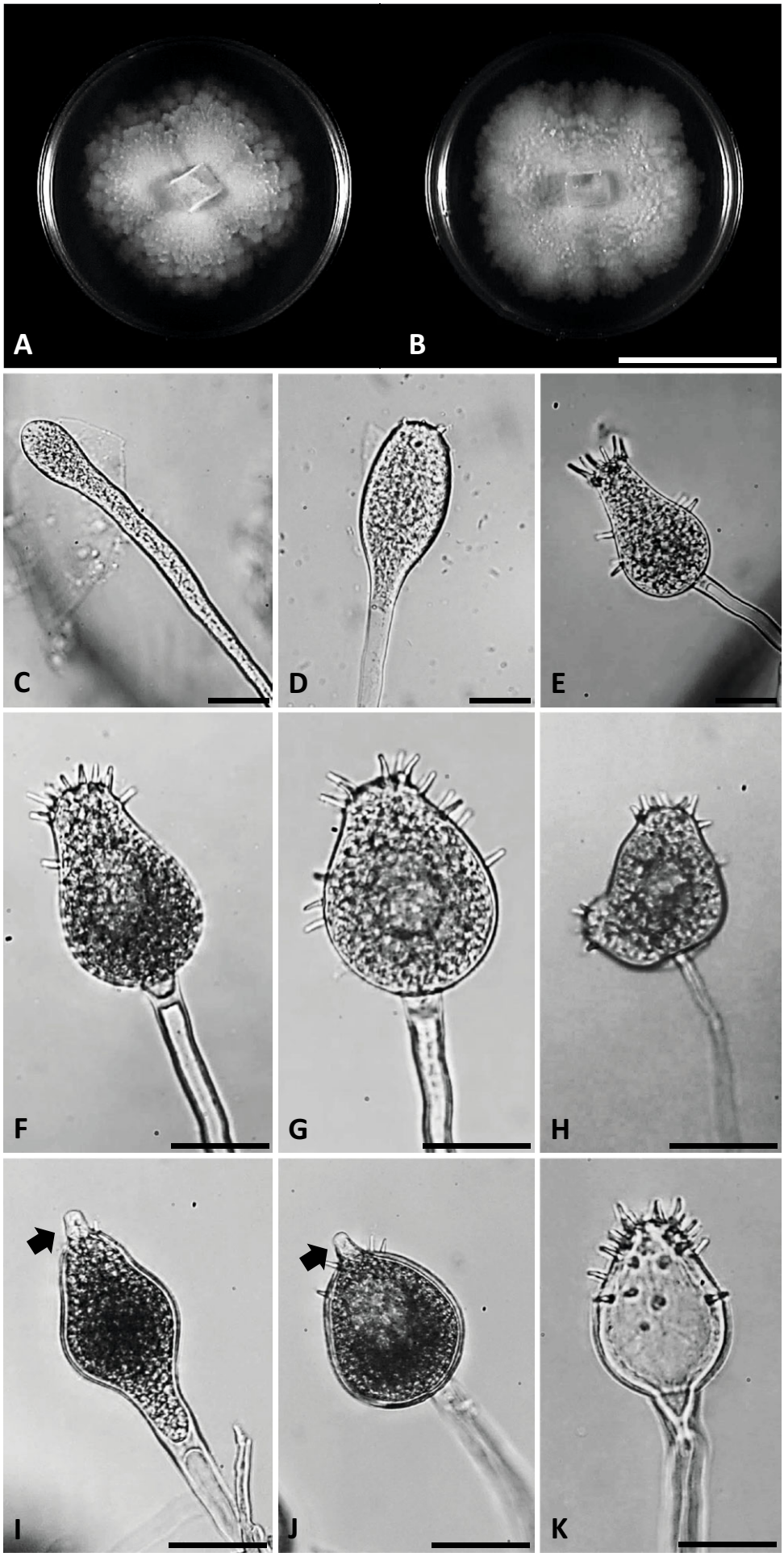

Fig. 1. Morphology of *Salispina hoi* (USTCMS 1611). Colony pattern on: **A.** Potato carrot agar (PCA), and **B.** Vegetable juice agar (VJA). **C.** Protosporangium. **D.** Immature or young sporangium. **E**–**H.** Mature sporangia; spines are forming at the apex of sporangia, while others are either having scattered spines on the surface of the sporangia or a smooth surface. **H.** Irregularly-shaped aculeolate sporangium. **I–J.** Sporangia with dehiscence tube (arrow), zoospores differentiate inside the sporangia. **K.** Empty sporangium. Bars: **A–B** = 30 mm, **C–K** = 20 µm.

Table 3. Morphology of *Salispina* species.

Structure	***Salispina hoi* sp. nov.** **USTCMS 1611**	***S. intermedia*** **(Li *et al.* 2016)**	***S. lobata*** **(Fell & Master 1975)**	***S. spinosa*** **(Fell & Master 1975)**
Colony pattern	Appressed and petaloid on VJA	Petaloid on PYGA	Appressed and petaloid on VJA	Appressed and rosette on VJA
Septa	Few, present at maturity	Few, present at maturity	Few, present at maturity	Non-septate at all ages
Hyphal diam (µm)	2–9	2.5–10	3–12	3–9
Sporangiogenic hypha	Undifferentiated from vegetative hypha, bears 1 terminal sporangium	Undifferentiated from vegetative hypha, bears 1 terminal sporangium	Undifferentiated from vegetative hypha, bears 1 terminal sporangium	Undifferentiated from vegetative hypha, bears 1 terminal sporangium
Sporangia				
Shape	Ovoid, clavate, globose, obpyriform, variable	Obovate, obpyriform, globose, elongate, variable	Obypriform to auriculate, botryose-like, similar to fused globose sporangia	Globose, ovate, obovate
Papilla	Non-papillate	ND	Inconspicuous, unipapillate	Inconspicuous, unipapillate
Size (µm)	(33.5–)43–57.6–77.5(–87) × (10.5–)20–36.6–66(–75.5)	33–197 × 25–183 (av. 82 × 62)	51–75 × 56–150 (av. 67 × 97)	60–107 (av. 80) diam.
Surface spines	Most spines at the apex of the sporangia, forming a crown-like appearance, Some sporangia have scattered spines or non-aculeolate	Smooth to spiny, variable degree of coverage from one at the tip to entirely aculeolate	Entirely, partially or non-aculeolate	Entirely, partially or non-aculeolate
Vacuole	Present	Present	Present	Present
Basal plug	Present, hyaline	Present, hyaline	Present, hyaline	Present, hyaline
Zoospore discharge	Through a thin-walled dehiscence tube, often inconspicuous after full release of zoospores	Through a persistent tube	Through a thin-walled, flask-shaped dehiscence tube	Through a thin-walled, flask-shaped dehiscence tube
Vesicle	Absent	Absent	Absent	Absent
Chlamydospores	Not observed	Not observed	Not observed	Not observed
Sexual structures	Not observed	Not observed	Not observed	Not observed

ND: No data provided.

from vegetative hyphae until the hyphal apex swells to form a protosporangium (Fig. 1C–D). The sporangia are ovoid, clavate, globose to obpyriform (Fig. 1E–J) but some were irregularly shaped (Fig. 1H); they measured (33.5–)43–57.5–77.5(–87) × (10.5–)20–36.5–66(–75.5) (*n* = 100). Spines were predominantly forming at the apex of the sporangia, resulting in a crown-like appearance (Fig. 1 D–E, J), while some sporangia were partially covered in spines, rarely entirely aculeolate (Fig. 1 F–H, J), or smooth-walled sporangia were observed (not depicted). The sporangia were non-caducous and non-papillate. The sporangial content was vacuolated. The inner base of the sporangia, where the basal plug is located, was concave (Fig. 1 I, K). The basal plug was observed to be hyaline, separating the sporangiogenic hypha from the sporangium. Zoospore release occurred only when mycelium with mature sporangia was placed in a saline solution with ≥ 3.5 % and incubated at 35 °C. The apex of the dehiscence tube (Fig. 1 I–J) deliquesces and zoospores swim directly out from the tube, i.e. no vesicle was observed. No chlamydospores and gametangia were observed. A summary of morphological features of known *Salispina* spp. is given in Table 3.

The mean colony radial growth of *Salispina* sp. USTCMS 1611 in VJA and PCA at different temperatures is given in Fig. 2A. The growth and sporulation of the three *Salispina* spp. in VJA in candle jar incubation at room temperature (~ 20–25 °C) are presented in Fig. 2B.

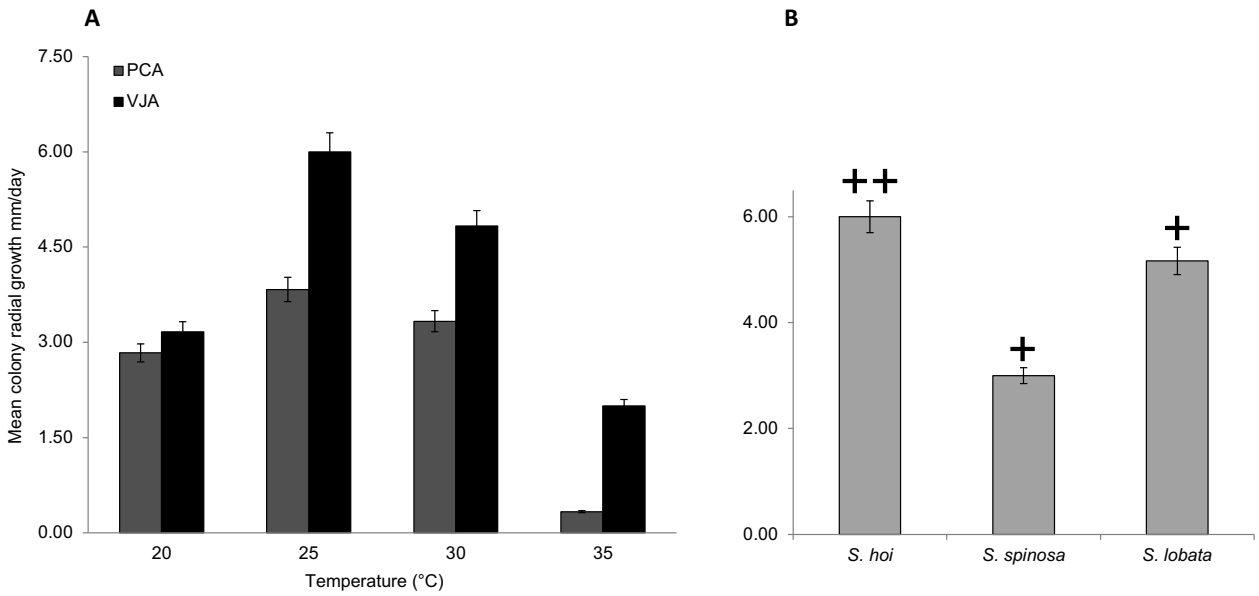

Fig. 2. Mean colony radial growth. **A.** Mean colony radial growth of *Salispina hoi* (USTCMS 1611) on VJA and PCA at different temperatures. **B.** Mean colony radial growth of the three *Salispina* species on VJA at room temperature in a candle jar. (++) = sporulation both under candle jar and ambient air conditions; (+) = sporulation under ambient air condition.

Phylogeny

The multigene phylogenetic analysis (Fig. 3) and the single-gene phylogenetic trees (Figs S1–S3) showed that USTCMS 1611 is a distinct member of the *Salispina* clade, with maximum support in all analyses. *Salispina* sp. USTCMS 1611 was not conspecific with any known species of *Salispina* (Figs S1, S3), and grouped as sister to *S. lobata* (Figs S1–S2). In addition, the genus *Salispina* was found to be sister to *Sapromyces elongatus* (*Rhipidiaceae*, *Rhipidiales*) with strong to maximum support in the phylogenetic reconstruction based on the concatenated dataset with nuclear and mitochondrial loci (Fig. 3).

DISCUSSION

The genus *Salispina* was proposed based on phylogenetics and sporangial characteristics with *Salispina intermedia* as the type species (Li *et al*. 2016). The two additional species, *S. spinosa* and *S. lobata*, were first considered to be members of *Phytophthora* (Fell & Master 1975; as *Ph. spinosa* var. *spinosa*, and *Ph. spinosa* var. *lobata*, respectively), and later transferred to *Halophytophthora* (Ho & Jong 1990; as *H. spinosa* var. *spinosa*, and *H. spinosa* var. *lobata*) based on their occurrence in estuarine environments. However, Nakagiri (2002) reported in a conference note that *S. spinosa* (referred to as *H. spinosa*) has close affinities to *Sapromyces* of *Rhipidiales*. Phylogenetic analyses in the present study revealed a strongly supported sister-group relationship between *Sapromyces elongatus*, which is the only species of *Rhipidiales* with sequences deposited at NCBI, and *Salispina*.

The family *Rhipidiaceae* includes *Araiospora* (Thaxter 1896), *Aqualinderella* (Emerson & Weston 1967), *Mindeniella* (Kanouse 1927), *Nellymyces* (Batko 1971), *Rhipidium* (Cornu 1871), and *Sapromyces* (Fritsch 1893). These taxa occur in freshwater habitats anchored to submerged twigs and fruits (Sparrow 1960, Beakes & Thines 2017). Most members of the family have arborescent thalli (except *Mindeniella* and *Sapromyces*) with a more or less distinct basal cell derived from a germinated zoospore (Minden 1916), a holdfast network, and all known members feature jointed or constricted hyphae, as well as stalked sporangia and gametangia (Sparrow 1960, Blackwell *et al*. 2015). Sporangia of members of the family are either aculeolate or smooth-walled. Examples with aculeolate sporangia include *Araiospora spinosa* (syn. *Rhipidium spinosum*) (Thaxter 1896), *A. coronata* (Linder 1926), *A. pulchra* (Kevorkian 1934), *A. streptandra* (Kevorkian 1934, Shanor & Olive 1942), *M. spinospora* (Kanouse 1927, Sparrow & Cutter 1941), *M. asymmetria* (Johnson 1951), and *N. megaceros* (Batko 1971). The formation of spines was believed to be influenced by the availability of nutrients in the substrate as outlined below. *Mindeniella* has the tendency to form aculeolate sporangia after colonies are well established in the substrate (Kanouse 1927, Sparrow & Cutter 1941). However, Sparrow (1960) mentioned that Ralph Emerson had informed him that there was a correlation between the formation of spines and the near absence of oxygen in axenic cultures. Zoospore release in the family is either directly through a discharge tube (e.g. *Aqualinderella fermentans*, *M. asymmetria*) or a vesicle (e.g. *Araiospora coronata*, *M. spinospora*, *R. americanum*). The discharge tube is generally formed at the sporangial apex, but its length varies in different species. Gametangia of *Rhipidiaceae* are often pedicellate, and some species apparently produce oospores parthenogenically (e.g. *Aqualinderella fermentans*, *M. spinospora*, *N. megaceros*, *R. parthenosporum*), similar to *Phytophthora insolita* (Ann & Ko 1980). Several members of *Rhipidiaceae* were reported to grow in low oxygen concentrations (e.g. *Aqualinderella*, *Mindeniella*, *Rhipidium*) (Emerson & Weston 1967, Gleason 1968, Dogma 1975, Natvig 1981) and, hence, can be considered as facultative anaerobes. Dick (2001) suggested in his diagnosis of the order *Rhipidiales* that members had either a facultative or an obligate fermentative metabolism.

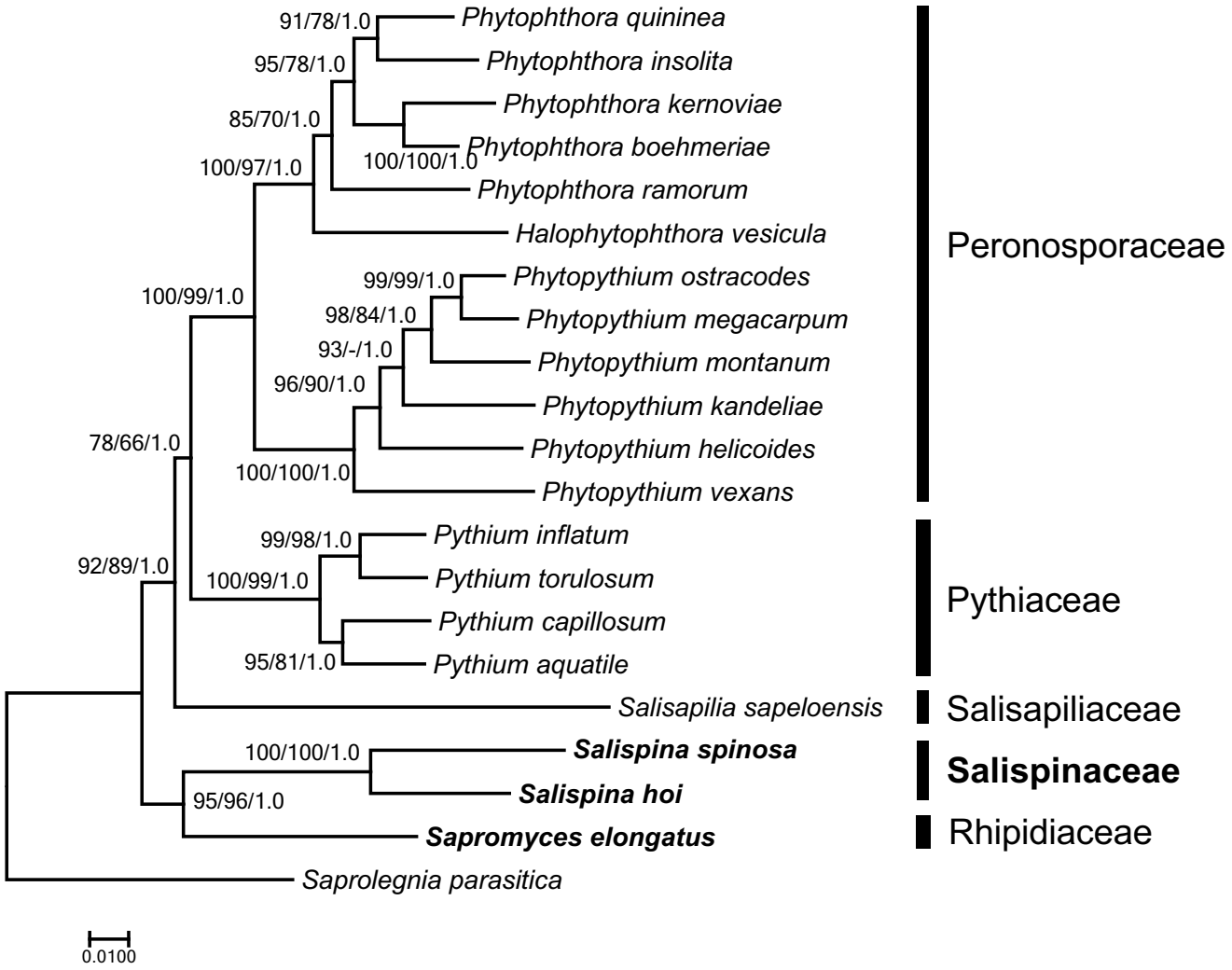

Fig. 3. Phylogenetic tree based on concatenated sequences of *cox*1, *cox*2, and LSU. Minimum Evolution (ME) was used as the primary tree with bootstrap support values from ME, and Maximum Likelihood (ML), and Bayesian posterior probability. (-) indicates bootstrap support values lower than 50 % or unsupported alternating topology from the corresponding primary tree. Scale bar indicates the number of substitutions per site.

In not displaying hyphal constrictions or stalked sporangia, *Salispina* is morphologically divergent from the accepted genera of *Rhipidiaceae*. Interestingly, Fell & Master (1975) inferred that nutrition plays an important role in the development of spines in *S. spinosa* (as *Phytophthora spinosa* var. *spinosa*), similar to the conclusions presented before by Kanouse (1927), Sparrow & Cutter (1941), and Sparrow (1960) for *Rhipidiaceae*. The three strains of *Salispina* (*S. lobata* CBS 588.85, *S. spinosa* CBS 591.85, and *Salispina* sp. USTCMS 1611) tested in this study were able to grow in a candle jar arrangement, where atmospheric oxygen is around 10–14 % and carbon dioxide about 2–5 % (Luechtefeld *et al*. 1982, El-Sherbeeny 1996). In a mangrove environment, abiotic factors (i.e. salinity, temperature, and oxygen concentration) constantly fluctuate (Leaño *et al.* 2000, Kathiresan 2004, Krauss *et al.* 2008). In particular, the oxygen concentration is often depleted during low tide, and gas production (e.g. CH_4, NH_3, H_2S) by anaerobic bacteria can be observed (Kathiresan 2004). This provides suitable conditions for both obligate or facultative anaerobes and microaerophiles. In line with the fermentative or microaerophilic habit observed for various members of *Rhipidiales*, *Salispina* sp. USTCMS 1611 showed normal vegetative growth in candle jars, but sporulation of members of *Salispina* was triggered by normal oxygen levels, and increased salinity and temperature, conditions that probably correspond to the early rise of the sea level after a low tide. While the physiological properties of *Salispina* support placement in *Rhipidiales*, the high morphological and phylogenetic divergence between *Salispina* and members of the *Rhipidiaceae* does not support a placement of *Salispina* in that family. Such a taxonomic classification would render the morphologically well-delineated family highly heterogenous. We therefore introduce the new family name *Salispinaceae* to accommodate the genus *Salispina*.

Salispina sp. USTCMS 1611 is a sister taxon to *S. lobata*, which has sporangia with a peculiar shape. Initially obpyriform, the sporangia of *S. lobata* subsequently develop lateral lobes until the sporangium looks botryose (Fell & Master 1975). However, USTCMS 1611 has ovoid, clavate, globose, to obpyriform sporangia, with some sporangia showing variations in shape, but not becoming botryose. In addition, the formation of spines appears to be different

between the two species, with most spines of USTCMS 1611 formed at the apex of the sporangium, while some sporangia have scattered spines or are even smooth-walled. In contrast, sporangia of *S. lobata* are either entirely or partially aculeolate (with no distinct pattern), or non-aculeolate (Table 3). Based on morphology and phylogenetic relationships, this strain cannot be assigned to any known taxon in *Salispina*, and so is described here as a new species.

This raises the number of known species in *Salispina* to four, but, given the still fragmentary knowledge regarding estuarine oomycetes in general and *Salispina* in particular, it seems likely that additional species of this genus will be discovered. In contrast to other orders of *Oomycota*, such as *Albuginales* (Choi *et al*. 2007, Thines *et al*. 2009, Ploch *et al*. 2010, Ploch & Thines 2011, Mirzaee *et al*. 2013), *Peronosporales* (Riethmüller *et al*. 2002, Voglmayr 2003, Voglmayr *et al.* 2004, Thines *et al*. 2006, 2007, Göker *et al*. 2007, Thines *et al*. 2008, 2015, Choi & Thines 2015), and *Saprolegniales* (Dick *et al*. 1999, Riethmüller *et al*. 1999, Leclerc *et al.* 2000, Spencer *et al.* 2002, Diéguez-Uribeondo *et al*. 2007, Hulvey *et al*. 2007, Steciow *et al.* 2013, Sandoval-Sierra *et al.* 2014, Steciow *et al.* 2014, Rocha *et al.* 2018), the *Rhipidiales* has received relatively little attention, probably owing to a lower degree of cultivation success from environmental samples due to their often microaerophilic to anaerobic nature. Thus, it seems promising to undertake targeted sampling in oxygen-depleted limnic environments in order to gain further insights into these understudied organisms which might play an important role in nutrient cycling.

TAXONOMY

Rhipidiales M. W. Dick, *Straminipilous Fungi:* 305 (2001).

Salispinaceae R. Bennett & Thines, **fam**. **nov**.
MycoBank MB824253

Diagnosis: Differs from *Rhipidiaceae* in the absence of conspicuous hyphal constrictions.

Type: *Salispina* Marano *et al.*, *Fungal Diversity* **78**: 198 (2016).

Salispina hoi R. Bennett & Thines, **sp. nov.**
MycoBank MB823076

Etymology: Dedicated to Hon Ho, for his pioneering studies into mangrove oomycetes.

Diagnosis: Differ from its sister taxon, *S. lobata* in sporangia that do not become botryose at maturity and from all species of the genus by a pronounced preference of spine formation at the apex and a quickly evanescing discharge tube.

Type: **Philippines**: Davao del Sur, 6.579667°N 125.453667°E, isolated from decaying mangrove leaf litter, 6 Sep. 2015, *R.M. Bennett*, *M.K. Devanadera*, *& G.R. Dedeles* (USTH 014145 – holotype; USTCMS 1611 – ex-type culture).

Description: *Mycelium* appressed on VJA and PCA. *Hyphae* 2–9 µm wide; septae forming at maturity, branching irregular; sporangiogenic hyphae not differentiated from vegetative hyphae, bearing a single terminal sporangium. *Sporangia*, shape ovoid, globose, obpyriform to variable; size (33.5–) 43–57.6–77.5(–87) × (10.5–)20–36.6–66(–75.5) µm; papilla absent, basal plug concave and hyaline; sporangial content vacuolate; surface aculeolate, with spines mostly forming at the apex of sporangia resulting in a crown-like appearance, some sporangia are smooth or with very few scattered spines. *Zoospores* discharge directly through a dehiscence tube; the apex of the tube deliquescent, allowing zoospores to escape from sporangia; vesicle absent. *Chlamydospores* not observed. *Gametangia* not observed.

Sequences: *cox*1 MG019399, *cox*2 MF991430, and LSU MG385863.

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RMB and MT conceived the study. RMB, MKD, and GRD arranged legal documents for collection, and conducted field sampling and isolation. RMB conducted laboratory work. RMB and MT analysed and interpreted the data. RMB and MT wrote the manuscript with contributions from the co-authors.

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Supplementary files can be found on the IMA Fungus website, http://www.imafungus.org/:

Fig. S1. Phylogenetic tree based on *cox*1 sequence data. Minimum Evolution (ME) was used as the primary tree with bootstrap support values from ME, and Maximum Likelihood (ML), and Bayesian posterior probability. (-) indicates bootstrap support values lower than 50% or unsupported alternating topology from the corresponding primary tree. Scale bar indicates the number of substitutions per site.

Fig. S2. Phylogenetic tree based on *cox*2 sequence data. Minimum Evolution (ME) was used as the primary tree with bootstrap support values from ME, and Maximum Likelihood (ML), and Bayesian posterior probability. (-) indicates bootstrap support values lower than 50 % or unsupported alternating topology from the corresponding primary tree. Scale bar indicates the number of substitutions per site.

Fig. S3. Phylogenetic tree based on LSU sequence data. Minimum Evolution (ME) was used as the primary tree with bootstrap support values from ME, and Maximum Likelihood (ML), and Bayesian posterior probability. (-) indicates bootstrap support values lower than 50 % or unsupported alternating topology from the corresponding primary tree. Scale bar indicates the number of substitutions per site.

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RMB and MT conceived the study; RMB and GRD processed all legal documents for collection, conducted field sampling and isolation of strains; RMB and BN conducted DNA extraction, PCR amplification, and sequencing; RMB conducted microscopy and taxonomic analyses; RMB and MT analyzed and interpreted data, and wrote the manuscript with contributions from the other authors

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ORIGINAL RESEARCH PAPER

Phytopythium leanoi sp. nov. and *Phytopythium dogmae* sp. nov., *Phytopythium* species associated with mangrove leaf litter from the Philippines

Reuel M. Bennett[1,2], Bora Nam[1,2], Gina R. Dedeles[3], Marco Thines[1,2,4,5]*

[1] Senckenberg Biodiversity and Climate Research Centre (SBiK-F), Georg-Voigt-Str. 14–16, 60325 Frankfurt am Main, Germany
[2] Department of Biosciences, Institute of Ecology, Evolution and Diversity, Goethe University Frankfurt am Main, Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany
[3] UST Collection of Microbial Strains, Research Centre for the Natural and Applied Sciences, College of Science, University of Santo Tomas, Manila, Philippines
[4] Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, 60325 Frankfurt am Main, Germany
[5] Integrative Fungal Research Cluster (IPF), Georg-Voigt-Str. 14–16, 60325 Frankfurt am Main, Germany

* Corresponding author. Email: m.thines@thines-lab.eu

Abstract
The genus *Phytopythium* is a monophyletic taxon of the Peronosporaceae with characteristics intermediate between *Phytophthora* and *Pythium*. In the Philippines, reports of *Phytopythium* are scarce, with the mangrove-swamp-inhabiting species *Phytopythium kandeliae* being the only species recorded to date. It was the aim of the current study to investigate the diversity of *Phytopythium* in mangrove habitats in more detail. Based on culture characteristics, morphology, and molecular phylogenetic position, two new species of *Phytopythium* are described from Philippine mangroves, *P. leanoi* USTCMS 4102 and *P. dogmae* USTCMS 4101. *Phytopythium leanoi* is a species morphologically similar to *P. kandeliae*, but with the ability to develop gametangia in a homothallic fashion. The other new species, *P. dogmae*, is characterized by having a short discharge tube, semipapillate to papillate sporangia and frequently exhibiting a clustering of two sporangia per sporangiogenic hypha. With the addition of the two species described in this study, the genus *Phytopythium* has grown from around 10 to beyond 20 recognized species over the past decade, and it seems likely that several more species of this genus await discovery.

Keywords
mangroves; Oomycetes; Peronosporaceae; *Phytopythium*

Introduction

Oomycetes are fungal-like organisms of the eukaryotic kingdom Straminipila. To date, approximately 2000 species striving in different habitats ranging from the arctic to tropics have been described [1]. The discovery of new lineages that branch outside the major genera of Peronosporales [2–7] has fueled research into the evolution and diversity of cultivable oomycetes of the Peronosporales [8]. Of the recently-described oomycete lineages, the former K-clade *Pythium* species [5,9] have attracted intense research and were segregated taxon from *Pythium* in 2010 [2] as members of the new genus *Phytopythium* on the basis of phylogenetic position and morphology. Uzuhashi et al. [7] as well inferred the polyphyly of *Pythium* based on large ribosomal subunit region (LSU) and *cytochrome oxidase* II (*cox*2) gene sequences and proposed four segregate genera, *Elongisporangium*, *Pilasporangium*, *Globisporangium*, and *Ovatisporangium*.

The genus *Ovatisporangium* was described specifically for the clade K of *Pythium*, however, this genus is a synonym of *Phytopythium*, as the print version of the latter genus description appeared earlier than the former. The characteristic features of *Phytopythium* as described by Bala et al. [2] are: globose to ovoid shaped sporangia that are often papillate, internal proliferation of sporangia, zoospore discharge similar to *Pythium*, smooth and large oogonia, thick-walled oospores and elongate to lobate lateral antheridia. *Phytopythium sindhum* is the type species of this group, isolated from a banana (*Musa paradisiaca* L.) field in Pakistan.

In the Philippines, mangrove oomycetes were reported by Leaño [10], including a single *Phytopythium* species, *P. kandeliae* [1] (basionym *Halophytophthora kandeliae*), which was isolated alongside *Halophytophthora vesicula*, *H. bahamensis*, *H. epistomium*, and *Salispina lobata* (homotypic synonym *H. spinosa* var. *lobata*) [10]. Considering that during the past decade several new species of *Phytopythium* have been discovered and that mangroves have been found to harbor a variety of oomycete species [10–12], it was the aim of this study to evaluate if additional species of the genus *Phytopythium* might be present in Philippine mangroves.

Material and methods

Isolation, growth on solid media, and *Phytopythium* strains

Fallen senescent mangrove leaves from various areas in the Philippines were collected and placed in sealed plastic bags. Leaves were blot-dried, cut into strips of approximately 1–2 mm width, and transferred onto clarified-vegetable juice agar (VJ) [medium No. 15, NBRC (http://www.nite.go.jp/en/nbrc/cultures/media/culture-list-e.html), using V8 Juice (Campbell, USA) or Gemüsesaft (Alnatura, Germany), with or without addition of seawater] with nystatin (500 mg/mL) and rifampicin (30 mg/mL) or streptomycin (0.5 mg/mL). Coenocytic hyphae growing from the edge of the leaf strips were cut and placed on new VJ media with antibiotics until axenic. Cultures were maintained in VJ with or without antibiotics. Incubation was done at room temperature in the dark for 7 days. Additional strains of *Phytopythium kandeliae* NBRC 32620, *Phytopythium* sp. CBS 113.91, *Phytopythium* sp. CBS 111.91, *P. chamaehyphon* CBS 259.30, and *P. helicoides* CBS 286.31 were either purchased from NITE Bioresource Centre (NBRC, Japan) or the Westerdijk Fungal Biodiversity Centre Culture Collection (KNAW, The Netherlands).

The mean radial colony growth of the Philippine strains at 20, 25, 30, and 35°C was assessed on VJ agar [medium No. 15, NBRC; using Gemüsesaft (Alnatura, Germany)] and three media as formulated by CBS [13]: potato carrot agar [PCA; based on Demeter Karotten mit Kartoffeln purée (Alnatura, Germany)], potato dextrose agar (PDA), and peptone yeast-extract glucose agar (PYGA). Colony radial growth was measured for 5 days and values were expressed as mm/day following the method outlined by Solis et al. [14]. For an initial testing at room temperature, isolates were also tested for growth on oatmeal and corn meal agar as formulated by CBS [13].

Induction of sporangia and gametangia

Sporangia and gametangia were induced using 6 mL saline (sea salt) solution at 0, 10, 20, and 30 g/L poured onto 3–7-day-old mycelia in VJ agar plugs in 60-mm Petri dishes. Plates were incubated at room temperature until sporangia and gametangia were formed. Alternatively, sporulation was induced in 90-mm culture plates using 6 mL unsterile soil extract (500 g soil in 500 mL distilled water, settled for 1–2 days and filtered with double-layered cheesecloth) and 6 mL saline solution as above. The set-up was incubated in a climate chamber (CMP 6010; Conviron, Germany) with continuous light and alternating constant temperature at 20°C for 6 h and 23°C for 18 h. Structures were observed and photos were taken using a Canon Digital Camera EOS 500D (Canon, Japan) attached to a Motic AE31 trinocular inverted microscope (Motic, Germany).

Tab. 1 Primer combinations used for PCR amplification.

Loci	Primer	Primer sequence (5′–3′)	Reference
ITS	ITS1-O	CGG AAG GAT CAT TAC CAC	[26]
	LR0	GCT TAA GTT CAG CGG GT	[27]
LSU	LR0R	ACC CGC TGA ACT TAA GC	[27]
	LR6-O	CGC CAG ACG AGC TTA CC	[28]
*cox*1	OomCox1_Levup	GCT TAA GTT CAG CGG GT	[29]
	OomCox1_Levlo	CYT CHG GRT GWC CRA AAA ACC AAA	[29]
*cox*2	cox2-F	GGC AAA TGG GTT TTC AAG ATC C	[30]
	cox2-RC4	TGA TTW AYN CCA CAA ATT TCR CTA CAT TG	[31]

DNA extraction, PCR, and phylogenetics

DNA extraction was performed on a BioSprint 96 Kingfisher flex robot (Thermo Fisher Scientific, USA) using a Qiagen plant tissue DNA extraction kit (Qiagen GmbH, Germany). Primer pairs for PCR amplification of the internal transcribed spacers (ITS), large nuclear ribosomal subunit (nrLSU), *cytochrome oxidase* I (*cox*1), and *cytochrome oxidase* II (*cox*2) are listed in Tab. 1.

The PCR reaction mix contained 1× PCR buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.8 µg BSA, 0.4 µM of each primer, 0.5 U *Taq* polymerase and 10–50 ng DNA. Cycling conditions for ITS were as follows: initial denaturation at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 20 s, and elongation at 72°C for 60 s. Subsequently, a final elongation at 72°C for 4 min was carried out. For LSU, initial denaturation was at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 53°C for 20 s, and elongation at 72°C for 120 s. Subsequently, a final extension was carried out at 72°C for 7 min. Cycling conditions for the *cox*2 region were: initial denaturation at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 40 s, annealing at 51°C for 40 s, and elongation at 72°C for 40 s. Subsequently, a final elongation was carried out at 72°C for 4 min. The *cox*1 locus was amplified with the following cycling conditions: initial denaturation at 95°C for 4 min, followed by 36 cycles of denaturation at 95°C for 40 s, annealing at 51°C for 40 s, and elongation at 72°C for 60 s. Subsequently, a final extension was carried out at 72°C for 5 min. PCR reactions were carried out on an Eppendorf Mastercycler Pro (Eppendorf AG, Germany). PCR products were sequenced at SBiK-F sequencing laboratory with the primers used for PCR. Sequences were analyzed, assembled into contigs, and edited using Geneious version 5.0.4 (Biomatters Ltd., USA). Edited contigs in FASTA format and other sequences obtained from the NCBI webserver (https://www.ncbi.nlm.nih.gov/) (Tab. S1) were uploaded to the TrEase webserver (http://www.thines-lab.senckenberg.de/trease/) for the alignment of sequences and phylogenetic tree construction. The sequence alignment was generated using MAFFT with the G-INS-i algorithm [15]. Phylogenetic trees were constructed for all loci using the following programs: FastTree for minimum evolution (ME) [16] with 1000 bootstrap replicates using the generalized time-reversible (GTR) model; RAxML for maximum likelihood (ML) with 1000 bootstrap replicates using the GTR-GAMMA model [17]; and MrBayes [18] for Bayesian inference and the calculation of Bayesian posterior probabilities (BPP) using the 6 GTR model and four incrementally heated chains run for 1 000 000 generations, sampling every 10 000th generation, with the first 30% of the trees discarded for ensuring sampling from the stationary phase. After confirming that no strongly supported alternate topologies were present, the individual alignments were concatenated into a single FASTA file and the phylogenetic inference was done on the combined dataset as described above. Phylogenetic trees were viewed and taxon labels were edited using MEGA, version 6 or 7 [19].

Results

Phytopythium cultures

The two *Phytopythium* isolates, USTCMS 4102 (Fig. 1) and USTCMS 4101 (Fig. 2), were isolated from mangrove leaf litter in the Philippines. Both isolates were obtained from yellow to brown leaves that were in direct contact with the water surface of the estuarine system.

Mean colony radial growth (Fig. 3) of *Phytopythium* sp. USTCMS 4101 and *Phytopythium* sp. USTCMS 4102 varied in relation to the agar medium used. Specifically, mean colony radial growth on vegetable juice agar was observed to be 11.25 and 6.75 mm/day at 25 and 30°C, respectively, for the former strain, 9.25 and 10.25 mm/day at 25 and 30°C, respectively, for the latter strain. However, USTCMS 4101 was also capable of growing at 35°C with a radial growth similar to the growth observed at 30°C. In contrast, *Phytopythium* sp. USTCMS 4102 showed limited growth on all culture media when incubated at 35°C.

Phytopythium sp. USTCMS 4102 sporulated within 1 day in saline solution at 10–30 g/L when incubated at room temperature (20–25°C) in the dark. *Phytopythium* sp. USTCMS 4101 sporulated in an unsterile soil extract solution with continuous light at 20–23°C.

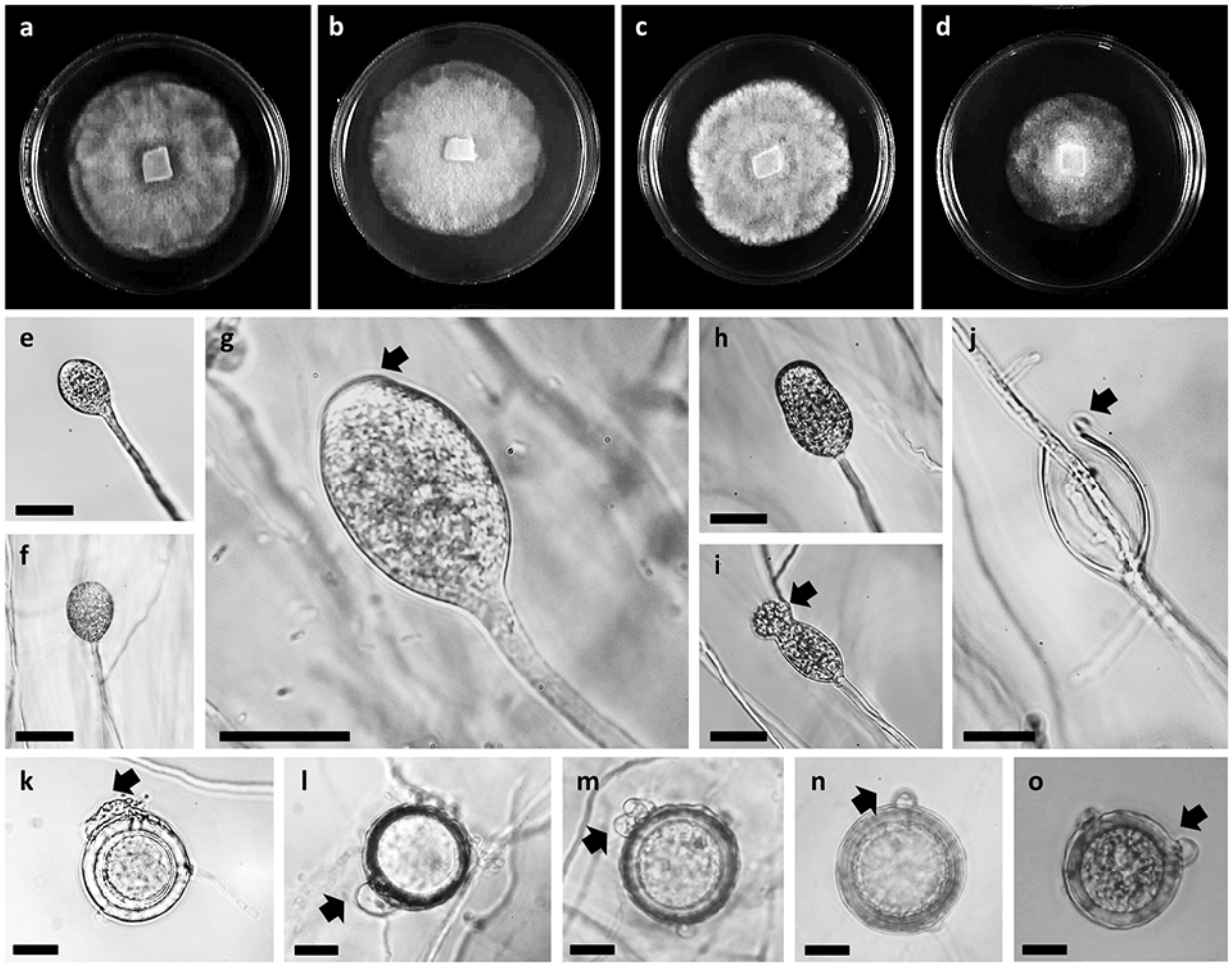

Fig. 1 Morphology of *Phytopythium leanoi* USTCMS 4102. Colony patterns on (**a**) vegetable juice agar, (**b**) potato carrot agar, (**c**) peptone yeast glucose agar, and (**d**) potato dextrose agar. **e,f** Immature sporangia. **g** Semipapillate sporangium, hyaline, and (**h**) a nonpapillate sporangium. **i** Zoospore release, zoospores develop both in the sporangium and vesicle (arrow). **j** Sporangium proliferation, arrow pointing to the operculum, which sometimes curls distally from the sporangium after some time. **k** Elongate, lobate antheridium with constrictions, oospore plerotic. **l** Lobate antheridium. **m** Antheridium with two lobes, oospores thick-walled, plerotic. **n,o** Single-lobed antheridia, oogonium with plerotic oospore. Scale bars: 20 μm.

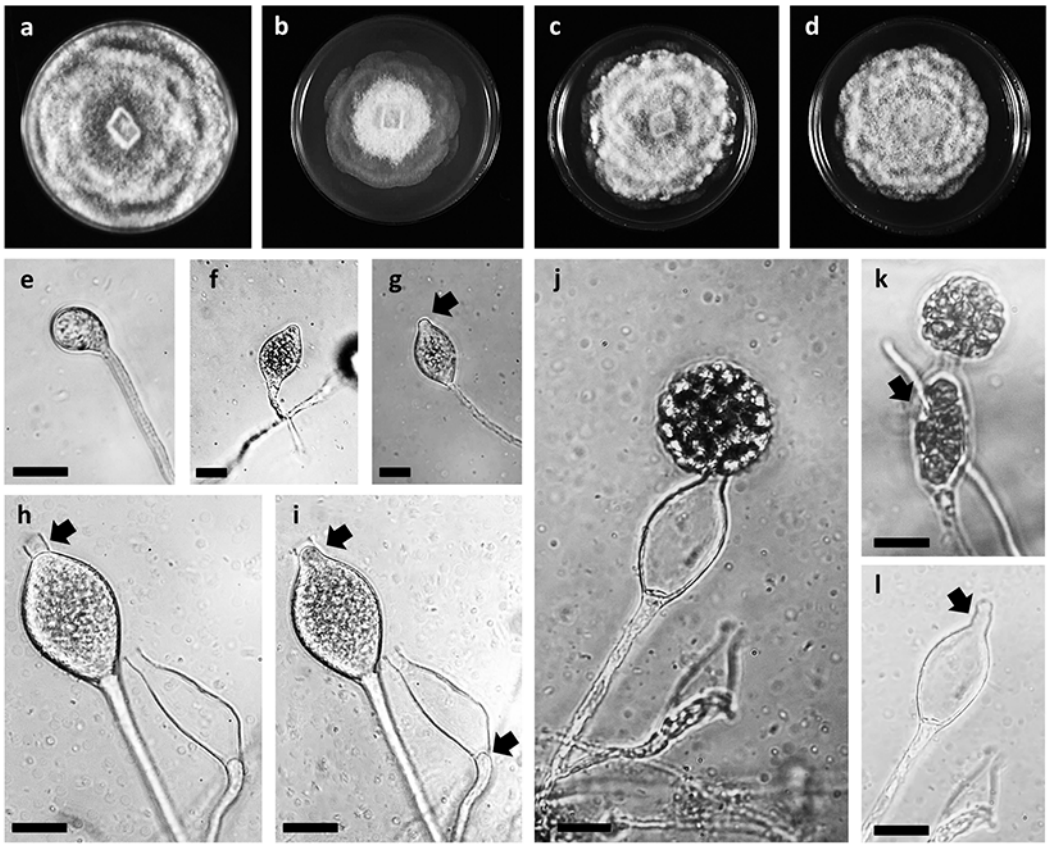

Fig. 2 Morphology of *Phytopythium dogmae* USTCMS 4101. Colony patterns on (**a**) vegetable juice agar, (**b**) potato carrot agar, (**c**) peptone yeast glucose agar, and (**d**) potato dextrose agar. **e** Immature sporangium. **f** Developing sporangium with a developing papilla. **g** Papillate sporangium (arrow). **h,i** Papilla develops into a discharge tube that guides the discharging protoplasma (arrow) forming an external vesicle that will nest at the apex of the sporangia. **i** Convex basal plug (arrow). **j** Development of zoospores in the vesicle. **k** Zoospores can similarly mature and develop inside the sporangia. **l** Empty sporangium; note the presence of the short discharge tube (arrow). Scale bars: 20 µm.

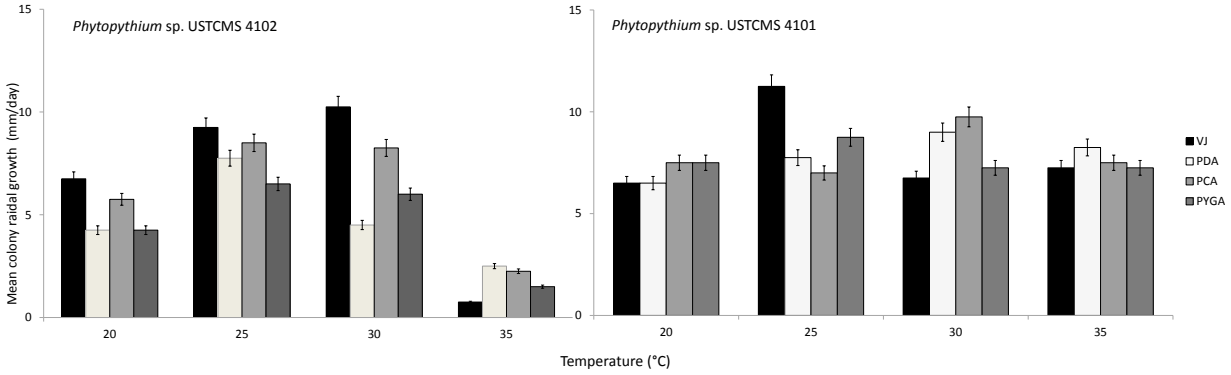

Fig. 3 Mean colony radial growth of *Phytopythium leanoi* USTCMS 4102 and *Phytopythium dogmae* USTCMS 4101.

Morphology

Phytopythium sp. USTCMS 4102 (Fig. 1) was initially identified as *Phytopythium* aff. *kandeliae* based on the mode of zoospore release and shape of sporangia. *Phytopythium* sp. USTCMS 4102 developed non- to semipapillate, obovoid to pyriform sporangia, with a size of 22–34 × 27–43 µm (average 28 × 35 µm; n = 100) within the standard deviation. Proliferation was both internal and external and branching sympodially. Zoospores of this strain developed both within vesicles and sporangia, and, upon

maturity, biflagellate cells moved rapidly inside the vesicle before rupture and release. An operculum was visible once sporangia were empty, which curled distally after some time. Interestingly, USTCMS 4102 is a homothallic species. Oogonia were formed either terminally or intercalary, with a size of 36–46 µm (average 41 µm; n = 100) within the standard deviation; oospores were mostly plerotic with a size of 31–41 µm (average 36 µm; n = 100) within the standard deviation. The antheridia were either lobate with constrictions, elongate, or bilobed and were attached at places all over the oogonial surface.

USTCMS 4102 is distinct from *P. kandeliae* NBRC 32620 (Tab. 2) on the basis of development of intercalary or terminal oogonia with an elongate to cylindrical antheridium and on the process by which sporangia proliferate – internal and external for the former and external for the latter.

Sporangia of USTCMS 4101 were obovoid, ovoid to pyrifom and limoniform, with a size of 25–27 × 29–37 µm (average 26 × 33 µm; n = 100) within the standard deviation. A papilla was formed at maturity, which further developed into a short dehiscence tube (mostly 2–10 µm long). Before zoospore release, the protoplasmic component of the sporangia gradually passed through the apical discharge tube until it came to a rest at

Tab. 2 Morphological differences between *P. kandeliae* and *P. leanoi*.

Characters	*P. leanoi* (USTCMS 4102)	*Phytopythium* sp. (CBS 113.91)	*P. kandeliae* (NBRC 32620 ex-type)	*Phytopythium* sp. (CBS 111.91)
Pattern on VJ plate	Abundant aerial mycelium, forming slightly petaloid colonies on VJ plate	Abundant aerial mycelium, forming slightly petaloid colonies on VJ plate	Abundant aerial mycelium, forming petaloid colonies on VJ plate	Abundant aerial mycelium, forming petaloid colonies on VJ plate
PDA, PYGA, PCA	Petaloid, rosette-like	Petaloid, rosette-like	Petaloid, rosette-like	Petaloid, rosette-like
Mature sporangium shape	Obovoid, pyrifom	Not observed	Turbinate, obovate, pyriform	Turbinate, obovate, pyriform
Papilla/apical projection	Non/semi papillate	Not observed	Non/semipapillate; presence of apical crescent-shaped translucent matrix	Non/semipapillate
Sporangium size	22–34 × 27–43 µm (average 28 × 35 µm)	Not observed	25–49 × 20–36 µm	22–56 × 20–45 µm
Proliferation type	Internal and external proliferation	Not observed	External proliferation	External proliferation
Branching pattern	Sympodial	Not observed	Sympodial	Sympodial
Operculum	Present	Not observed	Present	Present
Basal plug	Present	Not observed	Present	Present
Zoospore release	*Pythium*-like	Not observed	*Pythium*-like	*Pythium*-like
Antheridium	Elongate or cylindrical with constrictions; 1(–2) antheridia per oogonium	Not observed	Not observed	Not observed
Oogonium and oospores	Terminal or intercalary oogonia, 36–46 µm (average 41 µm); oospores plerotic, some are aplerotic; thick-walled, 31–41 µm (average 36 µm)	Not observed	Not observed	Not observed
Chlamydospores	Not observed	Not observed	Not observed	Not observed

its apex. Zoospores developed inside the vesicle and often also inside the sporangium. After some time, zoospores gradually began to move within the vesicle until the rupture of the vesicle and the subsequent release of zoospores took place. No gametangia were observed for USTCMS 4101. In Tab. 3, the morphological differences of species closely related to USTCMS 4101 are summarized.

Tab. 3 Morphology of Clade 2 species of *Phytopythium*.

Characters	*P. dogmae* USTCMS 4101	*P. helicoides* [32]	*P. chamaehyphon* [33,34]	*P. fagopyri* [23]	*P. paligenes* [35]
Pattern on VJ plate	Petaloid, rosette	No specific pattern, dense mycelia	No specific pattern, dense mycelia [33]	*Chrysanthemum*-like	*
Mature sporangium shape	Obovoid, ovoid, pyriform, limoniform	Ovoid to globose	Subspherical	Subglobose, pyriform	Subspherical, ovoid
Papilla/apical projection	Present, papilla develops into discharge tube at maturity (2–10 µm)	Present, papilla could grow approximately 20 µm serving as discharge tubes	Exit tube (dehiscence tube) 5 µm [33]	Present, papilla develops into discharge tube where vesicle exists	Present, develops into an evacuation tube (2–35 µm)
Sporangium size	25–27 × 29–37 µm (average 26 × 33 µm)	30.2–5.5 × 24.5–35.2 µm	15–30 µm [33]; 18–28 µm [34]	27–37 × 17–39 µm	24–42 × 18–36 µm
Proliferation type	External/internal (more frequent) proliferation	Internal proliferation	Internal and internally nested [34]	Internal or internally nested	Internal or internally nested
Operculum	Absent	Absent	*	*	*
Basal plug	Absent	Absent	*	*	*
Zoospore release	Zoospores formed in the vesicle or inside the sporangium (contains ~18–30 zoospores)	*Pythium*-like	*	Formation of vesicles (contained ~20 zoospores)	Zoospores formed in a vesicle, 6–30 zoospores
Antheridium	Not observed	Elongated, smooth, wavy, applied laterally to oogonia; 1–2 antheridia per oogonium	Not observed [33]; monoclinous, diclinous, applied laterally, broadly to the oogonium [34]	1–3 antheridia per oogonium, mainly diclinous	1–4 antheridia, average of 2, per oogonium, diclinous
Oogonium and oospore	Not observed	Terminal, intercalary, or lateral oogonia, 29.2–34.9 µm in diameter; oospores aplerotic, 23.7–32.5 µm in diameter	Not observed in artificial media, only on host [33]; oogonia smooth and globose, 33.5 µm in diameter on average, oospores aplerotic, 30.5 µm in diameter on average [34]	Present, oogonia globose, 28–31 µm in diameter; oospores aplerotic, 23–25 µm in diameter	Terminal, intercalary, or lateral oogonia, 19–41 µm in diameter; oospore 18–37 µm in diameter
Chlamydospores	Not observed	Not observed	Not observed	*	*
Radial growth on potato carrot agar at 25°C (mm/day)	7.75	15 [23]	22 [23]	10	*

* No data available.

Phylogeny

Based on the concatenated sequences of ITS, *cox*1, *cox*2, and LSU, USTCMS 4102 belongs to Clade 1 [20,21] of *Phytopythium* (Fig. 4, Fig. S1–Fig. S4). Interestingly, *P. kandeliae* is split into several distinct lineages. The ex-type specimen *P. kandeliae* NBRC 32620 [22] grouped together with the strain CBS 111.91 in trees based on all loci used, whereas USTCMS 4102 grouped with the strain CBS 113.91. Both CBS strains 111.91 and 113.91 were isolated from Taiwan and are referred to on the KNAW website as an uncharacterized species of *Phytopythium*. The strain *P. kandeliae* AJM 26, which was isolated from Brazil, clustered with the ex-type sequences of *P. kandeliae* in phylogenies based on ITS (Fig. S1), *cox*1 (Fig. S2), and LSU (Fig. S4). *Phytopythium* sp. USTCMS 4101 (Fig. 2) grouped with *P. fagopyri*, *P. palingenes*, *P. chamaehyphon* CBS 259.30, and *P. helicoides* CBS 286.31 (Fig. 4, Fig. S1–Fig. S4), all members of Clade 2 of *Phytopythium* [23,24].

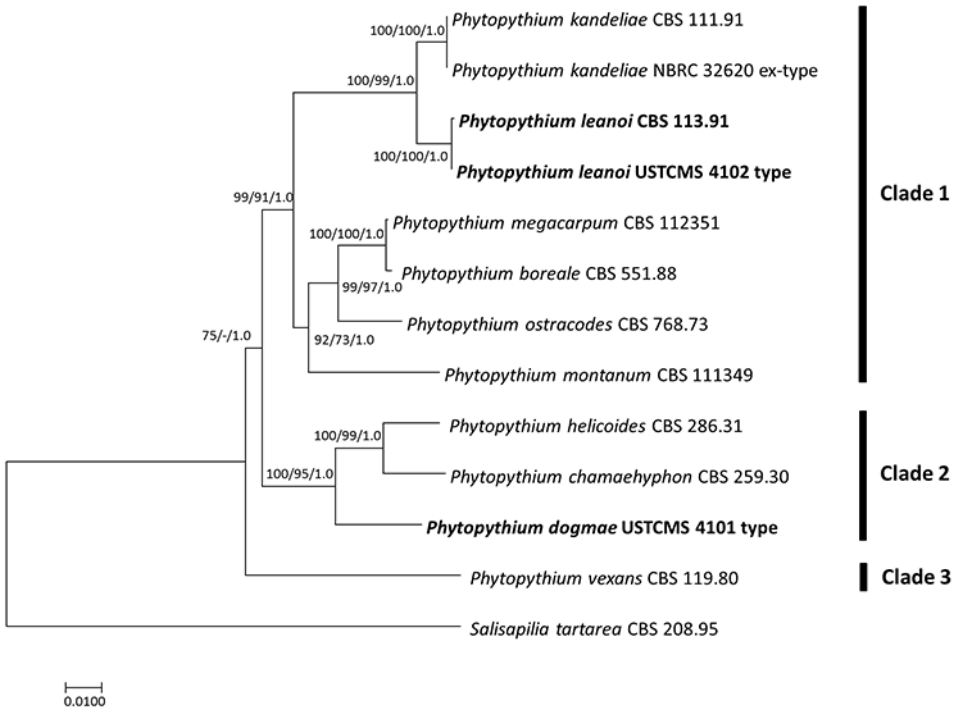

Fig. 4 Minimum evolution phylogenetic reconstruction generated from concatenated sequences of ITS, *cox*1, *cox*2, and LSU. Support values from minimum evolution, and maximum likelihood bootstrapping, as well as Bayesian posterior probabilities are given on the branches in the respective order. "-" denotes support values below 50 or alternate, unsupported topologies. The scale bar indicates the number of nucleotide substitutions per site.

Discussion

According to de Cock et al. [20], key characteristics for *Phytopythium* are the following: predominantly papillate sporangia and internal proliferation in most species as well as often constricted and elongate to cylindrical antheridia. However, there is a large degree of variation in sporangium characteristics. Phylogenetically, the *Calycofera-Phytopythium* lineage [25] is one of the early-diverging branches in the sister clade to *Pythium* that also harbors *Phytophthora* [2]. This is reflected by some morphological features shared with the one or the other of these genera, i.e., a sporangial shape similar to the majority of *Phytophthora* species and a process of zoospore release similar to most *Pythium* species.

The strains USTCMS 4102 and USTCMS 4101 constitute two new records of *Phytopythium* species from the Philippines, adding to *P. kandeliae* [10]. Both strains were isolated from mangrove leaf litter, where they acted as saprotrophs.

The strain USTCMS 4102 was inferred to be a member of the Clade 1 [23,24] group of *Phytopythium* (Fig. 4) and is almost sequence-identical to CBS 113.91 isolated from Taiwan. The ex-type strain of *P. kandeliae* NBRC 32620 is phylogenetically distinct from the aforementioned strains but sequence-identical to CBS 111.91 in all loci included in this study. Sporangia of *P. kandeliae* NBRC 32620 and CBS 111.91, as well as those of USTCMS 4102 and CBS 113.91, are operculate and non- to semipapillate [21] (also, this study). However, sporangia for the former two strains were turbinate, obovate to pyriform, and formed an apical translucent crescent-shaped matrix [22] (also, this study), while the sporangia of the latter two strains were obovoid to pyriform and without an apical crescent-shaped translucent matrix. The proliferation of the sporangia was external for *P. kandeliae* NBRC 32620 and CBS 111.91, while it was both internal and external for USTCMS 4102. With respect to colony morphology, there are also some differences between the groups, as USTCMS 4102 and CBS 113.91 formed rosette-petaloid-like colonies with rounded edges, whereas the colony edges of *P. kandeliae* NBRC 32620 and CBS 111.91 were acute (Tab. 4). Even if these differences might seem minor, they were consistently observed and thus seem stable enough to allow for differentiation.

Tab. 4 Colony patterns of *P. leanoi* and *P. kandeliae*.

Species	PCA	PGYA	VJ	PDA
Phytopythium leanoi USTCMS 4102 ex-type				
Phytopythium leanoi CBS 113.91				
Phytopythium kandeliae NBRC 32620 ex-type				
Phytopythium kandeliae CBS 111.91				

Phylogenetic analyses presented herein are in line with those of Baten et al. [23] and Jesus et al. [24], where *Phytopythium* was divided into three clades based on LSU, ITS, *cox*1, and *cox*2 sequences. Clade 1 includes 15 taxa and these are *P. aichiense*, *P. boreale*, *P. carbonicum*, *P. citrinum*, *P. delawarense*, *P. iriomotense*, *P. litorale*, *P. mercuriale*, *P. megacarpum*, *P. mirpurense*, *P. montanum*, *P. oedochilim*, *P. ostracodes*, *P. sindhum*, and *P. sterilum*. *Phytopythium chamaehyphon*, *P. fragopyri*, *P. palingenes*, and *P. helicoides* were the taxa of the monophyletic Clade 2. *Phytopythium cucurbitacearum* and *P. vexans* are those of Clade 3. Baten et al. [23] construed that the taxa of Clades 1 and 2 have papillate sporangia and proliferate internally (nested and extended) as well as externally; Clade 3 species have nonpapillate sporangia and do not proliferate internally.

USTCMS 4101 is a member of the Clade 2 of *Phytopythium* [23,24], and differs from all other members of the clade by featuring both internal and external proliferation, while other members of this clade are featuring extended or nested-internal proliferation [20]. USTCMS 4101 is similar to *P. chamaehyphon*, *P. fagopyri*, *P. helicoides*, and *P. palingenes*. However, it has a different combination of key morphological characters

(e.g., proliferation of the sporangia and the number of sporangia per sporangiogenic hypha; see also Tab. 3). The sister taxon to USTCMS 4101 is *P. palingenes*, from which it differs in terms of the proliferation of sporangia, the length of discharge tubes (which is longer in *P. palingenes*), and the number of sporangia per sporangiogenic hypha, which are often two in USTCMS 4101 but usually single in *P. palingenes*.

Based on phylogenetic distinctiveness and combination of morphological characters, neither USTCMS 4101 nor USTCMS 4102 can be assigned to any known species of *Phytopythium* and are thus introduced as new species in the "Taxonomy" section of this paper. Considering the recent discovery of some new species of *Phytopythium* from a variety of habitats and that only mangroves were sampled in the current study, it seems likely that additional species of *Phytopythium* await discovery in the Philippines and beyond.

Taxonomy

Phytopythium leanoi R. Bennett et Thines, sp. nov.
Mycobank No.: MB 819795

Type specimen. Isolated from the Philippines, Pagbilao, Quezon, ERDB Pagbilao Mangrove Forest (13.975698° N, 121.725363° E) from mangrove leaf-litter, collected on September 19, 2015, leg. RM Bennett & GR Dedeles, holotype herbarium specimen USTH 013930 (= PQ2YBP293) (University of Santo Tomas Herbarium, Manila, Philippines), ex-type strain USTCMS 4102 (University of Santo Tomas Collection of Microbial Strains).

Etymology. Dedicated to Eduardo Leaño, in recognition of his contributions to the knowledge on oomycetes of the Philippines.

Description. Straminipila, Oomycota, Peronosporales, Peronosporaceae.Hyphae hyaline, aseptate. Sporangiophores undifferentiated, branching sympodially. Sporangia nonpapillate to semipapillate, obovoid to pyriform, noncaducuous, typically 22–34 × 27–43 µm (average 28 × 35 µm), proliferation predominantly external submerged in 10–30 g/L saline solution. Basal plug present, hyaline. Zoospore release *Pythium*-like, operculum present. Homothallic. Oogonia globose, lateral, terminal or intercalary, typically 36–46 µm (average 41 µm) in diameter. Oospores plerotic, sometimes aplerotic, typically 31–41 µm (average 36 µm) in diameter, oospore wall thickness 2–5 µm. Antheridia one(–two) per oogonium, often elongate, cylindrical and constricted, laterally attached to the oogonium, monoclinous or diclinous. Chlamydospores not observed.

Colony characteristics. Grows on corn meal agar (CMA), oatmeal agar (OA), peptone yeast glucose agar (PYGA), potato carrot agar (PCA), and vegetable juice agar (VJ). Growth on VJ with abundant aerial mycelium. Colonies stellate to rosette-like with rounded edges on all culture media used; except OA and CMA, and when old in VJ agar.

Known distribution. Philippines, Taiwan.

Phytopythium dogmae R. Bennett et Thines, sp. nov.
Mycobank No.: MB 819796

Type specimen. Isolated from the Philippines, Padada, Davao del Sur (6.659833° N, 125.380733° E), from decaying mangrove leaf-litter, collected on the September 5, 2015, leg. RM Bennett & GR Dedeles, holotype herbarium specimen USTH 013931 (= P1YBL1144) (University of Santo Tomas Herbarium, Manila, Philippines), ex-type strain USTCMS 4101 (University of Santo Tomas Collection of Microbial Strains, Philippines).

Etymology. Dedicated to Irineo J. Dogma Jr., for his pioneering research on "zoosporic fungi" in the Philippines.

Description. Straminipila, Oomycota, Peronosporales, Peronosporaceae. Hyphae hyaline, aseptate. Sporangiophores undifferentiated, branching sympodially, producing one to two terminal sporangia. Immature sporangia nonpapillate, ovoid to obovoid. Mature sporangia papillate, limoniform, obovoid, obpyriform, pyriform, noncaducuous, typically 25–27 × 29–37 µm (average 26 × 33 µm). Proliferation internal and external. Basal plug convex in empty sporangia. Zoospore release *Pythium*-like, papilla developing into a discharge tube (~2–10 µm); after the dissolution of the tip of the dehiscence tube, the protoplasmic component ejects and nests at the apex of the tube enclosed by a vesicle; zoospores maturing typically inside the vesicle or sometimes inside the sporangium. Operculum absent. Gametangia not observed. Chlamydospores not observed.

Colony characteristics. Petaloid or rosette-like on VJ agar and PCA media with or without marine water. Grows on CMA, OA, PYGA, and PCA. Mycelia on VJ, PDA, and PYGA are typically with abundant aerial mycelium.

Known distribution. Philippines.

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Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/am/rt/suppFiles/am.1103/0:

Tab. S1 GenBank accession numbers of various loci used in this study.

Fig. S1 Phylogenetic tree based on minimum evolution using ITS sequence data with support values from ME, ML, and BPP, in the respective order.

Fig. S2 Phylogenetic tree based on minimum evolution using *cox*1 sequence data with support values from ME, ML, and BPP, in the respective order.

Fig. S3 Phylogenetic tree based on minimum evolution using *cox*2 sequence data with support values from ME, ML, and BPP, in the respective order.

Fig. S4 Phylogenetic tree based on minimum evolution using LSU sequence data with support values from ME, ML, and BPP, in the respective order.

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ORIGINAL ARTICLE

Calycofera gen. nov., an estuarine sister taxon to *Phytopythium*, Peronosporaceae

Reuel M. Bennett[1,2] · **Arthur W. A. M. de Cock**[3] · **C. André Lévesque**[4] · **Marco Thines**[1,2,5]

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Abstract The genus *Halophytophthora* is a predominantly marine or estuarine genus of the oomycete family Peronosporaceae. This genus has been revealed as a highly polyphyletic assemblage of largely unrelated species. A new genus of the Peronosporaceae, *Calycofera*, is introduced in this manuscript to accommodate *Halophytophthora operculata*. *Calycofera* is distinct compared to *Phytopythium* s. str. and to *Halophytophthora* s. str. on the basis of sporangium morphology, sporangiogenous hyphae, and the development and release of zoospores. Phylogenetic analyses using ITS, *cox*2 and *cox*1 sequences strongly supported the establishment of this taxon. In addition, a second species of the genus was found, which is genetically more divergent from *C. operculata* than most sister species in the sister genus *Phytopythium*, and is described as *C. cryptica* based on diagnostic nucleotides.

Keywords Cryptic species · DNA-based diagnosis · DNA-based species description · *Halophytophthora* · Peronosporaceae · *Phytopythium*

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✉ Marco Thines
marco.thines@senckenberg.de

[1] Biodiversity and Climate Research Centre (BiK-F), Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, 60325 Frankfurt am Main, Germany

[2] Department of Biosciences, Institute of Ecology, Evolution and Diversity, Goethe University Frankfurt am Main, Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany

[3] Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584, CT Utrecht, The Netherlands

[4] Ottawa Research and Development Centre, Agriculture and Agri-Food Canada, Ottawa, ON K1A 0C6, USA

[5] Integrative Fungal Research Cluster (IPF), Georg-Voigt-Str. 14-16, 60325 Frankfurt am Main, Germany

Introduction

The genus *Halophytophthora* was described in 1990 to accommodate *Phytophthora*-like species thriving in mangrove or estuarine ecosystems. Ho and Jong (1990) argued that these species are better accommodated into a genus of their own, *Halophytophthora*, not only on the basis of their ecological niche but also based on differences in sporangial morphology (e.g. proliferation, shape and sporangial apical structure), mode of zoospore release (e.g. through the formation of an evanescent, semi-persistent and persistent vesicle), and some other characteristics (e.g. hyphal diameter, development of gametangia). However, the addition of some *Phytophthora*-like species to *Halophytophthora* (e.g. *H. tartarea* and *H. kandeliae*) was primarily based on habitat and substrate, i.e. the estuarine system and mangrove leaf litter, and species with similar morphological characteristics are also present in other genera containing facultative saprotrophs, e.g. *Phytophthora* and *Phytopythium*. With the advent of molecular phylogenetics, *Halophytophthora* was inferred to be polyphyletic, and some species classified to *Halophytophthora* were transferred to different genera, e.g. *Salisapilia tartarea* (basionym: *H. tartarea*) (Hulvey et al. 2010) and *Phytopythium kandeliae* (basionym: *H. kandeliae*) (Thines 2014). Likewise, the species *H. operculata* has been shown not to belong to the genus *Halophytophthora* s. str., but to have close affinities with the genus *Phytopythium* (de Cock et al.

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2015), from which it differs in terms of sporangial shape, as well as proliferation and morphology of sporangiogenous hyphae. However, as no strong support for a placement of the species within or apart from *Phytopythium* could be obtained in previous studies (de Cock et al. 2015; Marano et al. 2014), *H. operculata* had neither been transferred to *Phytopythium* (which would have rendered the otherwise morphologically well-defined genus rather heterogeneous) nor to a genus of its own. Thus, it was the aim of this study to clarify the phylogenetic position of *H. operculata* to resolve its generic placement.

Materials and methods

Specimen identification and phylogenetic analysis

Halophytophthora operculata strains NBRC 32629 (=ATCC 44952, CBS 241.83) and NBRC 32865 were investigated in this study. Cultivation, sporulation assays, and light microscopy was carried out as described in Bennett and Thines (2017). DNA was extracted using a phenol–chloroform–isoamyl alcohol (Carl Roth, Karlsruhe, Germany) method as outlined in Bennett et al. (2017). PCR amplification for *cox*2 was done using the primers (Table 1) described in Choi et al. (2015a), *cox*1 was amplified with the primers reported in Robideau et al. (2011), and ITS regions were amplified using the primer combination given in Choi et al. (2011). PCR reactions were carried out in 25-μl reaction mixtures containing 1× PCR Buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.8 μg BSA, 0.4 μM of each primer, 0.5 U *Taq* polymerase, and 10–50 ng DNA. Cycling conditions for ITS were as follows: initial denaturation at 94 °C for 4 min, followed by 36 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 20 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 4 min. Cycling conditions for the *cox*2 region were as follows: initial denaturation at 94 °C for 4 min, followed by 36 cycles of denaturation at 94 °C for 40 s, annealing at 51 °C for 40 s, elongation at 72 °C for 40 s, and final elongation at 72 °C for 4 min. *cox*1 was amplified with a cycling program of initial denaturation at 95 °C for 4 min followed by 36 cycles of denaturation at 95 °C for 40 s, annealing at 51 °C for 40 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. PCR reactions were carried out on an Eppendorf Mastercycler equipped with a vapoprotect lid (Eppendorf, Hamburg, Germany).

PCR amplicons were sequenced at the BiK-F sequencing laboratory with the primers used for PCR and sequences were edited using Geneious, v.5.0.4 (Biomatters, USA). FASTA files of edited sequences together with manually selected published sequences in NCBI from strains with available ITS, *cox*1, and *cox*2 sequences were uploaded to the TrEase webserver (http://www.thines-lab.senckenberg.de/trease/) (Mishra et al. 2017). Sequences were aligned using MAFFT with the G-INS-i model (Katoh et al. 2002). Phylogenetic trees for single loci were generated using FastTree for Minimum Evolution (Price et al. 2009) with 1000 bootstrap replicates, FastTree2 for Maximum Likelihood (Price et al. 2010) with 1000 bootstrap replicates and MrBayes for Bayesian posterior probabilities (Ronquist et al. 2012) were run for 10 M generations with every 1000th tree sampled and subsequently discarding the trees from the first 3 M generations to ensure that trees were only sampled from the stationary phase. Substitution models were the standard models of the TrEase server for FastTree and MrBayes and of PAUP 4.0a151 (Swofford 2002) for Maximum Parsimony analysis. Heuristic searches in Maximum Parsimony were done using the Tree Bisection and Reconstruction (TBR) branch swapping algorithm. For Maximum Parsimony, maxtrees were set to 100, gaps were treated as missing data, and branches were collapsed if the maximum branch length was zero. For testing the robustness of the phylogenetic reconstruction, 1000 bootstrap replicates were carried out. Alignments of the single loci (ITS, *cox*1, and *cox*2) were concatenated into a single fasta alignment and a phylogenetic tree following the above-mentioned algorithms was generated. Single gene-based phylogenetic trees and the concatenation-based phylogenetic tree were viewed using MEGA, version 6 or 7 (Tamura et al. 2013).

Results

Morphology

Halophytophthora operculata is a taxon with a very distinct sporangium shape. Mature sporangia are cylindrical to tulip-glass-shaped (Fig. 1e–g), and zoospores develop within the sporangia (Fig. 1e–h). Sporangia of *H. operculata* have an operculum (Figs. 1i, j) which is seen during zoospore release. Once zoospores are completely released, the operculum curls (Fig. 1h). A hyaline protruding basal plug (Fig. 1j) is another feature of *H. operculata* (*Phytopythium kandeliae* is another species with a basal plug; however, there is no proximal protrusion as in *H. operculata*). Similarly, some *Phytophthora* spp. have a conspicuous basal plug, e.g. *P. quercina* (Jung et al. 1999), *P. citricola* (Jung and Burgess 2009), and *P. gemini* (Man In 'T Veld et al. 2011).

Another well-defined feature of *H. operculata* is the formation of twisted sporangiogenous hyphae (Fig. 1k, o). This structure is evident in both immature (Fig. 1o) and mature sporangia (Fig. 1h). For the latter there is a basolateral outgrowth thus forming a sympodial and usually monochasial type of proliferation (Fig. 1m, n). Often, a secondary hypha develops from a sporangiogenic hypha right at the base of a previously-formed sporangium and sometimes develops again into a sporangiogenous hypha (Fig. 1k, o). While immature sporangia (Fig. 1a–d) are ovoid, globose, ellipsoid, or obovoid, as the sporangia of *H. operculata* mature, they develop into cylindrical to tulip-glass shapes and with a flat and broad

Table 1 Primer pairs used for PCR amplification

Locus	Primer	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*Cox*1_Levup	GCT TAA GTT CAG CGG GT	Robideau et al. 2011
	Oom*Cox*1_Levlo	CYT CHG GRT GWC CRA AAA ACC AAA	Robideau et al. 2011
*cox*2	*cox*2-F	GGC AAA TGG GTT TTC AAG ATC C	Hudspeth et al. 2000
	*cox*2-RC4	TGA TTW AYN CCA CAA ATT TCR CTA CAT TG	Choi et al. 2015a

apex (Fig. 1e–h). Zoospores fully mature within the sporangia (Fig. 1e–h). Upon sporangial germination, no vesicle or discharge tube was observed for the ex-type strain NBRC 32629 and zoospores were released as the operculum opens at the broad apex of the sporangia. The strain NBRC 32865 showed a vegetative growth similar to the ex-type strain of *H. operculata*, but did not sporulate during this study.

Phylogenetic analyses

Based on ITS, *cox*2, and *cox*1, *Halophytophthora operculata* is the sister taxon to *Phytopythium*, see Figs. S1, S2, and S3, respectively. As no strongly supported conflicting topologies were present, the three loci were concatenated to produce a multigene phylogenetic reconstruction (Fig. 2). In the multigene analyses, the sister-group relationship of *Phytopythium* and *H. operculata* received high to maximum support (Fig. 2). The strain NBRC 32865, which had been assigned as *H. operculata*, is genetically different from the ex-type strains of *H. operculata* (NBRC 32629, CBS 241.83) by 6.6% in ITS, 4.5% in *cox*2, and 5.4% in *cox*1, a divergence greater than for most sister species in the sister genus, *Phytopythium*. There are several diagnostic bases at various specific positions in the alignments for the three loci, as highlighted in Fig. 3 and given in the Taxonomy section.

Discussion

In the phylogenetic analyses of de Cock et al. (2015), an ex-type strain of *Halophytophthora operculata*, CBS 241.83, was found to be basal to *Phytopythium* s. str. using LSU (large subunit) and ITS markers. However, *cox*1 and SSU (small subunit) analyses showed alternate topologies, with the ex-type specimen nested within *Phytopythium*. However, de Cock et al. (2015) did not suggest a new combination nor propose a separate genus for this taxon, since its morphology is strongly deviating from *Phytopythium* and there were

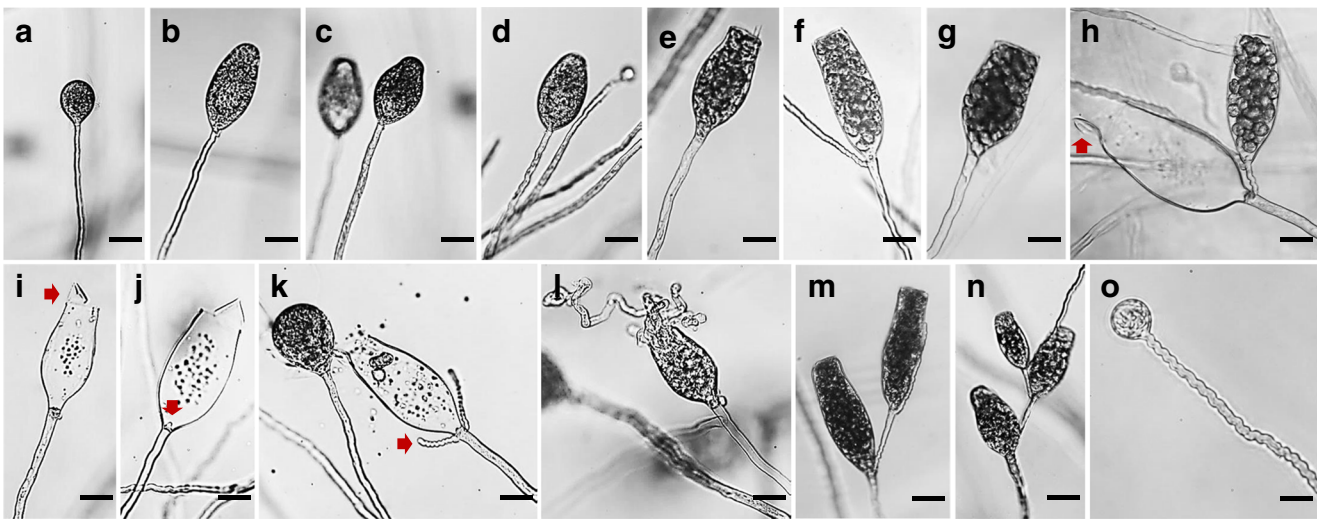

Fig. 1 Morphology of an ex-type strain (NBRC 32629) of *Calycofera operculata*. **a** Immature sporangium. **b**–**d** Developing sporangia. **e**–**h** Mature sporangia. The shape is often narrowly ellipsoid, cylindrical, or elongated, with a narrow base and a flat and broad apex; non-caducous and smooth-walled. **h** Operculum curls away from the main axis of the sporangium. **i** Empty sporangium with an operculum. **j** Sporangium showing the operculum and a distinct protruding basal plug. **k** Empty sporangium with developing basal hyphae (*arrow*); the sporangium proliferates externally. **l** Unreleased zoospores germinating inside a sporangium. **m**, **n** External proliferation of sporangia. **o** Immature sporangium on a twisted sporangiogenous hypha. Scale bars equal 20 μm

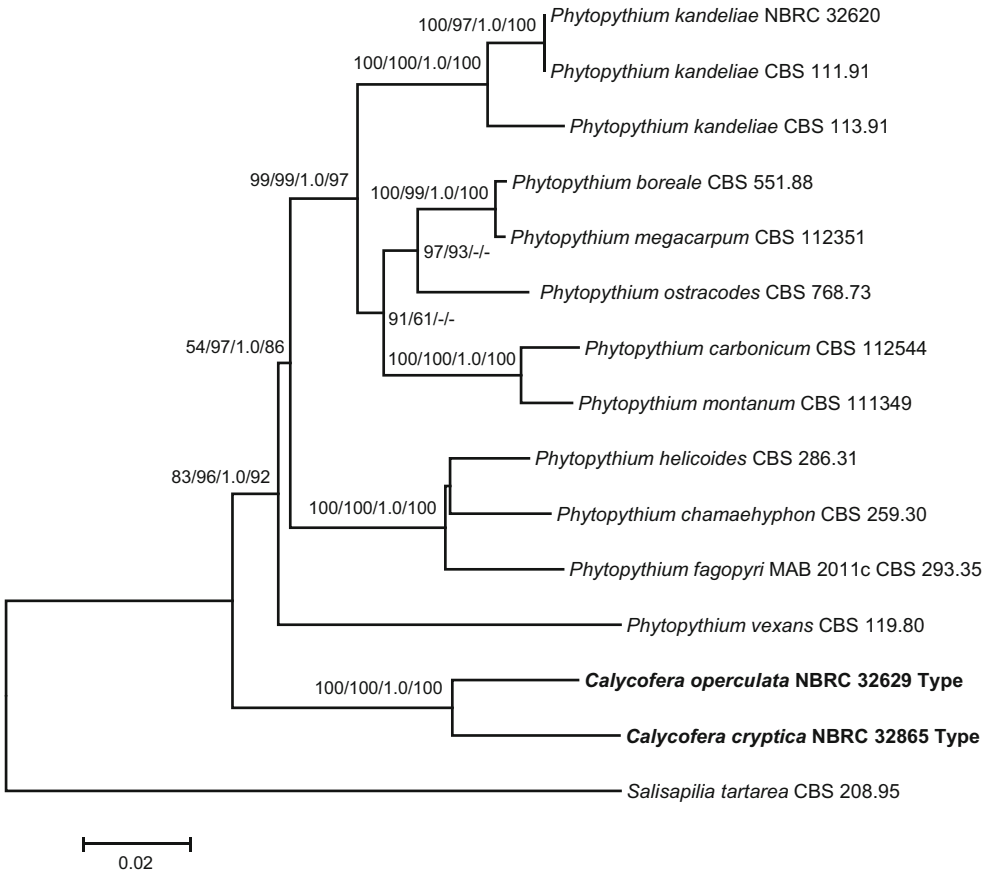

Fig. 2 Multigene phylogeny of concatenated *cox*1, *cox*2, and ITS sequences based on Minimum Evolution phylogenetic reconstruction with bootstrap support values from Minimum Evolution and Maximum Likelihood analyses, Bayesian posterior probabilities, and bootstrap support values from Maximum Parsimony analysis, in the respective order. The *scale bar* indicates the number of substitutions per site in ME analysis; (−) denotes support values <50% or an unsupported conflicting topology in the respective analysis

conflicting tree topologies, respectively. Marano et al. (2014) included *H. operculata* WPC 15747C1319 in an SSU phylogeny, in which it was placed basal to *Phytopythium* s. str. But the SSU sequences were 700 bp shorter and the sampling covered only 5 of the 15 species of the genus as compared to de Cock et al. (2015), and no further analyses using additional genetic markers were given. In the present study, using a combined analysis of ITS, *cox*2, and *cox*1 sequences, the sister-group relationship of *H. operculata* and *Phytopythium* received high to maximum support. Considering the short genetic distance between the proposed genus and *Phytopythium*, and the strong morphological evidence provided below, there is sufficient phylogenetic evidence to propose a new genus in this particular case. However, it should be noted that these markers are primarily for species identification, and thus care has to be taken when applying these loci at higher levels because of potential homoplasy.

The morphology and development of *H. operculata* differs markedly from species of the sister genus, *Phytopythium*. While sporangia are cylindrical to tulip-glass-shaped in *H. operculata* (Pegg and Alcorn 1982; this study), they are globose to ovoid in the genus *Phytopythium* (Bala et al. 2010; de Cock et al. 2015). The mode of zoospore discharge for this taxon is unique and distinct when compared to *Phytopythium*. Members of *Phytopythium* typically develop zoospores in a *Pythium*-like manner within a vesicle or occasionally inside the sporangia in some species. *Phytopythium sindhum*, the type species of *Phytopythium*, forms a vesicle in which the undifferentiated protoplasmic component develops into zoospores (Bala et al. 2010). Similarly, *P. mirpurense*, like most *Phytopythium* species, develops its zoospores in a vesicle (de Cock et al. 2015). Other *Phytopythium* species develop a short or longer discharge tube, e.g. *P. helicoides* and *P. palingenes* (Chen et al. 2016; de Jesus et al. 2016). In *Phytopythium kandeliae* zoospores develop within the sporangia and are discharged through an extruded vesicle. However, in *H. operculata*, the zoospores fully mature within the sporangia and are discharged through an operculum at the broad-apex of sporangia. Proliferation is internal to external in species of *Phytopythium* (Abdul Baten et al. 2014; Bala et al. 2010; Chen et al. 2016; de Cock et al. 2015; de Jesus et al. 2016; Ho et al. 1991), while it is right from the base of sporangia by spiral hyphae in *H. operculata*.

Thus, the morphology of *H. operculata* does neither agree with the consensus morphology of *Phytopythium* s. str. in terms of morphological characteristics of the sporangiogenous hyphae and sporangia, nor in terms of the development and release of zoospores. As a consequence of its placement as the sister group

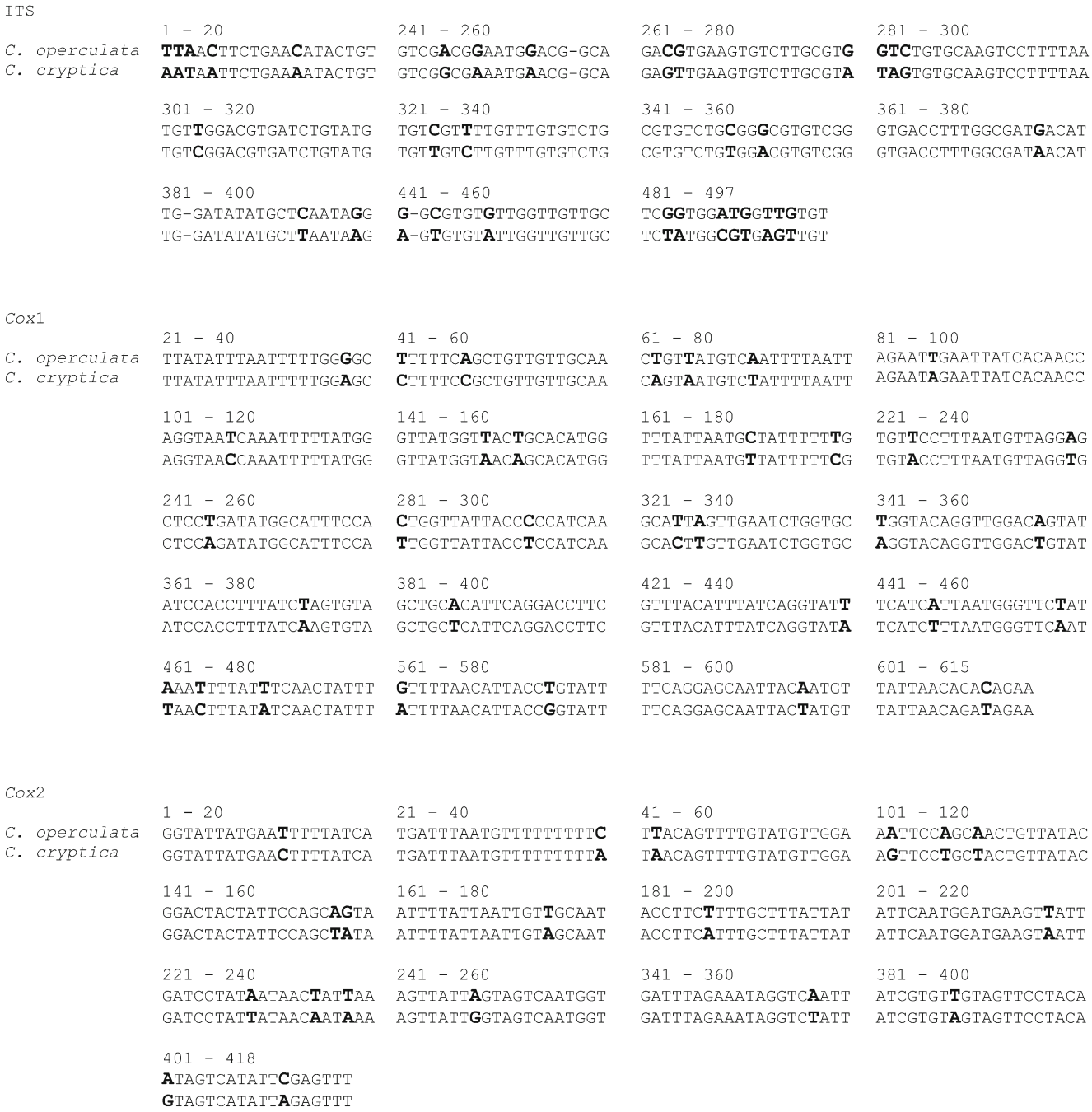

Fig. 3 Sequence alignment of ITS, *cox*1, and *cox*2 loci used to determine diagnostic bases differentiating between *C. operculata* and *C. cryptica* (*in bold*)

to *Phytopythium* and various morphological features unique to *H. operculata*, this species should be accommodated in a genus of its own instead of transferring it to the genus *Phytopythium*. This taxonomic treatment also conserves *Phytopythium* as a morphologically well-delineated genus.

The presence of species complexes that are difficult to resolve based on morphology is common in oomycetes due to the limited amount of available morphological characteristics, e.g. in the biotrophic genera *Peronosclerospora* (Telle et al. 2011) and *Plasmopara* (Rouxel et al. 2013), as well as in the hemibiotrophic *Phytophthora citricola* species complex (Jung and Burgess 2009). Such cryptic species can be identified using phylogenetic approaches alongside host specificity. However, host specialisation cannot be used to delineate saprotrophic oomycetes that do not cause diseases. However species complexes were also found in saprotrophs feeding on litter of mangrove tree species, e.g. in *Phytophthora insolita* (Bennett and Thines 2017), a

pathogen of citrus (Ann and Ko 1980; Puglisi et al. 2017) and *Phytophthora elongata* (Bennett et al. 2017), a pathogen of *Eucalyptus marginata* (Rea et al. 2010). There is no fixed threshold or measure of the barcoding gap or the genetic distance to define species boundaries that would be applicable throughout oomycetes or true fungi. In many biotrophic genera, a difference of less than 1% in ITS or *cox*2 sequences will delineate well-defined species in terms of genetic distinctiveness and ecological differentiation (Choi and Thines 2015; Choi et al. 2015b). However, the host-specific *Pythium tracheiphilum* shows up to 1% of intraspecific variation in ITS (Schroeder et al. 2013). For saprotrophic species without substrate specialisation, it is not clear which thresholds are useable, if any. There are some ecologically and often morphologically well-defined species of facultative saprotrophs, e.g. in the genera *Phytophthora*, *Phytopythium*, and *Pythium* that are separated by less than 2% of sequence divergence between the sister species (Allain-Boulé et al. 2004; Goodwin et al. 1999; Jung and Burgess 2009; Rea et al. 2010). The strain NBRC 32865 is phylogenetically more divergent from the ex-type strain of *H. operculata* than most sister species within the sister genus *Phytopythium*, with around 5% divergence in several barcoding loci (Robideau et al. 2011; Choi et al. 2015a; Schroeder et al. 2013). The unusually high genetic distance in markers normally used primarily for identification in this case is a solid basis for the description of a new species. Such variation is in the intraspecific range of species complexes as represented by *P. vexans*, as well as species complexes in *Pythium* s.l., as represented by *Pythium irregulare* and *Py. ultimum*, which likely need to be split. However, using thresholds alone is risky, as there might be species with significant intraspecific variation. Thus, such delineation should only be done after careful consideration and after a thorough search for differentiating morphological characters and/or for markers that can assess gene flow. The potential for crosses and gene flow still need to be evaluated as for most sister species of facultative and obligate saprotrophic oomycetes.

Even with the recognition of *H. operculata* and its cryptic sister species as members of a new genus, the genus *Halophytophthora* is still largely polyphyletic (Lara and Belbahri 2011; Hulvey et al. 2010; Nigrelli and Thines 2013) and will require further substantial revision.

Taxonomy

Calycofera R. Bennett & Thines, **gen. nov.**, MB 819429.

Type: *Calycofera operculata* (K.G. Pegg & J.L. Alcorn) R. Bennett & Thines.

Etymology: *Calyx*, chalice; *−ferre*, to bear; to bear sporangia that are shaped like a narrow chalice or tulip-glass.

Diagnosis: *Calycofera* is the sister genus to *Phytopythium* but differs significantly from the consensus morphology of *Phytopythium* on the basis of sporangium shape, which is globose, ovate, obovate to obovoid for *Phytopythium*, but cup-like for *Calycofera*. In addition, the proliferation of sporangia is internal, sometimes nested, or external with non-spiral sporangiogenous hyphae in *Phytopythium*, but by external, initially spiral sporangiogenous hyphae, leading to monochasial sympodia in *Calycofera*.

Calycofera operculata (K.G. Pegg & J.L. Alcorn) R. Bennett & Thines, **comb. nov.**, MB 819430.

Basionym: *Phytophthora operculata* K.G. Pegg & J.L. Alcorn, Mycotaxon 16: 99 (1982).

Homotypic synonym: *Halophytophthora operculata* (K.G. Pegg & J.L. Alcorn) Ho & Jong, Mycotaxon 36: 381 (1990).

Type: A herbarium specimen, BRIP 13362, was deposited at the Queensland Plant Pathology Herbarium (BRIP). The culture was isolated from *Avicennia marina* at Moreton Bay, Queensland on 18 June 1980. Ex-type strains are deposited as ATCC 44952 (USA), CBS 241.83 (The Netherlands) and NBRC 32629 (Japan). (GenBank numbers of ex-type sequences: ITS KY604973, *cox*1 KY604974, *cox*2 MF598482).

Calycofera cryptica R. Bennett, J. Kruse & Thines, **sp. nov.**, MB 819431.

Type: A dried specimen of the culture of *C. cryptica* NBRC 32865 stored in the herbarium Senckenbergianum at Görlitz (international acronym GLM) under the accession number GLM-F110480. The initial isolation of the culture was from a submerged yellow leaf of *Avicennia* sp. at Belize on 4 September 1996.

Etymology: refers to the cryptic nature of the species.

Known distribution: Belize.

Diagnosis: Differs from *Calycofera operculata* in several diagnostic bases as highlighted in Fig. 3, i.e. in having transitions G ↔ A at positions 245, 248, 253, 280, 352, 376, 399, 441, 449, and 484 in the ITS alignment, at positions 102, 158, 248, and, 401 in the *cox*2 alignment, and at positions 38 and 561 in the *cox*1 alignment, as well as T ↔ C at positions 304, 324, 327, 349, 394, and 444 in the ITS alignment, at position 12 in the *cox*2 alignment, and at positions 41, 107, 171, 179, 281, 293, 324, 464, and 611 in the *cox*1 alignment, and transversions T ↔ A at positions 1, 2, 3, 282, and 492 in the ITS alignment, 42, 107, 110, 157, 175, 187, 217, 229, 235, 238, 357, and 388 in the *cox*2 alignment, and 62, 65, 71, 86, 149, 152, 224, 239, 245, 326, 341, 356, 374, 386, 440, 446, 458, 461, 470, and 596 in the *cox*1 alignment, as well as C ↔ A at positions 5, 13, and 488 in the ITS alignment, at positions 40 and 412 in the *cox*2 alignment, and at position 47 in the *cox*1 alignment, as well as C ↔ G at positions 263 and 283 in the ITS alignment, as well as T ↔ G at positions 264, 281, 483, 489, 490, 493, and 494 in the ITS alignment, and at position 575 in the *cox*1 alignment. (GenBank numbers of ex-

type sequences: ITS KY604972, *cox*1 KY604975, *cox*2 MF598483).

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Discussion

Mangrove oomycetes: A diverse group in the estuarine environment

Oomycetes are fungal-like protists that are distributed world-wide (Thines 2014). The majority of well-studied oomycetes are those that are found in the terrestrial and freshwater environments. Some terrestrial oomycetes are either pathogens of plants, e.g. downy mildews (e.g. *Bremia*, *Peronospora* and *Plasmopara*), white blister rusts (e.g. *Albugo*), blight (*Phytophthora*) and stem and root damping-off (*Pythium*) (Lévesque 2011) or are pathogens of mammals (e.g. *Pythium insidiosum*) (De Cock et al. 1987). Furthermore, the genus *Pythium* is often found in soil and grasses (Plaats-Niterink 1981). On the other hand, several pathogenic oomycetes from the freshwater biome have also been well investigated, including the freshwater crayfish pathogen *Aphanomyces astaci* (Schikora 1903, Filipova 2013) and several species of the *Saprolgenia* group (Humphrey 1892). Of the different environments colonized by oomycetes, the mangrove environment is the least explored biome in contrast to the terrestrial and freshwater environments.

Mangroves are plant species that are highly abundant in several tropical regions (e.g. the Philippines). These plant species are mainly present in the estuarine environment; an estuary is said to be situated between a freshwater and a marine environment (Kristensen 2008, Ghizelini et al. 2012). Of the different microflora thriving in a mangrove ecosystem, oomycetes have been underexplored or neglected for several years as compared to true fungi (e.g. Ascomycetes and Basidiomycetes). Mangrove oomycetes, or estuarine oomycetes, are common colonizers of fallen mangrove leaf litter (Pegg and Forsberg 1981, Nakagiri et al. 1989, Tan and Pek 1997, Nakagiri 2000, Nakagiri et al. 2001, Leaño et al. 2000, Leaño 2001, Bennett and Thines 2017, Bennett et al. 2017a, 2017b, 2017c, 2018, Bennett and Thines 2019). Furthermore, it has been inferred that mangrove oomycetes are considered to be the first colonisers of fallen mangrove leaves (Newell et al. 1987, Nakagiri et al. 1989, Leaño 2001) and other substrates (e.g. driftwoods and decayed roots) (Pegg and Foresberg 1981, Leong et al. 1988, Tan and Leong 1992). The symbiotic relationship between a mangrove plant and an oomycete has always been construed to be saprotrophic, while no literature has dealt with the

capability of mangrove oomycetes to act as pathogens of mangrove plants, not even those from known species of *Phytophthora* from mangrove areas (Table 2).

Of the recorded mangrove oomycetes in recent literature and from those presented in this study (summarized in Table 2), mangrove oomycetes are said to be distributed into three oomycete families: Peronosporaceae, Salisapiliaceae and Salispinaceae. Based on existing records, there are thirty known species of mangrove oomycetes (Table 2), mainly reported from the tropical and subtropical regions (Leaño et al. 2000, Leaño 2001). Papers presented in this thesis, alongside other publications (Ho and Jong 1990, Bala et al. 2010, Hulvey et al. 2010), have contributed to the establishment of families (e.g. Salispinaceae and Salisapiliaceae), genera (e.g. *Calycofera*, *Halophytophthora*, *Phytopythium*, *Salispina* and *Salisapilia*), species (e.g. *Calycofera cryptica*, *Phytopythium dogmae*, *Phytopythium leanoi*, *Salispina hoi* and *Salisapilia coffeyi*), combinations (e.g. *Phytopythium kandeliae*, *Salisapilia bahamensis*, *Salisapilia elongata*, *Salisapilia epistomium*, *Salisapilia masteri*, *Salisapilia mycoparasitica* and *Salisapilia tartarea*) and even emendations (e.g. *Salisapilia nakagirii*) in order to establish a coherent taxonomy and phylogeny of mangrove oomycetes. Oomycetes from the mangrove environment are regarded as a phylogenetically rich group since some studies (Hulvey et al 2010, Nigrelli and Thines 2013) revealed lineages that are potentially new species of *Halophytophthora* (Peronosporaceae) and *Salisapilia* (Salisapiliaceae). *Phytopythium* and *Phytophthora* are groups that should be studied further since some congeners of these taxa are pathogens of terrestrial plants and could hinder the conservation of mangrove plants. It is, therefore, proposed that the mangrove environment is a cryptic habitat harboring *Phytophthora* spp. and possibly other novel species (Bennett and Thines 2017, Bennett et al. 2017b).

Table 2. Mangrove oomycetes recorded in literature.

	Substrate	Country	Reference
Peronosporaceae			
Phytophthora			
Ph. elongata	Mangrove leaf litter	Australia, Philippines	Bennett et al. 2017a
Ph. estuarina	*Laguncularia racemosa* *Rhizophora mangle*	Brazil	Li et al. 2016
Ph. insolita	Mangrove leaf litter	India, South China, Philippines, Taiwan, USA	Bennett and Thines 2017
Ph. inundata	*Zostera marina*	The Netherlands	Man In 'T Veld et al. 2011
Ph. gemini	*Zostera marina*	The Netherlands	Man In 'T Veld et al. 2011
Ph. rhizophorae	*Rhizophora mangle*	Brazil	Li et al. 2016
Halophytophthora			
H. avicenniae	*Avicennia marina*	Australia, Taiwan	Gerrettson-Cornell and Simpson 1984, Pang et al. 2014
H. batemanensis	Mangrove leaf litter *Avicennia marina*	Australia, Japan, Philippines	Gerrettson-Cornell and Simpson 1984, Nakagiri 2000
H. exoprolifera	Mangrove leaf litter *Rhizophora mangle*	Bahamas, Japan, Philippines	Ho et al. 1992
H. polymorphica	*Eucalyptus* sp.	Australia, Taiwan	Gerrettson-Cornell and Simpson 1984, Pang et al. 2014
H. porrigovesica	Mangrove leaf litter *Sonneratia alba*	Japan, Philippines	Nakagiri et al. 2001
H. vesicula	*Prunus laurocerasus* (in seawater) *Avicennia*, *Rhizophora*, *Sonneratia*, *Xylocarpus*	Canada, Japan, Philippines, Taiwan	Anastasiou and Chuchland 1969 Nakagiri 2000, Leaño 2001, Pang et al. 2014, Caguimbal et al. 2019
Phytopythium			
Pp. dogmae	Mangrove leaf litter	Philippines	Bennett et al. 2017b
Pp. kandeliae	*Avicennia lanata* *Rhizophora apiculate* *Sonneratia* sp. *Kandelia candel*	Brazil, Japan, Philippines, Taiwan	Nakagiri 2000, Leaño 2001, Marano et al. 2014a, Ho et al. 1991
Pp. leanoi	Mangrove leaf litter	Philippines	Bennett et al. 2017b

Calycofera			
C. cryptica	*Avicennia* sp.	Belize	Bennett et al. 2017c
C. operculata	*Avicennia marina*	Australia	Pegg and Alcorn 1982 Bennett et al. 2017c
Salisapiliaceae			
Salisapilia			
S. bahamensis	Mangrove leaf litter	Bahamas, Philippines	Bennett and Thines 2019
S. coffeyi	*Rhizophora mangle*	Bahamas	Bennett and Thines 2019
S. elongata	Mangrove leaf litter	Philippines, Taiwan	Bennett and Thines 2019
S. epistomia	*Rhizophora*, *Sonneratia*, *Xylocarpus* Decaying leaf, *Rhizophora*	Japan, Philippines, USA	Nakagiri 2000, Leaño 2001, Bennett and Thines 2019 Fell and Master 1975
S. nakagirii	*Spartina alterniflora*	USA	Hulvey et al. 2010
S. masteri	*Avicennia germinans*	Bahamas	Nakagiri et al. 1994, Bennett and Thines 2019
S. mycoparasitica	*Rhizophora*	Japan, Malaysia	Fell and Master 1975, Nakagiri 2000, Bennett and Thines 2019
S. sapeloensis	*Spartina alterniflora*	USA	Hulvey et al. 2010
S. tartarea	*Spartina alterniflora*	USA	Nakagiri et al. 1994, Hulvey et al. 2010, Bennett and Thines 2019
Salispinaceae			
Salispina			
S. hoi	Mangrove leaf litter	Philippines	Bennett et al. 2018b
S. intermedia	*Rhizophora mangle* *Laguncularia racemose*	Brazil	Li et al. 2016
S. lobata	*Xylocarpus* *Rhizophora*	Japan, Malaysia, Philippines, Seychelles, Singapore, Taiwan, Thailand, USA, Vietnam	Fell and Master 1975, Nakagiri 2000, Leaño 2001, Li et al. 2016,
S. spinosa	*Rhizophora*	Bahamas, Colombia, Grand Cayman, Haiti, Japan, Philippines, Thailand, Taiwan, The Netherlands, Trinidad and Tobago	Fell and Master 1975, Pang et al. 2014, Li et al. 2016, Caguimbal et al. 2019

Mangrove oomycetes have several advantages over true fungi. One of their main advantages is their ability to produce free-swimming biflagellate zoospores. Zoospores are considered as dispersal agents that give rise to new vegetative thalli (Dick 2001). The development and release of zoospores are somewhat distinct upon careful observation for some taxa, e.g. the observation of an exogenous evanescent or persistent vesicle (e.g. *Phytopythium*, *Phytophthora* and *Salisapilia*) or the direct zoospore release through the exit tube or exit pore (e.g. *Halophytophthora* and *Salispina*). Zoospores are capable of colonizing different substrates due to positive chemotaxis (or true chemotaxis) and are also capable of avoiding areas with chemical gradients that are potentially harmful for zoospores, i.e. negative chemotaxis (Leaño 2001). Positive, or true chemotaxis, is described as the direct attraction of zoospores to specific chemical exudates or diffusible substances from plant parts (e.g. roots and leaves) which leads to zoospore infestation. This is followed by encystment where a zoospore retracts its flagella, thus becoming immobile and the release of adhesins to a suitable substrate in preparation for germination. The germination stage occurs when the cells form a germ tube which penetrates a specific substrate. This feature is considered as an advantage of mangrove oomycetes over marine fungi as it renders oomycetes to be the first colonisers of fallen mangrove leaf litter (Nakagiri et al. 2001, Leaño et al. 2000, Leaño 2001). Chemotaxis has been studied for *Phytophthora* spp. (Allen and Newhook 1973, Allen and Harvey 1974, Halsall 1975, Khew and Zentmyer 1973, Morris et al. 1992, 1995, Tyler et al. 1996), *Pythium* spp. (Donaldson and Deacon 1993, Jones et al. 1991) and *Halophytophthora* spp. (Leano et al. 1998).

Another physiological feature of mangrove oomycetes is their ability to tolerate a wide range of salinity (or salt concentration). It has been inferred that mangrove oomycetes can sporulate and grow within a wide range of salinity when compared to true fungi (either terrestrial, freshwater or marine fungi) (Leaño et al. 2000, Leaño 2001). Marine fungi, such as *Asteromyces cruciatus*, *Corollospora maritima*, *Paradendryphiella salina* (≡*Dendryphiella salina*) and *Zalerion maritimum*, are capable of vegetative growth and sporulation at 0-100% salinity, whereas some terrestrial fungi, such as *Absidia glauca*, *Aspergillus* spp., *Chaetomium globosum*, *Gelasinospora retispora*, *Penicillium* spp., *Sordaria fimicola*, *Stachybotrys atra* and *Tetracladium setigerum*, sporulate at 0-50% salinity (Jones and Jennings 1964, Byrne and Jones 1975). This

data is comparable to that of the ability of mangrove oomycetes to sporulate at 0-35% salinity and to grow vegetatively at 0-50% salinity (e.g. *Calycofera*, *Halophytophthora* spp., *Phytopythium dogmae*, *Phytopythium leanoi*, *Phytopthora elongata*, *Phytophthora insolita*, *Salispina* spp. and *Salisapilia* spp.) (Leaño et al. 2000, Leaño 2001, Bennett and Thines 2017, Bennett et al. 2017a, 2017b, 2017c, Bennett et el., 2018, Bennett and Thines 2019). This, therefore, proves that mangrove oomycetes are capable of competing with other true fungi in the mangrove and marine environments when colonizing substrates under fluctuating salinity.

Fungal succession in a mangrove leaf litter was studied by Nakagiriet et al. (1989). *Halophytophthora* spp. and some mangrove oomycetes reported in this manuscript can colonize mangrove leaf litter at the yellow senescent stage. Most of the time, the collected leaves were those from the above mentioned stage, where the leaves were expected to produce exudates once they had reached the water system due to prior infestation by phyllosphere fungi (e.g. *Acremoium* sp., *Pestalotiopsis* sp., *Cladosporium* sp., *Cylindrocarpon* sp. and *Phyllosticta* sp.). Once a yellow leaf falls and floats on the surface of the water system, mangrove oomycetes (e.g. mainly *Halophytophthora*, *Phytopythium*, *Salispina* and *Salisapilia*) are able to infest and colonise the leaf surface. It was found that green leaves that were still attached to the mangrove tree, but were dipped in the water system during high tide, yielded no oomycete isolates, but instead *Fusarium* sp., *Gliocladium* sp. and *Trichoderma* sp. were isolated (Nakagiri et al. 1989).

It has been proven that mangrove oomycetes are also capable of producing extracellular enzymes that degrade the leaf material, therefore providing a source of nutrients for oomycetes and other marine and mangrove microflora (Fell and Master 1980, Nakagiri et al. 1989, Raghukumar et al. 1994). Furthermore, it has been proven that mangrove oomycetes are highly adapted for the capture of cellulose-rich substrates (such as leaf litter) by pervasion and digestion from within, thus being able to degrade the cellulosic components of lignocellulose faster (Kristensen et al. 2008). Mangrove oomycetes are expected to continuously persist during the entire decay stage of mangrove leaves (from the yellow decay stage to the yellow-brown decay stage and to the brown decay stage) (Nakagiri et al. 1989), alongside other fungal-like groups (e.g. *Labyrintula* and Thraustochytrids) and true fungi (e.g. *Acremonium* sp.,

Gliocladium sp., *Fusarium* sp., *Scolecobasidium* sp. and *Trichoderma* sp.). The latter two groups are considered to be almost exclusively saprotrophic (Dighton 2013). This proves that the competitive ability of mangrove oomycetes to compete against other microorganisms (i.e. fungi and fungal-like protists), does not lie just in their tolerance to salinity, but also in their ability to colonize and degrade mangrove leaves.

A mangrove environment has two distinct layers: the sediment area which is primarily anaerobic and the aerobic, or top surface area, where oxygen is available (Kristensen 2008, Ghizelini et al. 2012). In addition, it can be noted that an intermediate layer exists, the microaerophilic layer. The oxygen concentration of the mangrove area is constantly fluctuating due to the change of sea level and, likewise, on the production of various anaerobic gases (e.g. ammonia, methane, sulfide) (Kathiresan 2004). The majority of mangrove leaf litter samples, from previous literature and similarly from the papers presented in this manuscript, have always been collected from the upper surface layer of the water system or simply from the oxygenic layer. This is due to the fact that most mangrove oomycetes probably require oxygen for vegetative growth and sporulation (i.e. the development of sporangia, gamentangia and release of zoospores) or, at least, those that are considered as facultative aerobes. However, *Salispina* spp. was inferred to grow and sporulate under reduced oxygen concentrations, similar to Rhidipiaceae, a group from the freshwater biome with pedicellate gametangia and sporangia (Bennett et al. 2018). The genus *Salispina* was introduced to accommodate those mangrove oomycetes having aculeolate sporangia (Li et al. 2016). The higher taxonomic rank of *Salispina* was resolved based on combined morphology, physiology and phylogeny and was found to be closely related to Rhipidiaceae (Bennett et al. 2018). The latter taxon is similarly composed of members having aculeolate to smooth sporangia. Several members of Rhipidiaceae are also capable of growing at reduced oxygen concentrations (e.g. *Aqualinderella*, *Mindeniella* and *Rhipidium*) (Emerson and Weston 1967, Gleason 1968, Dogma 1975, Natvig 1981) similar to *Salispina* and have even been considered as facultative anaerobes (Natvig 1981, Dick 2001, Bennett et al. 2018). Congeners of *Salispina* are mangrove dwelling, whereas members of the Rhipidiaceae are primarily isolated from freshwater streams and stagnant water. This gives rise to the question as to whether an ecological shift occurred for members of

Rhipidiaceae since, based on recent phylogeny, Rhipidiaceae is situated between Salisapilaceae and the mangrove oomycete group of Peronosporales. However, the targeted isolation and DNA sequencing for members of Rhipidiaceae would be required since *Sapromyces elongatus* is the only organism under this family with available sequences in repository DNA banks.

Challenges of Mangrove oomycetes: Studying morphology, phylogeny and diversity

Morphology and Phylogeny

The current taxonomic arrangement of the oomycetes is fundamentally based on the work and reviews of Sparrow (1960, 1976) and Dick (2001) and was recently updated by Beakes and Thines (2017). Early phylogenetic studies of several mangrove oomycetes were conducted by Cooke et al. (2000) and Nakagiri (2002) and it was inferred that these organisms are members of the "peronosporalean galaxy" of the phylum Oomycota (Hudspeth et al. 2000, Petersen and Rosendahl 2000).

The use of morphology in describing an oomycete is common practice in setting boundaries between closely related species. All species described in this manuscript, except *Calycofera cryptica*, were described morphologically coupled with phylogenetic analysis. The morphological characteristics used are: sporangium (e.g. shape, size, exit tube, presence or absence of vesicle, type of vesicle if present, sporangium morphometry, presence or absence of papilla, basal plug and proliferation), sporangiogenic hyphae (e.g. branching pattern), process of zoospore release, zoospore shape, antheridium (e.g. attachment, shape, size), oogonium (e.g. wall, shape, size and coloration), oospore (e.g. wall, number of oospores formed after fertilization and coloration), chlamydospores (e.g. presence or absence, size and shape), colony pattern and radial growth on a certain agar medium and the hyphae morphology. These constitute the common morphological characteristics noted when describing oomycetes. Some taxa have a distinct morphology, e.g. *Calycofera operculata* (distinct shape of sporangia, morphology of sporangiogenic hyphae, branching pattern and zoospore release), *Phytopythium* (*Phytophthora*-like sporangia and a zoospore release similar to *Pythium*), *Salisapilia* (formation of a distinct hyaline dehiscence plug or apical plug) and *Salispina* (aculeolate sporangia,

microaerophilic growth and sporulation), whereas some groups lack synapomorphic characters (e.g. *Phytophthora* and *Pythium*). Furthermore, the classical grouping for some taxa, e.g, *Phytophthora* spp. (Waterhouse 1963, Stamps et al. 1990), appears arbitrary and does not reflect natural groupings. Overlapping morphologies were observed for *Halophytophthora* (e.g. *Halophytophthora avicenniae* and *Halophytophthora batemanensis*), *Phytophthora* (e.g. clades 8, 9 and 10) and *Pythium* spp., while some species even exhibit morphological plasticity (e.g. *Halophytophthora exoprolifera*, *Halophytophthora polymorphica* and *Phytophthora elongata*) (Bennett et al. 2017b). Some organisms for reasons unknown tend to stop producing sporangia (e.g. *Calycofera cryptica*, *Calycofera operculata* and *Salisapilia nakagirii*) (Hulvey et al. 2010, Bennett et al. 2017a, Bennett and Thines 2019) which hinders species description. As the number of described species increases over time (e.g. *Phytophthora*, *Phytopythium* and *Pythium*), limitations on the use of morphology arise, such that finding a species-specific morphological trait becomes too difficult, thus electron micrograph approaches (e.g. SEM and TEM) could be an appropriate method in describing mangrove oomycetes similar to Chytridiomycetes (James et al. 2006, Letcher and Powell 2014, Seto et al. 2020).

With the advent of molecular phylogenetics, the use of genetic markers in describing oomycetes, including the mangrove oomycetes in this study, became helpful. The use of universal primers, e.g. Internal Transcribed Spacers (White et al. 1990, Moncalvo 1995), nuclear Large Subunit (Riethmueller 1999), *Cytochrome oxidase* 1 (Robideau et al. 2011) and *Cytochrome oxidase* 2 (Hudspeth et al. 2000, Choi et al. 2015), helped in establishing several groups and even pointed out problems in the natural grouping of mangrove oomycetes. Nakagiri (2002) proposed some clade issues for *Halophytophthora* spp., particularly *Salispina spinosa* (≡*Halophytophthora spinosa* var. *spinosa*, basionym *Phytophthora spinosa* var. *spinosa*). This was consequently followed by several phylogenetic studies (Hulvey et al. 2010, Lara and Belbahri 2011, Nigrelli and Thines 2013, Marano et al. 2014b), where *Halophytophthora* was inferred to be a polyphyletic group and so requires reanalysis of both the morphology and natural groupings. The genus *Phytophthora* is a highly paraphyletic group based on molecular phylogenetic analyses and is composed of roughly 8 to 10 clades (Cooke et al. 2000, Kroon et al. 2004). As research on mangrove oomycete progressed, the combined morphological and

phylogenetic analyses became a trend in establishing natural groupings of some taxa. The family Salisapiliaceae was proposed in 2010 wherein some of its members were isolated from the saltmarsh biome (e.g. *Salisapilia sapeloensis*) (Hulvey et al. 2010) and is now composed of nine species (Bennett and Thines 2019). The genus *Phytopythium*, proposed by Bala et al. (2010), was formerly part of the clade K of the genus *Pythium* and is roughly composed of 27 species (www.indexfungorum.org). Similarly, the proposal of the genus *Calycofera* preserved the morphological integrity of *Phytopythium* (Bennett et al. 2017a, 2017c). The family Salispinaceae (Bennett et al. 2018) was proposed as a sister taxon to Rhipidiaceae where the analyses were based on combined physiological (i.e. sporulation at reduced oxygen concentration), morphological and phylogenetic approaches. The use of morphology may be the standard approach when describing a species, however, it would be better if analysis were to be coupled with phylogeny to clearly delineate species boundaries and to come up with a stable, natural grouping for some members of the mangrove oomycetes. Despite these recent advances in molecular phylogenetics, mangrove oomycetes are still relatively underexplored in contrast to Albuginales (Choi et al. 2007, Thines et al. 2009, Ploch et al. 2010, Ploch and Thines 2011, Mirzaee et al. 2013), downy mildews (Riethmüller et al. 2002, Voglmayr 2003, Voglmayr et al. 2004, Thines et al. 2006, 2007, Göker et al. 2007, Thines et al. 2008, 2015, Choi and Thines 2015, Thines and Choi 2016, Kara et al 2020, Hoffmeister et al. 2020) and Saprolegniales (Dick et al. 1999, Riethmüller et al. 1999, Leclerc et al. 2000, Spencer et al. 2002, Diéguez-Uribeondo et al. 2007, Hulvey et al. 2007,Filipova et al. 2013, Steciow et al. 2013, Sandoval- Sierra et al. 2014, Steciow et al. 2014, Rocha et al. 2018, Choi et al. 2019).

Studying diversity: The cultivation approach and metabarcoding

The fungal community structure of the mangrove ecosystem has been studied using both cultivation and metagenomic approaches (Hyde 1991, Arfi et al. 2012, Simoes et al. 2015, Li et al. 2016, Loganathachetti et al. 2017), where several Ascomycetes, Basidiomycetes, Chytridiomycetes, Glomeromycetes and Zygomycetes were reported as occurring in both the rhizosphere and mangrove leaf and root systems.

All isolates explored in this thesis resulted from the cultivation approach/method. It is believed that this approach is efficient in terms of establishing a concrete phylogeny since the isolate in question is at hand. It is a known fact that not all organisms are cultivable and there are several species that are too fastidious and require selective media or baits for targeted isolation. Some *Salispina* spp. (e.g. *Salispina hoi* and *Salispina spinosa*) grow appressed in agar media (e.g. vegetable juice agar) with compact mycelia, growing at a slow rate. Furthermore, *Salispina* spp. do not prefer carbohydrate-rich media (e.g. Potato Dextrose Agar) for isolation and growth due to reasons unknown. This means that during the initial stage of cultivation, one should look at the reverse side of the agar plate and even check for hyphae to a wide diameter under the microscope. *Halophytophthora porrigovesica* (Nakagiri et al. 2001) is also difficult to isolate; this organism prefers cornmeal agar over the commonly used vegetable juice agar. This may be due to the fact that *Halophytophthora porrigovesica* prefers high carbohydrate-based agar media (in contrast to other mangrove oomycetes), at near neutral pH over a media with low pH and high vitamins and other organic vegetable extracts (which come mainly from tomato and carrot). Soil extracts and pond water are useful media since these contain compounds and biotic components that can trigger growth and sporulation. Biotic components, such as bacterial cells, have been suggested to produce specific metabolites that can trigger sporulation, e.g. in *Phytophthora*, *Pythium* (Chee and Newhook 1965) and *Phytopythium dogmae* (Bennett et al. 2017c). Marine salt solution can also be considered at the start of the isolation process and, likewise, in attempting to sporulate an organism. It has been intensively used to sporulate *Halophytophthora* (Leaño et al. 2000, Leaño 2001) and for most isolates presented in this manuscript. However, there is a need to determine which concentration would produce the greatest number of sporangia and, likewise, if other external factors (such as abrupt changes in temperature or increased salinity) would be required to sporulate an organism, e.g. *Salispina* (Bennett et al. 2018) and *Phytopythium* (Bennett et al. 2017c). In general, these are the factors to consider when attempting to isolate, grow and sporulate an organism. Unfortunately, a synoptic isolation approach of mangrove oomycetes seems non-existent and the possibility of not isolating cultivable organisms remains.

Metagenomics tend to capture sequences from an array of microorganisms that are both cultivable and non-cultivable. This approach can determine the vast diversity of microorganisms in a certain biome by taking advantage of sequencing technologies. This has paved the way for the high exploration of fungal communities in the mangrove environment (Arfi et al. 2012, Simoes et al. 2015, Imchen et al. 2017, Loganathachetti et al. 2017, Haldar et al. 2019, Lee et al. 2019, Luis et al. 2019). Taheri et al. (2017) conducted a metagenomic analysis of an oomycete community in a field pea rhizosphere using ITS-based metabarcodes; they identified 105 operational taxonomic units (OTUs) where 45 and 16 OTUs were identified at the species and genus levels, respectively. Species annotation took place at a minimum of 90% query coverage and at 97% sequence similarity, whereas for the genus annotation, 80% query coverage and identity were used. It was inferred that *Pythium w*as the dominant genus and that *Pythium heterothallicum* was the most prevalent species. However, in their metagenomic data, *Apodachlya* (a freshwater oomycetes) and *Atkinsiella* (a crustacean pathogenic marine oomycetes), both from the order Leptomitales, were reported; these were considered as unexpected since the sampling area was a field pea rhizosphere. A study using the high-throughput sequencing approach based on the SSU rRNA v9 region of a peat bog, an oligotrophic and acidic environment, revealed 34 phylotypes where 3 of the 34 phylotypes resulted in 100% similarity to known barcoded taxa (*Peronospora schachtii* X1510, *Phytophthora* sp. X1794 and *Saprolegnia parasitica* X1602), while the remaining 70% of the samples had less than 98% similarity (Singer et al. 2016). Several taxa were likewise detected, e.g. *Albugo*, *Aphanomyces*, cf. *Atkinsiella*, *Haptoglossa*, *Lagenidium*, *Leptolegnia*, *Peronospora*, *Phytophthora*, *Phytopythium*, *Pythium*, *Saprolegnia*, some undescribed basal clades, Peronosporales and Saprolegniales (Singer et al. 2016). Metagenomics data usually lump sequences into a consensus sequence that do not often correspond to real species. Furthermore, designation of a certain consensus sequence to a putative species, genus or any taxonomic rank is dependent on sequence similarity (or percentage match) to deposited DNA sequences. As for the case of *Atkinsiella* and *Apodachlya*, a physical isolate would definitely result in a comprehensive phylogeny, since Leptomitales itself is seen to be enigmatic and contains some brackish and marine oomycetes (Nakamura and Hatai 1995, Buaya and Thines

2020). Both techniques have their own advantages and disadvantages. It would be of great benefit if both cultivation and metagenomic approaches could be fused in order to produce a good understanding of the diversity of microorganisms (metagenomics) in a certain biome (the mangrove area in this case) and to have isolates (cultivation approach) that would be useful in establishing a phylogeny.

Where else can we explore oomycetes?

It remains a known fact that the mangrove environment is persistently underexplored for oomycete diversity despite recent proposals in its taxonomy and phylogeny. As presented in Table 2, the number of reported mangrove oomycete species is lower when compared to the species-rich and well-studied biotrophic and pathogenic members of oomycetes. Primarily, this may be attributed to the low cultivation success of mangrove oomycetes (especially those that are too difficult to maintain in the axenic state) and the difficulty in bringing some isolates to sporulation (e.g. *Calycofera cryptica*, *Calycofera operculata*, *Halophytophthora porrigovesica*, *Phytopythium dogmae*, *Salisapilia nakagirii*, *Salispina lobata and Salispina spinosa*). There are also cases where a mangrove oomycete will stop sporulating (e.g. *Phytophthora insolita*, *Calycofera operculata* and *Salisapilia nakagirii*).

The members of *Phytophthora* and *Phytopythium* should be thoroughly investigated since efforts are being made to conserve the mangrove habitat in some tropical countries (e.g. the Philippines). Furthermore, it is a known fact that the majority of *Phytophthora* spp. and a few members of *Phytopythium* are omnivorous pathogens of terrestrial plants (e.g. crops and other ornamental plants). Interestingly, for the part of *Phytophthora*, since this group is primarily soil/terrestrial-dwelling, an ecological shift to or from the mangrove or marine environment should be taken into consideration; this would help to understand the ecology of the organism, not just as a potential pathogen or saprotroph, but also the paraphyly of the genus (Bennett and Thines 2017, Bennett et al. 2017b).

The anaerobic and microaerophilic areas of the mangrove environment is, therefore, seen as an opportunity to explore oomycete diversity. Bennett et al. (2018) established the growth conditions for *Salisapilia* spp. in an artificial incubation set-up at a reduced oxygen

concentration, thus, the possibility that the mangrove sediment area, or the anaerobic layer, in addition to the microaerophilic area, can serve as other cryptic habitats for oomycetes can be explored. Through this approach, the role of these microaerophilic or facultative anaerobic oomycetes in the decay of mangrove leaf litter, drift woods or decayed rootlets can first be unraveled and secondly, this will help in establishing the phylogeny, not just of Salispinaceae, but also of the Rhipidiales and Peronosporales in general.

Current status: Concluding statements and future directions

Of the approximately 1700 species of known oomycetes (Thines 2014, Wijayawardene et al. 2020), 30 species are considered as mangrove or saltmarsh oomycetes (Table 2). This number is approximately 1.7% of the estimated total number of species for mangrove or saltmarsh oomycetes. Species classified in this group are distributed into three oomycete families: Peronosporaceae (*Calycofera*, *Halophytophthora*, *Phytophthora* and *Phytopythium*), Salispinaceae (*Salispina*) and Salisapiliaceae (*Salisapilia*). The genus concept of *Halophytophthora* as *Phytophthora*-like species in estuarine environments has been rejected since several groups of saprotrophic oomycetes are found in the mangrove environment (i.e. *Calycofera*, *Phytophthora*, *Phytopythium*, *Salisapilia* and *Salispina*) (Lara and Belbahri 2001, Thines 2014, Nigrelli and Thines 2013, Bennett et al. 2017a, 2017b, 2017c, 2018, 2019). In addition, the premise “mangrove or estuarine oomycetes” is arbitrary as it does not represent any phylogenetic significance and several genera include species from both freshwater and marine habitats (Figure 5).

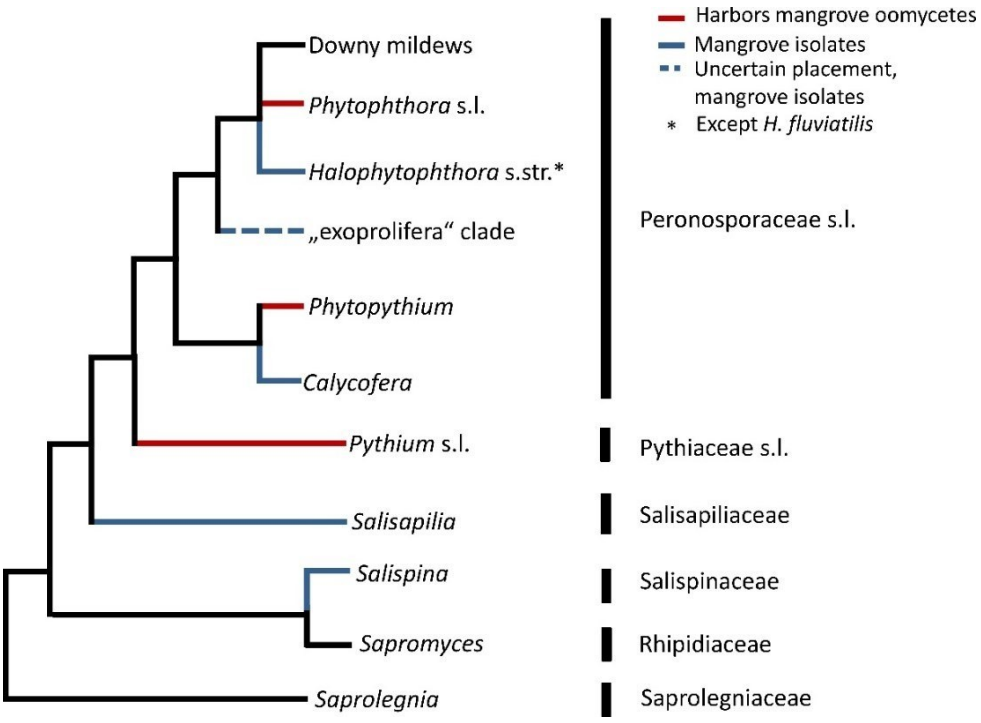

Figure 5. Proposed phylogeny for mangrove oomycetes.

Several taxa previously classified as *Halophytophthora* were transferred to *Salisapilia* (Bennett and Thines 2019), which now contains 9 species and can be considered as a rather species-rich genus. In addition, the phylogenetic and morphological distinctiveness of the species justifies the recognition of Salisapiliaceae as a family independent from Pythiaceae and Peronosporaceae (Figure 5). The genus *Salispina* was previously considered as an *incertae sedis* taxon but was resolved based on combined morphological and phylogenetic analyses (Bennett et al. 2018). This genus is now accommodated to a family of its own, Salispinaceae; it is phylogenetically related to Rhipidiaceae. These two families are both placed in the order Rhipidiales – a group capable of growing in an environment with reduced oxygen concentration. A fourth congener was introduced for *Salispina*, *Salispina hoi*. *Salispina hoi*, is, after *Salispina* spinosa, the second species of the genus reported for the Philippines.

Apart from the three genera mentioned above, the genus *Phytopythium* also appears to be a frequent inhabitant of mangroves (Lara and Belbahri 2012, Thines 2014). From the Philippines, *Phytopythium leanoi*, a sister taxon to *Phytopythium kandeliae* and *Phytopythium dogmae* has recently been described. *Phytopythium leanoi* is morphologically divergent from

Phytopythium kandeliae based on several combined characteristics. The third reported species for the Philippines, *Phytopythium dogmae*, is considered to be a member of the clade 2 group of the genus *Phytopythium*. The sister genus to *Phytopythium* is *Calycofera*; the latter genus was described to preserve the well-delineated morphological diagnosis of *Phytopythium*. Apart from the type species, *Calycofera operculata* (≡*Halophytophthora operculata*), a second congener, *C. cryptica*, was described based on nucleotide sequence divergence (Bennett et al. 2017a).

Despite the recent efforts outlined above, there are still taxa that remain enigmatic and require further analysis, for example, the genus *Phytophthora*. This genus is an assemblage of unrelated species and is paraphyletic or polyphyletic with respect to the downy mildews and grouped into approximately 8 to 10 clades without clear-cut synapomorphies. *Phytophthora* harbors mangrove oomycetes (Bennett et al. 2017b, Bennett and Thines 2017) (i.e. in clades 9/10, 6 and 2) but it is unclear if these species act as opportunistic plant pathogens of mangroves or are purely saprotrophic in this environment. In addition, the family Pythiaceae remains problematic despite the proposed changes of Uzuhashi et al. (2010) to resolve the polyphyly of *Pythium* by describing the genera *Elongisporangium*, *Globisporangium*, *Pilasporangium* and *Ovatisporangium* (as a later synonym of *Phytopythium*, Bala et al. 2010) apart from *Pythium* in a strict sense. The phylogeny presented by Uzuhashi et al. (2010) was not well-resolved and still harbors mangrove oomycetes of unclear phylogenetic affinity.

After the revisions outlined above, there are only five congeners of *Halophytophthora* remaining (*H. avicenniae*, *H. batemanensis*, *H. polymorphica*, *H. vesicula* and the freshwater isolate *H. fluviatilis*). In addition, two species, *Halophytophthora exoprolifera* and *H. porrigovesica*, are not clearly nested within the *Halophytophthora* s.str. clade but may represent a sister taxon to Halophytophthora or belong to either *Nothophytophthora* (Jung et al. 2017) or the paraphyletic genus *Phytophthora*. Since there is a lack of morphological support for *H. exoprolifera* as a new genus of its own, is has so far not been transferred from *Halophtophthora*. Similarly, *Halophytophthora porrigovesica* was not officially transferred to *Phytophthora* since the latter taxon itself is unresolved and the morphology of

Halophytophthora porrigovesica does not fit well into either *Nothophytophthora* or clades 9 and 10 of *Phytophthora*.

Marano et al. (2016), as well as Beakes and Thines (2017), emphasized the importance of saprotrophic Peronosporomycetes (including mangrove and saltmarsh oomycetes) for the better understanding of the evolutionary patterns of oomycetes in general. This group is phylogenetically nested between the downy mildews and Saprolegniomycetes and is key for understanding ecological shifts (i.e. habitat change), lifestyle transitions (e.g. biotrophy and saprotrophy) and thallus organization in oomycetes. Therefore, more research into these interesting species is likely to reveal more insights into oomycete evolution, diversity and ecological functions in the years to come.

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Ggfs. Unterschrift *corresponding author*: Marco Thines
Datum/Ort: _____

Title: Confirmation that *Phytophthora insolita* (Peronosporaceae) is present as a marine saprotroph on mangrove leaves and the first report of the species in the Philippines

Status: Published

Name der Zeitschrift: Nova Hedwigia

Beteiligte Autoren: Reuel M. Bennett (RMB), Marco Thines (MT)

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Title: A revision of *Salispina*, with its placement in a new family, Salispinaceae (Rhipidiales), and the description of its fourth species, *S. hoi* sp. nov.

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Ggfs. Unterschrift *corresponding author*: Marco Thines
Datum/Ort: _____

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

Status: Published

Name der Zeitschrift: Mycosphere

Beteiligte Autoren: Reuel M. Bennett (RMB), Gina R. Dedeles (GRD), Marco Thines (MT)

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