

## ABSTRACT

Title of Document: **DIVERSITY OF PHYTOPHTHORA SPECIES  
IN COSTA RICA'S TROPICAL FORESTS**

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The fungus-like organism *Phytophthora* includes more than 130 species, most of which are destructive plant pathogens. Information about the occurrence and diversity of *Phytophthora* species in forest and protected areas are largely unknown. The purpose of this study was to fill in the scientific gap in species biodiversity by conducting a survey in tropical forest ecosystems. The survey was conducted at 10 sites across Costa Rica. Leaves with lesions attached to live plants (LP), fallen from tree canopies (LF), submerged in forest streams (LS) and soil was sampled. Isolate identification was based on a multi-locus (4 mitochondrial and 4 nuclear) phylogeny, and examination of morphological features. In total 258 isolates were characterized into 21 species: eight known and 13 novel species. Phylogenetically, species discovered were placed in seven different clades and clade 9 included the most number of species. Species were more common in transitional forests. The number of unknown species discovered suggests that tropical forests are a “hotspot “for *Phytophthora* diversity.

Keywords: Multilocus, phylogeny, Oomycete, tropics, plant pathogens.

DIVERSITY OF *PHYTOPHTHORA* SPECIES IN COSTA RICA'S  
TROPICAL FORESTS

by

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## **Dedication**

This Thesis is dedicated to, Sebastian, Nathan, Joshua, Elias and Ivy.

Up and forward

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## Chapter 1 Introduction

Natural ecosystems have many indigenous pathogenic organisms associated with them. However, parallel to the increase in commerce, an exponential increase in invasive, exotic organisms is emerging in natural forests and plantations around the world. Tropical areas in developing countries are most vulnerable, because of the lack of resources for comprehensive research. Among the invasive plant pathogens that received the most attention is *Phytophthora* (plant destroyer from Greek). Several species of the genus cause important diseases in vegetable crops, forest trees and nursery crops. The disease known as late blight of potato caused by *Phytophthora infestans* is a clear example of the devastation that this organism can cause: The great Irish famine is caused by this organism that resulted with starvation and large scale migration. *Phytophthora cinnamomi* the cause of severe eucalyptus dieback on Jarrah in Australia, *Phytophthora lateralis*, a disease of Port-orford-cedar (*Chamaecyparis lawsoniana*) on the west coast of North America, and *Phytophthora alni* and its subspecies on alder (*Alnus* spp.) in Europe are examples of the genus causing disease problems in forest and urban ecosystems (Brasier et al., 2004; Hansen, 2008).

The emergence of *P. ramorum* in 2000 on the west coast of the United States, the causal agent of the disease known as Sudden Oak Death, has re-emphasized the destructive role played by this important plant pathogenic group. Because of its destructive impact in California's oak ecosystems and nursery industry, it has generated an interest in identifying existing species of *Phytophthora* and has motivated systematic surveys to determine the distribution of the organism throughout Europe and the US (Balci & Halmshlager, 2003a,b; Balci et al., 2007,

Brasier et al., 2005; Delatour, 2003; Moralejo et al., 2009; Werres et al., 2001). A survey of diseased ornamental plants in Spain demonstrated 17 putative species of *Phytophthora* with 37 new host/pathogen combinations (Moralejo et al., 2009). Thirteen of these isolates could not be assigned to any known or formally described species. Similarly, more than a dozen species, including *P. ramorum*, were isolated when nurseries were surveyed in California and North Carolina for *Phytophthora* (Warfield et al., 2008; Yakabe et al. 2007; Yeshi et al., 2008). A more recent survey in Maryland nurseries resulted with similar findings with direct evidences of how outstate plant imports contributed to introduction of new strains (e.g. mating types) and species into a new environment (Bienapf and Balci 2012).

Findings from these surveys suggest the presence of a diverse population of *Phytophthora*, which largely remains uninvestigated around the world. Knowledge of native and naturally occurring *Phytophthora* species, their biology and host range can be helpful in monitoring species movement and introduction into new areas.

### **Biodiversity of *Phytophthora***

The genus *Phytophthora* belongs to the family of Pythiaceae, order Peronosporales, and the class Oomycete. Oomycetes are within the Kingdom Chromista which includes water molds, diatoms and brown algae. *Phytophthora* species resemble true fungi because they grow by means of fine filaments, called hyphae, and produce spores. But unlike true fungi, their cell walls contain cellulose instead of chitin, their hyphae lack cross-walls, and the diploid phase, rather than the haploid phase, dominates their life cycle. Another striking feature of *Phytophthora* is

that it produces zoospores, during one phase of its life cycle, a vestige of its evolutionary origins in water (Erwin et al., 1983). There are currently more than 130 described species of *Phytophthora* worldwide, and the vast majority of them are plant pathogens (Martin et al., 2012). During the last decade a significant number of new species has been described, sophisticated tools for species identification and recent disease outbreaks has generated a spike in interest as well as accelerated description of new genera (Kroon et al., 2011).

Recently, it was proposed that the number of existing *Phytophthora* species might be several times more than the number of known and described species (ca. 600 species) (Brasier 2007). This estimate is perhaps not exaggerated considering that only about 10% of the world total fungi are described. Because of the great diversity in tropical ecosystems and variable habitats in Costa Rica, it is to assume that the country could harbor a large number of species waiting to be discovered.

### ***Phytophthora* Diseases in Tropical Environments**

*Phytophthora* diseases have been well studied in the temperate regions of the world; however they are also very common throughout the tropical regions. Except banana, on tropical plantation crops such as cacao, coconut, rubber, oil palm, citrus, durian, jackfruit, papaya, pineapple, mango and avocado, *Phytophthora* have major global impacts. The damage varies from root and stem rots and cankers, leaf and flower blights, root and bud rots and fruit rot. Losses to *Phytophthora* in the tropics are not well documented; however, annual global losses to cocoa industry are valued at US\$1 billion (Guest 2007). In South-east Asia the *Phytophthora* associated

damages (up to 25% depending on crop) on cocoa, durian, rubber, coconut, pepper, citrus and potato was estimated to be at least US\$ 2.3 billion (Drenth and Sendall 2004). The impact of *Phytophthora* is also quite dramatic in oil palm (*Elaeis guineensis*) production, a tree crop which the area under cultivation is steadily increasing. Two recent outbreaks of bud rot of oil palm in Colombia have been estimated to cause losses of 250 million dollars (Mosquera Montoya et al. 2014).

In Costa Rica, reports of diseases caused by *Phytophthora* have always been associated with agricultural crops (Acebo-Guerrero et al., 2012). *P. palmivora* is frequently cited as the causal agent of diseases such as black pod of cacao, heart rot in oil palm, gummosis canker in citrus and root rot in papaya plantations (Acebo-Guerrero et al., 2012). Other *Phytophthora* species that were reported with a disease in Costa Rica includes *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. nicotianae*, *P. palmivora*, *P. phaseoli*, *P. porri* (Villalobos Calderon et al. 2009). Among these species perhaps most impact is associated to *Phytophthora palmivora* which is thought to have a Central American origin (Dick, 2001). This organism has been reported on wild and cultivated plants in almost all tropical regions of the world and in large numbers in subtropical regions (Erwin and Ribeiro 1996). Black pod of cacao was very influential in the overall destruction of the once thriving cacao industry in Costa Rica (Galindo, 1985; Phillips and Galindo, 1991).

## **Project Justification**

Products and natural resources unique to different parts of the world are now traded globally at an amazing rate. Despite the benefits this provides, increased globalization and international trade can open the doors for the introduction and spread of new exotic invasive pests and diseases that can severely damage our native ecosystems, agricultural crops, and possibly human health (Brasier, 2011). Diseases caused by *Phytophthora* have played a significant role in human history and some species are the causal agent of some of the most destructive plant pathogens that continue to threaten ecosystem health, and global food supplies. The origins of some of the most devastating plant pathogenic species of this organism are still unknown because due to the lack of information and surveys on the diversity of this group in natural ecosystems. Particularly, protected areas of Costa Rica ecosystems have never been systematically studied for Oomycetes. *Phytophthora* and *Pythium* are known as major plant pathogens, but our knowledge of their biology is currently limited to disease outbreaks, typically in agricultural systems. Knowledge of their behavior and function in their endemic environment is largely unknown limiting our efforts to effectively prevent and manage plant disease outbreaks.

The trend in invasion of alien plant pathogens around the world and the US demonstrated the increasing chances of new introduction; there is a need to develop new tools to reduce the threat posed by alien plant pathogens (Hulme 2009, Desprez-Loustau 2009). International concerns over the emergence of numerous, previously unknown plant pathogens within the orders Peronosporales and Pythiales provides the justification for this research proposal. High levels of diversity are expected in

tropical and subtropical ecosystems; however, the diversity of these pathogens in undisturbed environments remains largely unknown. This lack of knowledge is a serious weakness in current international plant biosecurity protocols (Brasier, 2008).

The threat posed by emerging *Phytophthora* species associated with woody plants around the world, the lack of information and surveys on the biodiversity of this group in Costa Rica tropical and subtropical ecosystems provided the impetus for this research. In order to prevent or reduce the incidence and spread of this group of pathogens, we need to develop information on conditions required for inoculum buildup and new infections to occur. The starting point for an effective management is a correct identification of species, but unknown species could undermine such efforts. The aim of this study is to identify the diversity of *Phytophthora* species present in diverse niches (e.g. forest canopy, soil, and streams) in ten different geographical sites across Costa Rica and determine their phylogenetic relationship to known species in temperate regions

## **Objectives**

The threat posed by emerging *Phytophthora* species associated with woody plants around the world and the scientific gap on species assemblage provided the impetus for this research proposal. In order to reduce or eliminate the incidence and spread of this group of pathogens, we need to develop information on by widening the knowledge base of tropical species of *Phytophthora*. Whether they can be more effectively managed using an integrated approach starting with a correct identification needs to be evaluated by taking into consideration the various unknown species.

The objectives of this study were to;

- 1- Determine *Phytophthora* species present in diverse ecosystems in Costa Rica's tropical forests, and
- 2- Describe new species assemblages to formal taxa and identify their phylogenetic relationship to known species of *Phytophthora*

## Chapter 2: The occurrence of *Phytophthora*

**Tentative title:** A hidden danger in the jungle. Neotropical forests harbor major *Phytophthora* pathogens.

Products and natural resources unique to different parts of the world are now traded globally at an amazing rate. Despite the benefits this provides, increased globalization and international trade can open the doors for the introduction and spread of new exotic invasive pests and diseases that can severely damage our native ecosystems, agricultural crops, and possibly human health (Brasier, 2011). At an accelerating rate, many new diseases are emerging in natural forest and plantations around the world (Aukema et al., 2010). Tropical areas perhaps are the most vulnerable, because of the lack of resources for comprehensive research.

Among the plant pathogens, species of *Phytophthora* emerged as significant pathogens of natural ecosystems (Hansen 2015). For example, *Phytophthora cinnamomi* causes severe eucalyptus dieback and decline of native flora in Australia, involved in oak declines in Mediterranean Europe and north eastern US (Balci et al., 2007). Most recently, the emergence of Sudden Oak Death pathogen (*P. ramorum*) in west coast of the United States and larch (*Larix decidua*) decline in UK has re-emphasized the destructive role played by this plant pathogenic group. During the last decade, the list of species of *Phytophthora* causing outbreaks in forests with significant declines has grown progressively to include *P. alni*, *P. austrocedrae*, *P. pinifolia* and *P. pluvialis* (Brasier et al., 2004, Dick et al., 2014, Durán et al., 2008, Greslebin et al. 2007).

The devastating impact of *P. ramorum* in California has motivated systematic surveys in forest and nurseries for *Phytophthora* species of regulatory concern in various countries. However, these surveys were primarily done in temperate regions (Balci et al., 2007 and references therein). Nevertheless, these surveys revealed the endemic and widespread species assemblages with exotic origin. The list in nurseries was remarkably diverse (Bienapfl & Balci 2013 and references therein, Park et. al., 2014). Infested nursery stock is suspected as the main pathway of introduction for many of these new outbreaks (Brasier et al., 2004; Roy et al., 2014). Indeed, recent population genetic studies indicate that some of the new *Phytophthora* spp. are probably either introduced, or they have emerged through hybridization and/or spread via horticultural trade and forest plantations (Brasier 2008).

One challenge that we face with managing invasive pathogens (e.g. *Phytophthora*) is that almost all of the species causing forest declines were unknown to science prior to their outbreak. This can be largely attributed to the lack of information and surveys on the biodiversity of Oomycetes and, in particular, lack of surveys for plant pathogens prior causing any significant damages. Therefore, our aim was to explore the native ecosystems of Costa Rica for biodiversity of *Phytophthora*. Our goal was to establish knowledge of tropical biodiversity for this notoriously pathogenic group that still remains largely unexplored for tropical ecosystems.

## **Materials and methods**

### **Study sites**

A well-defined mountain system divides Costa Rica in five predominant forest types:

1) Tropical wet forests, the most dominant forest type, is characterized by the high percentage of rainfall (4,000 to 6,000 mm/year) and absence of well-defined dry season; 2) Pre Montane very humid forest (2,000-4,000 mm/year) with no defined dry season, 3) Low Montane pluvial forest, characterized by high humidity and three months of dry period; 4) Pre Montane pluvial forest, and; 5) Tropical humid forests with 0 to 5 months of dry season (Janzen 1983).

In total, 10 sites were selected to represent different forest types across Costa Rica's National Parks (Figure 1, Table 1). The first field trip took place between July 5 and July 15, 2012 and the second from January 15 to January 25, 2013.

### **Sampling and isolation**

Sampling was aimed to explore different ecological niches including aerial, soil and aquatic environments. Sampling material consisted of four different sources; 1) Leaves of understory plants with necrotic lesions that are still attached to the plant (LP); 2) Newly dropped leaves from tree canopy that had dark, brown necrotic lesions (LF); 3) Leaves submerged in water with water-soaked lesions (LS), and 4) soil samples that were collected from around trees (S). A *Phytophthora* characteristic infection on foliage typically included a dark, brown lesion that primarily start from petiole or leaf tip with an expanding blotch-like lesion (Figure 2). Sampling material was collected while hiking on a forest trail, which typically required 1-3 hour of

hiking distance depending on the terrain. Samples were primarily collected along trails by entering the forest up to 30 m off the trail. Efforts were made to sample diverse plants, and only those with suspect *Phytophthora* infection were collected. Each leaf sample was wrapped in a t-fold paper towel in the field to eliminate cross contamination. Soil samples were collected from randomly selected trees at a distance of 1-2m from tree stem (Balci et al., 2007; Martin et al., 2012).

Soil samples were placed in containers and flooded with tap water twice the amount of the soil (Martin et al., 2012). Once surface was cleaned of organic debris mango leaflets (*Mangifera indica* collected in La Sabena Park in the City of San Jose, Costa Rica) were placed on the surface and incubated in room temperature. Once water-soaked lesions were visible they were processed as leaves collected in the field (see below).

Leaves collected in the field were processed the same day after returning from field trips. Before isolation, leaves (both collected in the forest and used during baiting) were first washed with tap water, blotted dry and then, 3-5 mm sized necrotic tissues were placed on the surface of clarified V8 juice based PARPNH growth media (Martin et al., 2012). Petri dishes were incubated in room temperature and after 2 days, outgrowing hyphae that is characteristic of *Phytophthora* was transferred onto corn meal agar (CMA) test tubes (1ml CMA in a 1.5 ml conical tube) using a stereomicroscope. Subculturing was based on hyphal morphology and growth pattern; anything that deemed different in hyphal or colony morphology was sub-cultured. Once isolates arrived at the University of Maryland Forest Pathology lab (3-5 days shipment duration) all isolates were sub-cultured onto a clarified V8 juice media and

then passed through the PARPNH selective media to eliminate bacterial contamination using the pancake method (Martin et al., 2012). All isolates were maintained in CMA agar slants at 15 °C.

### **Identification of *Phytophthora***

Isolates were first grouped using sequence homology based on the internal transcribed spacer (ITS) region of the rDNA (primers ITS 4 & 5; White et al., 1990). Each isolate within a group was examined for their key morphological features using a lucid key (Martin et al., 2012). When morphology and ITS based sequence identification were found in agreement no additional loci was sequenced. Isolates within a group with varying morphology and sequence identity that was less than 99.9% to any *Phytophthora* sequences available in GenBank and/or morphological features supporting variation from the closest species were deemed as potentially novel species and further sequenced using an additional seven loci (see below).

Morphological classification was based on observation and measurements of asexual and sexual structures as indicated in Martin et al., (2012). To calculate average sizes and size variations, 50 randomly selected structures were measured. Colony growth pattern on Potato Dextrose Agar (PDA) and clarified V8 juice agar (cV8A), and growth-temperature requirements (10 to 40 °C) using cV8A (19 ml in a 9 cm Petri dish) were recorded. Growth-temperature requirements were repeated twice with three replicates.

## **DNA Extraction**

Two DNA extraction methods were used: 1) Lyse and Go polymerase chain reaction (PCR) Reagent (Thermo Scientific Pierce) during initial ITS sequencing and 2) AccuPrep® Genomic DNA Extraction Kit (Bioneer Corporation) for multilocus sequencing. For ITS analysis, isolates were inoculated into 1.5ml tubes containing Potato Dextrose Broth (PDB) (Difco Laboratories) and allowed to grow for 7 days at room temperature. A toothpick-tip-sized piece of mycelium was taken directly from the PDB from each isolate using a sterile toothpick and transferred into a 0.2 ml PCR tubes containing 10µL of Lyse and Go PCR Reagent (Pierce Biotechnology) to release genomic DNA. According to the manufacturer's protocol, the PCR tubes were placed in a thermal cycler (S1000 or C1000, Bio-Rad) and cycled using the following program: 65°C for 30s, 8°C for 30s, 65°C for 90s, 97°C for 180s, 8°C for 60s, 65°C for 180s, 97°C for 60s, 65°C for 60s, hold at 80°C. After cycling, the released DNA product was immediately diluted 1:10 with molecular-grade water and stored at -20°C.

For the multilocus study, all mycelium grown in PDB was removed from the broth with a sterile toothpick; it was homogenized using lysing matrix A test tube in a FastPrep-24 (MP Biomedicals). DNA was extracted from the homogenized mycelium using an AccuPrep® Genomic DNA Extraction Kit (Bioneer Corporation, CA) according to the manufacturer's protocol. Elution was performed with 100µL of elution buffer rather than 200µL to increase DNA concentration. In a few cases where an isolate did not grow well in broth, homogenization and extraction were performed using a 0.5cm x 0.5cm slice of agar taken directly from the cV8 plate.

## PCR Amplification & Sequencing

PCR amplifications were set up in 30 $\mu$ L reactions as follows 2 $\mu$ L of extracted DNA, 19.7  $\mu$ L molecular-grade water, 0.2 $\mu$ L (total: 1 unit) Platinum Taq DNA polymerase (Life Technologies), 3 $\mu$ L 10X PCR buffer (Life Technologies), 1.5 $\mu$ L 50mM MgCl<sub>2</sub> (final concentration: 2.5mM), 0.6 $\mu$ L 10 $\mu$ M dNTPs (Affymetrix) (final concentration: 200 $\mu$ M), 1.5 $\mu$ L 10 $\mu$ M forward primer (final concentration: 0.5 $\mu$ M), 1.5 $\mu$ L 10 $\mu$ M reverse primer (final concentration: 0.5 $\mu$ M) (see Supplemental Table S1 for primers; all from Integrated DNA Technologies). The cycling protocols for every locus were as follows: initial denaturation at 95°C for 45s, then 36 cycles of [95°C for 45s, annealing for 30s (annealing temperatures varied, see Table S1), 72°C extension (extension times varied, see Table S1), final extension at 72°C for 5 minutes. For isolates which did not amplify well on the first attempt, retries were done using 40 cycles rather than 36 cycles, with the annealing temperature dropped by 1°C.

PCR product size and quality were checked via horizontal gel electrophoresis. Before sequencing, 10 $\mu$ L of each PCR product was cleaned of leftover primers and dNTPs by adding 2 $\mu$ L FastAP Alkaline Phosphatase, 1 $\mu$ L FastAP buffer, and 0.4 $\mu$ L Exonuclease I (Thermo Scientific) and thermal-cycling as follows: 37°C for 30 minutes, then 80°C for 15 minutes. The cleaned PCR products were sent to Molecular Cloning Laboratories for Sanger sequencing (MCLab, San Francisco, CA). Sequencing was done using the same primers as were used for PCR amplification. Sequence data was analyzed using the software Geneious Pro v. R6 (Biomatter Ltd.).

## Phylogenetic classification

For the phylogenetic analysis, sequence data from five nuclear loci were generated as described by Blair et al (2008). Sequences for the nuclear loci included; internal transcribed spacer (ITS) region 4 & 5,  $\beta$ -tubulin ( $\beta$ -tub), translation elongation factor 1 $\alpha$  (Efl $\alpha$ ), and approximately 1200 bp of the 5' end of the 28S rDNA (LSU). In addition, four other mitochondrial loci were amplified including cytochrome oxidase locus (cox1 and cox2), NADH Dehydrogenase subunit 9 and Flanking Regions (nad9), and rps10 (Martin et al., 2014).

All sequence data was processed using the software Geneious v6.1.6 (Kearse et al. 2012). The forward and reverse read of each locus were quality-trimmed, de novo assembled, and ambiguities were resolved with visual analysis of the chromatograph and re-sequencing when necessary. The consensus sequence was extracted from each assembly and sequences were aligned using the Geneious alignment option. Aligned sequences for each locus then were concatenated and used to conduct Bayesian analysis with MrBayes v3.1 with a plugin in the software Geneious.

## Results

In total, 417 isolates of *Phytophthora* were recovered. Of this assemblage, 257 were unique to a particular source material. The additional 159 isolates were identified as repeats hence excluded from the final assemblage list (i.e. isolates that were identified as same species from the same source material). In addition, despite selective media being used we identified 314 isolates as *Pythium* spp. (including

*Phytophthora* spp.) and 16 isolates as *Plasmopara* sp. These isolates were only identified based on ITS sequences, no morphological examinations were carried thus no species ID is given here. The 257 *Phytophthora* isolates were characterized into 21 species including *P. capsici*, *P. cinnamomi*, *P. cryptogea*, *P. hevea*, *P. macrochlamydospora*, *P. palmivora*, *P. tropicalis*, *P. taxon "PgChlamydo"* and 13 novel species (Table 2). The formal descriptions of novel taxa are proposed elsewhere. The greatest number of isolates consisted of *P. taxon "lagoariana"* (40 isolate), followed by *P. taxon "vulcanica"* (36). The isolation frequency was about similar (~20 isolates) for *P. capsici*, *P. palmivora*, *P. tropicalis* and *P. taxon "myristicae"* and *P. taxon "costaricensis"* (Table 2). The most widespread species was *P. capsici*, *P. taxon "myristicae"* and *P. taxon "lagoariana"* which were found in half of the sampling sites (Table 3). The second most common species were *P. hevea*, *P. tropicalis*, *P. taxon vulcanica* and *P. taxon frondescens*.

Seventeen species were found associated with aerial parts of the plants (Table 2). Of these 14 were associated with leaves that were still attached to the plants (LP). With exception of two instances, when a species was found on attached leaves (LP) it was also found on leaves that were dropped from canopy (LF). The exceptions were with taxon sp3, sp7, sp10 and sp13. All of the airborne species were also discovered in streams, the exception was only with *P. taxon sp10*. Species that were only found in streams (LS) were *P. cryptogea*, *P. taxon sp2* and "*cuyabensis*" (Table 2). While some species occurred in both aerial parts of the plants and water, their isolation frequency indicate a preference to a particular habitat. For example, the taxon "*lagoariana*" and "*vulcanica*" primarily were isolated from water whereas *P. capsici*,

*P. palmivora*, *P. tropicalis*, taxon "PgChlamydo", and "myristicae" were isolated primarily from canopy leaves (LF) (Table 2). *P. cinnamomi* was only found in soil and was never isolated from LP, LF or LS.

Typically only one *Phytophthora* species was isolated from a given leaf sample. However, in 26 instances (LP= 2; LF= 13; LS= 11) two species, 13 instances (LP= 2; LF= 4; LS= 6) three species, 6 instances four species (LP= 3; LF= 3), and one instance five species (LS= 1) were isolated from a single foliage. Isolation success also varied based on the sampling site and source material (Table 4). Overall, while leaves submerged in streams (LS) yielded 51% isolation success, isolation success was lower with leaves fallen from canopy (FL) (41%). Soil samples were the least successful and most bait were colonized by *Pythium* spp. and in only two instances *Phytophthora* was isolated (Table 4).

Species diversity depended on the sampling site. At site CR5, 15 species was discovered (Table 3). This was in great contrast to sites CR3 and CR6-10 where 1 to 5 different species were isolated. When sites were grouped by forest types, lowland areas with humid/dry climate (known as transitional forests) had the greatest species diversity (Table 5). Similarly, in high elevation Montane sites (known as cloud forests) a greater number of species diversity was found (e.g. sites 1, 2, 3, 6). Species diversity was the lowest where humid condition persisted throughout the year or considered tropical dry climates (e.g. sites 4, 8, 7, 9, 10).

### **Phylogenetic placement.**

The multilocus phylogeny placed the species in eight different clades (Figure 3). The clade 2 and 9 were represented with six species whereas only two species were found within the clade 4, 5, 6 and 8. Only one species was found in clade 7 (e.g. *P. cinnamomi*). Of the novel species, 4 were found in clade 9, and 3 in clade 2. Only one species were found in all other clades, except the clade 7 (Figure 3). There was some consensus in some morphological features when species were grouped based on their molecular clade. In clade 9, all species found had non papillate sporangia. Similarly, all species found in clade 2 had semi papillate sporangia. However, there was no consensus with morphology and ecological niche.

### **Discussion**

This study is among the first systematic biodiversity survey of *Phytophthora* spp. in tropical forests. The finding of 22 taxa including 13 novel species at only ten survey sites demonstrates that these forests harbor much greater *Phytophthora* diversity than temperate forests. At a temperate forest, typically one or two species dominate species diversity and the total number in a given survey ranged from 4 to 10 species (Balci and Halmschlager 2003a, b; Balci et al., 2007, Delatour, 2003; Jung et al., 2000, McConnell and Balci 2012). The diversity is also very high in the tropical forest if one single site is considered. We identified 15 species in Carara National Park (CR5). This is in great contrast in temperate forests where the number of species range from one to four.

Given the infamous status of *Phytophthora* species as plant pathogens, in this study, we have shown that they are readily to be found in diverse ecological niches without any obvious damage to the plant community. With exception of one case when *P. palmivora* was isolated from dying saplings on understory plants, in no other instances plant mortality was observed. Live plant infections, at the time of sampling, consisted of small necrotic sections on the leaves. However, all of the known species found in this study are well-known major plant pathogens with a worldwide distribution (Erwin and Ribeiro 1996). With exception of *P. macrochlamydo*, which is only reported from soybean (*Glycine max*) in Australia, *P. capsici*, *P. cinnamomi*, *P. cryptogea*, *P. hevea*, *P. palmivora* and *P. tropicalis* have an extensive host list exceeding hundreds of plant species (Erwin and Ribeiro 1996, Farr and Rossman 2015). Of this assemblage *P. palmivora*, *P. capsici* and *P. tropicalis* were the most common. Their frequent presence parallels their impact in the tropics, where they rank as the most significant infectious *Phytophthora* species affecting almost all agricultural tree crops in the tropics except banana (Drenth and Guest 2013; Uchida and Kadooka 2013).

The widespread occurrence of the provisionally named species, *P. taxon* “PgChlamydo” is noteworthy because this is the first time it was found in a tropical setting. This species has been discovered in multiple countries with adaptations to varying ecosystems (Brasier et al. 2003, Greslebin et al., 2005, Burgess et al. 2009, Reeser et al., 2011, Hüberli et al., 2013). While it was primarily isolated from streams and regarded as “weak” pathogen, incidental reports demonstrate its potential causing diseases on diverse plants in nursery systems (Blomquist et al., 2012;

Schwingle et al., 2007). In our study, it was found as airborne, soil borne and waterborne. In other ecosystems, it is listed among the most frequent waterborne *Phytophthora* community (Balci unpublished). While its frequent occurrence in streams is noted in the tropical Costa Rica, the airborne behavior was never been observed. Whether it reached its southern limit or if this is an introduction to Costa Rica's natural environments is not clear. Its remarkably adapted to diverse ecosystems and deserve further study to reveal its biology behind such plasticity.

The isolation of *P. cinnamomi* only from soil is a further evidence that this species is primarily restricted to soil environment. *P. cinnamomi* was only found in soil environment and absent from stream and waterways in diverse surveys (Balci et al. unpublished, Balci et al., 2007, Oh et al., 2013, Reeser et al., 2011, Huberli et al., 2013). This species is also largely absent from aquatic environments in nurseries where plenty of opportunities exist to establish beyond the soil environment (Bienapfl and Balci 2013, Parke et al., 2013). The reason behind its niche adaptation and why it cannot persist in an aquatic environment is a mystery for a "water mold". In contrast, airborne species are readily found in soil and streams (Drenth and Guest 2013; Erwin and Ribeiro 1996; Uchida and Kadooka 2013). While we have detected several of the airborne species in streams, soil sampling yielded limited success. Our soil sampling was very limited, thus more sampling is needed to conclude whether the newly recognized airborne species assemblages in this study are truly absent from soil environment.

The list of *Phytophthora* species reported from Costa Rica includes *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. nicotianae*, *P. palmivora*, *P.*

*phaseoli*, *P. porri* (Villalobos Calderon et al., 2009). Of the known species, *P. hevea*, *P. macrochlamydo*, *P. tropicalis* and *P. cryptogea* and the provisional taxon “*PgChlamydo*” are new records for Costa Rica. It is of surprise that *P. tropicalis* and *P. hevea* were never reported in Costa Rica because these species exist in numerous tropical countries in association with various diseases (e.g. Erwin and Ribeiro 1996). We believe many species possibly have been misidentified as *P. palmivora* due to their cultural similarities or not identified to species level as indicative from the plant health reports that contain a large list of unidentified *Phytophthora* associated diseases (Villalobos Calderon et al. 2009).

Niche adaptations in *Phytophthora* also became evident in our study. The species diversity was greatest at the high elevation tropical humid forests (cloud forests) that are subject to great temperature fluctuations and lowland sites that are considered as transitional forests with humid/dry cycles. Based on our finding we could hypothesize that in tropical forests, areas with varying climate provides a greater advantage to the *Phytophthora* community compared to areas with rather constant climatic conditions. One particular reason behind such niche adaptations could be the less competitive ability of *Phytophthora* species when diverse organisms are present (Erwin and Ribeiro 1996). A dry cycle and largely fluctuating temperature could reduce the inoculum of antagonistic organisms and prove adventitious for *Phytophthora*.

Seventeen species were found associated with aerial parts of the plants. This was never been demonstrated in any other survey involving *Phytophthora* spp. While airborne species exist in temperate regions, they are considered almost conclusively

as exotics both for those that cause damage on woody plants or agricultural crops. Several studies have demonstrated how *P. palmivora* is spread to canopy through rain splash, various beetles and ants, mollusks and vertebrates (Drenth and Guest 2013; Truong et al. 2010), a similar mechanism might account for the species found in this study. While tropical areas appear to be hotspots of aerial *Phytophthora* their role as early leaf colonizer and studies looking into their “cryptic biology” promises new insights on their functional roles.

Of the 21 species found, only five were homothallic and produced readily oogonium and all others were heterothallic with nine being sterile. From a taxonomical standpoint, these structures are important to delineate species from one another (Martin et al., 2012). We determine the absence of these structures based on the artificial growth media (e.g. V8 juice agar, corn meal agar), however, we do not know whether sexual structures are produced in different plant substrates and/or in the presence of diverse microbial communities. For example, despite *P. palmivora* and *P. cinnamomi* appear as heterothallic in artificial growth media, oospores are formed in naturally infected tissue or when paired with common soil fungi such as *Trichoderma* sp. (Brasier 1972, Crone et al., 2013, Jung et al., 2013). Whether most tropical species function primarily as asexual organisms, and if sexual structures form routinely but our methods are not suitable, requires further research. On the other hand, from an biological stand point, scant information exist of an sexual recombination of tropical species despite two mating types exist in the same environment (Drenth and Guest 2013; Truong et al., 2010), suggesting a biology that evolves around asexual life cycle with sporadic sexual recombination. Previously, it

has been postulated that in the wet tropics *Phytophthora* species do not need thick-walled chlamydospores or oospores to survive dry or cold periods like those in temperate climate types (Drenth and Guest 2013).

On the practical side, for plant pathologist to identify which of the novel taxa would represent a plant health threat is a difficult task. Considering that vast majority of the species within the genus being plant pathogens, perhaps all could potentially cause diseases. However, which of the novel taxa would be a “forest killer” like *P. ramorum* or *P. cinnamomi* would be impossible to predict based on morphology, phylogeny or ecology alone. Nevertheless, our sampling approach provided some clues on their behavior and potential habitats they prefer. Most species found on foliage dropped from canopy, which were also capable to infect live plants. Despite some of these species being isolated occasionally in streams such as *P. capsici*, *P. palmivora*, *P. tropicalis* and *P.* taxon “myristicae”, they were primarily associated with airborne parts of the plants. *P. cinnamomi*, on the other hand only is found in soil. This type of ecology knowledge could help tremendously in refining diagnostic and management approaches and survey efforts to determine presence of these pathogens if they become introduced into new environments.

**Figure 1.** Study sites across Costa Rica



**Table 1** Study sites in Costa Rica.

Site Code	Site Name	Geographic coordinates	Forest Types (I)	Sampling Altitude	Rainfall range (mm/yr.)	Temp day-night (°C)	Date sampled
CR1	Tapantí National Park	9°44'58.6"N; 83°46'54.4"W	Pre Montane (humid)	1288	3000-7000	18-12	7/9/12
CR2	Volcán Poás National Park	10°09'23.3"N; 84°13'17.9"W	Montane (humid)	2641	3000	12-6	7/8/12
CR3	Guayabo-National Monument	9°57'50.2"N; 83°41'32.0"W	Pre Montane (humid)	1040	3500	18-24	7/11/12
CR4	Las Palmas National Park	10°09'42.1"N; 83°56'16.3"W	Low land (Very humid)	500	4500	>24	7/7/12
CR5	Carara National Park	9°47'08.2"N; 84°36'56.8"W	Low land (humid/dry)	90	4000	>25	7/10/12
CR6	Los Quetzales National Park	9°36'46"N 83°49'05"W	Pre Montane (humid)	1200	2500	24-18	7/12/12
CR7	Manuel Antonio National Park	9°22'32"N 84°08'09"W	Low land (dry)	17	3500	>24	1/15/13
CR8	Corcovado National Park	8°33'00"N 83°35'00"W	Low land (Very humid)	25	5000	>24	1/17/13
CR9	Barra Honda National Park	10°10'30"N 85°20'00"W	Low land (dry)	85	2000	>24	1/19/13
CR10	Santa Rosa National Park	10°53'01"N 85°46'30"W	Low land (dry)	85	1500	>24	1/20/13

**Table 2** Number of isolates that were recovered based on the isolation source

<i>Phytophthora</i> spp.	N	Source			
		Live plant	Fallen leaves	Stream	Soil
<i>P. capsici</i>	22	4	12	6	
<i>P. cinnamomi</i>	1	-	-	-	1
<i>P. cryptogea</i>	5	-	-	5	
<i>P. hevea</i>	13	4	5	4	
<i>P. macrochlamydospora</i>	6	2	2	2	
<i>P. palmivora</i>	20	6	10	4	
<i>P. tropicalis</i>	20	3	12	5	
<i>P. taxon PgChlamydo</i>	16	1	10	4	1
<i>P. taxon vulcanica</i>	36	2	6	28	
<i>P. taxon aquatropicalis</i>	5	-	-	5	
<i>P. taxon cararaensis</i>	2	-	1	1	
<i>P. taxon lagoriana</i>	39	3	16	20	
<i>P. taxon tapantiensis</i>	6	2	4	-	
<i>P. taxon frondescens</i>	11	2	6	3	
<i>P. taxon montana</i>	7	-	2	5	
<i>P. taxon aerium</i>	5	1	3	1	
<i>P. taxon myristicae</i>	18	2	14	2	
<i>P. taxon pteridophyta</i>	3	3	-	-	
<i>P. taxon costaricensis</i>	19	1	13	5	
<i>P. taxon cuyabensis</i>	1	-	-	1	
<i>P. taxon rarus</i>	3	-	2	1	
Total	258	36	118	102	2

**Table 3** Distribution of *Phytophthora* species across sampling sites

<i>Phytophthora</i> spp.	Sampling sites									
	CR1	CR2	CR3	CR4	CR5	CR6	CR7	CR8	CR9	CR10
<i>P. capsici</i>	x	x	x		x	x				
<i>P. cinnamomi</i>				x						
<i>P. cryptogea</i>		x			x					
<i>P. hevea</i>		x		x	x			x		
<i>P. macrochlamydospora</i>	x				x		x			
<i>P. palmivora</i>	x	x			x					
<i>P. tropicalis</i>	x	x	x		x					
<i>P. taxon PgChlamydo</i>		x			x					
<i>P. taxon vulcanica</i>		x			x			x	x	
<i>P. taxon aquatropicalis</i>							x			
<i>P. taxon cararaensis</i>		x			x					
<i>P. taxon lagoariana</i>		x			x		x	x	x	x
<i>P. taxon tapantiensis</i>	x				x					
<i>P. taxon frondescens</i>	x	x	x		x					
<i>P. taxon montana</i>	x	x	x							
<i>P. taxon aerium</i>				x	x					
<i>P. taxon myristicae</i>	x	x		x	x			x		
<i>P. taxon pteridophyta</i>	x									
<i>P. taxon costaricensis</i>	x		x	x						
<i>P. taxon cuyabensis</i>				x						
<i>P. taxon rarus</i>				x	x					
# species at a site	9	12	5	7	15	1	3	4	2	1

**Table 4** Total number of samples collected/samples yielded *Phytophthora* spp.

Site	Source			
	Live plant (LP)	Fallen leaves (LF)	Stream (LS)	Soil (S)
CR1	16/5	38/18	11/6	na
CR2	10/1	22/10	29/28	5/0
CR3	10/1	17/12	15/4	na
CR4	8/1	39/7	4/4	5/1
CR5	29/10	29/18	25/11	5/1
CR6	0/0	0/0	2/2	
CR7	3/0	12/2	13/12	
CR8	0/0	22/7	37/5	
CR9	0/0	22/13	12/4	
CR10	0/0	0/0	5/2	
Total	76/18	213/87	153/78	15/2

**Table 5** Number of different *Phytophthora* species in different forest/ecological settings

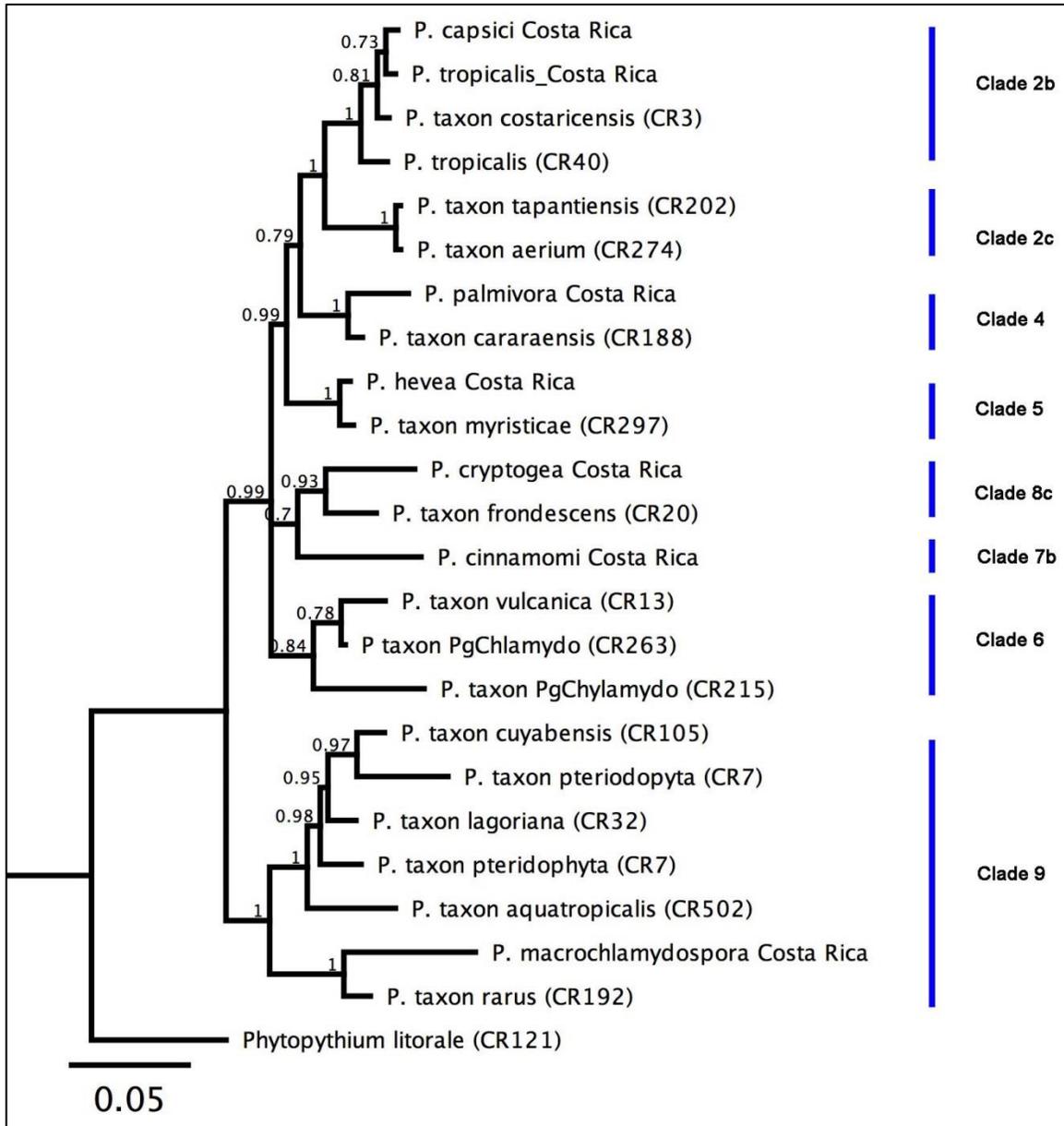
Forest Types (I)	Number of sites	Av. Sampling Altitude	# of different species
Pre Montane (humid)	4	1542	27
Basel (Very humid)	2	262	11
Basel (humid/dry)	1	90	15
Basel (dry)	3	62	6

**Figure 2** Sampling material and isolation methods. Sampling material consisted of four different sources; A-C) Leaves submerged in water with water-soaked lesions; D-K Newly dropped leaves from tree canopy that had dark, brown necrotic lesions; L Leaves of understory plants with necrotic lesions that are still attached to the plant; and M soil samples that were collected from around trees and baited with mango (*Mangifera indica*) leaflets. Typical *Phytophthora* colonies (N) and setting demonstrating isolation tools and media (selective media, CMA filled microtubes, dissecting needle and cork borer, paper towel, ethanol, light source and a compact stereomicroscope).



**Figure 3** Bayesian inference tree based on 8 markers.

Based on four nuclear and four mitochondrial sequences showing phylogenetic relationship of *Phytophthora* species found in this survey. *Phytophthora litorale* from Maryland streams were used as outgroup. Number above the branches represent a bootstrap support >70% based on parsimony analysis. Molecular clades as shown in Martin et al. 2014 are shown.



**Supplemental Table 1** Primers used for amplification and sequencing for the multilocus phylogeny.

<b>Marker</b>	<b>Amplification primers</b>	<b>Annealing Temp. (°C)</b>	<b>Extension time</b>	<b>Reference</b>
ITS	ITS5 GGAAGTAAAAGTCGTAACAAGG ITS 4 TCCTCCGCTTATTGATATGC	56	60 s	White et al. 1990-
$\beta$ -tubulin	Btub_F2 CGGTAACAACCTGGGCCAAGG Btub R1 CCTGGTACTGCTGGTACTCAG	65	60 s	Blair et al. 2008
Elongation Factor 1 $\alpha$	EF1A_for TCACGATCGACATTGCCCTG EF1A-rev ACGGCTCGAGGATGACCATG	65	60 s	Blair et al. 2008
28S rDNA	LROR-O ACCCGCTGAACTYAAGC LR6-O CGCCAGACGAGCTTACC	60	90 s	Blair et al. 2008
Cox1	Cox1-F GGTGCACCTGATATGGCTTT Cox1-R ACAGGATCACCTCCACCTGA	56	60 s	Quesada-Ocampo et al. 2011
Cox2 + spacer	FM35 CAGAACCTTGGAATTAGG Phy10b GCAAAAGCACTAAAAATTAAYATAA	54		Martin et al. 2014
Nad9	Nad9-F TACAACAAGAATTAATGAGAAC Nad9-R GTTAAAATTTGTACTACTAACAT	61		Martin et al. 2014
rps10	Prv9-F GTATACTCTAACCAACTGAGT Prv9-R GTTGGTTAGAGTAAAAGACT	59		Martin et al. 2014

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### Chapter 3 Description of novel *Phytophthora* taxa found

This chapter describes the morphological features of novel taxa and compares the species to the phylogenetically closest species. This component of the study has been drafted to be submitted for the peer-reviewed journal *Persoonia*. Tentative title: Multiple new *Phytophthora* species from tropical ecosystems of Costa Rica.

#### Abstract

A survey of *Phytophthora* species was conducted in several National Parks and Conservation Areas across Costa Rica. Based on morphological features and phylogenetic placement using eight nuclear or mitochondrial markers, in total 13 novel species were identified. These include; *P. vulcanica*, *P. aquatropicalis*, *P. cararaensis*, *P. lagoriana*, *P. tapantiensis*, *P. frondescens*, *P. montana*, *P. aerium*, *P. myristicae*, *P. pteridophyta*, *P. costaricensis*, *P. cuyabensis*, and *P. rarus*. The molecular clades where new species were placed included clade 2 (3 species), clade 4 (1 species), clade 5 (1 species), clade 6 (1 species), clade 8 (2 species) and clade 9 (5 species). Only four species were homothallic and all others heterothallic. Several of these species were found infecting foliage but also found in streams. *P. pteridophyta* was only found infecting foliage of live plants whereas *P. aquatropicalis* was only found in freshwater streams.

Keywords: Oomycete, Biodiversity, ITS, multilocus, tropics, plant pathogens

## **Introduction**

*Phytophthora* species are primarily known as plant pathogens. Therefore, for about a century, they have been studied only when they caused diseases. Merely after late 1990's attempts were made to discover their biodiversity in natural ecosystems without them being involved in obvious disease involvement. These studies revealed dozens of new species (Appel et al., 2004; Balci and Halmshlager, 2003; E. Moralejo, 2009; Hong et al., 2005; Leonberger et al., 2013; Oh et al., 2013) and also sparked expeditionary surveys where researchers randomly sampled soils or baited/filtered stream water to isolate *Phytophthora*. This resulted, as expected, with an exponentially increase in novel species (Martin et al., 2012). Perhaps, one short come of these studies were that almost all were carried out in temperate forest; the tropical areas still remains largely undiscovered. In this study, we describe novel species found in tropical forest ecosystems of Costa Rica. Attempts were made to isolate these organisms from different niches such as fresh water, soil, live plants and foliage that dropped from tree canopies.

## **Materials and Methods**

### **Sampling and isolation of *Phytophthora***

Sampling material included four different sources; 1) Leaves of understory plants with necrotic lesions that are still attached to the plant (LP); 2) Newly dropped leaves from tree canopy that had dark, brown necrotic lesions (LF); 3) Leaves submerged in water with water-soaked lesions (LS), and 4) soil samples that were collected from around trees (S). A *Phytophthora* characteristic infection on foliage

typically included a dark, brown lesion that primarily starts from petiole or leaf tip with an expanding blotch-like lesion. Leaf samples were primarily collected along trails and efforts were made to sample diverse plants at different distances. Each leaf sample was wrapped in a t-fold paper towel in the field to eliminate cross contamination. Soil samples were collected from randomly selected trees at a distance of 1-2m from tree stem (Balci et al. 2007; Martin et al. 2012).

Isolation from leaves was targeted to necrotic areas on the leaf and small sections were plated on a *Phytophthora* selective media (PARPNH) as described in Martin et al., (2012). Isolation from soil involved baiting soils with *Mangifera indica* leaflets (collected in La Sabana Park in the City of San Jose, Costa Rica) as described previously (Martin et al., 2012). Petri dishes were incubated at room temperature and after 2 days, outgrowing hypha that is characteristic of *Phytophthora* was transferred onto corn meal agar (CMA) test tubes (1ml CMA in a 1.5 ml conical tube) using a stereomicroscope. Sub-culturing was based on hyphal morphology and growth pattern; anything that deemed different in hyphal or colony morphology was sub-cultured and shipped to the University of Maryland Forest Pathology lab (3 days shipment duration). Once arrived to the lab, all isolates were sub-cultured onto a clarified V8 juice media and then passed through the PARPNH selective media to eliminate bacterial contamination using the pancake method (Martin et al., 2012). All isolates were maintained in CMA agar slants at 15 °C.

## **DNA isolation, amplification and sequencing**

DNA was extracted either using the Lyse and Go Reagent (Thermo Scientific Pierce) or AccuPrep® Genomic DNA Extraction Kit (Bioneer Corporation) and by following manufacturer's protocols. Mycelia was harvested after isolates were grown in 1.5ml tubes containing Potato Dextrose Broth (PDB) (Difco Laboratories) and allowed to grow for 7 days at room temperature. In a few cases where an isolate did not grow well in broth, homogenization and extraction were performed using a 0.5cm x 0.5cm slice of agar taken directly from the V8 juice agar plate.

PCR amplifications were set up in 30 $\mu$ L reactions as follows 2 $\mu$ L of extracted DNA, 19.7  $\mu$ L molecular-grade water, 0.2 $\mu$ L (total: 1 unit) Platinum Taq DNA polymerase (Life Technologies), 3 $\mu$ L 10X PCR buffer (Life Technologies), 1.5 $\mu$ L 50mM MgCl<sub>2</sub> (final concentration: 2.5mM), 0.6 $\mu$ L 10 $\mu$ M dNTPs (Affymetrix) (final concentration: 200 $\mu$ M), 1.5 $\mu$ L 10 $\mu$ M forward primer (final concentration: 0.5 $\mu$ M), 1.5 $\mu$ L 10 $\mu$ M reverse primer (final concentration: 0.5 $\mu$ M) (see Supplemental Table S1 for primers and annealing temperatures; all from Integrated DNA Technologies). For isolates which did not amplify well on the first attempt, retries were done using 40 cycles rather than 36 cycles, with the annealing temperature dropped by 1°C. PCR product size and quality were checked via horizontal gel electrophoresis. Before sequencing, 10 $\mu$ L of each PCR product was cleaned of leftover primers and dNTPs by adding 2 $\mu$ L FastAP Alkaline Phosphatase, 1 $\mu$ L FastAP buffer, and 0.4 $\mu$ L Exonuclease I (Thermo Scientific) and thermal-cycling as follows: 37°C for 30 minutes, then 80°C for 15 minutes. The cleaned PCR products were sent to Molecular

Cloning Laboratories for Sanger sequencing (MCLab, San Francisco, CA).

Sequencing was done using the same primers as were used for PCR amplification.

### **Phylogenetic analysis**

The forward and reverse read of each locus were quality-trimmed, de novo assembled, and ambiguities were resolved with visual analysis of the chromatograph and re-sequencing when necessary using the software Geneious Pro v. R6 (Biomatter Ltd.) (Kearse et al., 2012). The consensus sequence was extracted from each assembly and sequences were aligned using the Geneious alignment option.

Alignments were then concatenated and used to conduct Bayesian analysis with MrBayes v3.1 with a plugin in the software Geneious. All dataset and trees derived from Bayesian analyses are available in TreeBASE ([www.treebase.org/](http://www.treebase.org/))

### **Characterization of isolates to species level**

Isolates were first grouped using Internal Transcribed Spacer (ITS) region of the rDNA (ITS 4 & 5) (White et al., 1990). For this purpose ITS sequence data was compared with other species in GenBank using BLAST searches. Each group then examined for their key morphological features using a *Phytophthora* lucid key (an early version of the lucid key is published in Martin et al., 2012). When morphology and ITS based sequence identification were in agreement, the species identification was deemed final. Any isolate that had mismatch between ITS sequence identity and morphological features were deemed as potentially novel species and further sequenced using an additional seven loci (see below).

After multi-locus sequence analysis, isolates representing a novel species were subject to extensive morphological evaluation that included various sexual and asexual features (Martin et al., 2012). Morphological observations were made on clarified Campbell's V8 juice agar media (cV8A). For any morphological feature, at least 25 measurements were made and the average size along the standard deviation was provided. Subsets of select isolates were also subject to growth-temperature requirements and colony pattern determination. Isolate daily growth rate was determined at temperature ranging from 10 C to 40 °C using cV8A (19 ml) on 9-cm Petri dishes. Growth pattern was determined after 10-days of growth on cV8A and Potato Dextrose Agar (PDA) at 20 °C.

## **Results**

Genbank accession numbers for various markers, culture accession numbers and Mycobank numbers are pending for all species described below.

## **Phylogenetic analysis**

The new species were found in six different molecular clades (Figure 1). Most species resided in clade 9 (5 species) followed by clade 2 (3 isolates), clade 8 (2 isolates). One species each were placed in clades 4, 5, 6 and 7.

## **Taxonomy**

Morphological and physiological characters of the 13 new species are summarized in Tables 3 and 4.

***Phytophthora vulcanica* Y. Balci & S. Wallace, sp. no.**

-MycoBank no: MBxxxxxxx

**Etymology** “vulcanica” refers to its frequent occurrence in the close vicinity to Volcán Poás where this species was first isolated.

**Type:** Costa Rica Parque Nacional Volcán Poás, 10°09'23.3”N; 84°13'17.9” W. Isolate code CR 13 (Site code CR 2- LS 20A); ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx); collection date: July 2012.

**Ecology:** Most isolates were recovered from Montane (holotype isolate) and pre Montane humid tropical forests. Type species was isolated from leaves submerged in freshwater streams (LS) but additional isolates were recovered from small necrosis on live plants (LP), and newly fallen leaves from tree canopy (LF).

Additional isolates were recovered at national parks throughout Costa Rica in July 2012 or January 2013. Isolation sites include; Volcán Poás National Park (CR2-LS= 26 isolates), Carara National Park (CR5-LP= 2 isolates), Corcovado National Park (CR8-LS=1 isolate), (CR9-LF= 4 isolates), Barra Honda National Park (CR9-LS= 2 isolates).

**Gametangia.** *Phytophthora vulcanica* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A 2 tester strains of *P. cinnamomi*.

**Sporangia.** The majority of isolates including the type species produced sporangia around 24 hours after flooding with soil extract water. Sporangia were non-papillate,

ovoid, ovoid-elongated in shape, with simple sympodial sporangiophore and nested extended proliferation. Sporangial sizes (length x width) for the most common shape (ovoid-elongated) was  $41.9 \pm 4.7$  (35.1 - 50.8)  $\mu\text{m}$  length x  $28.2 \pm 3.4$  (21.87 – 35.3)  $\mu\text{m}$  width and a length: width ratio of  $1.5 \pm 0.1$  (Fig.2). Sporangia were non-caducous.

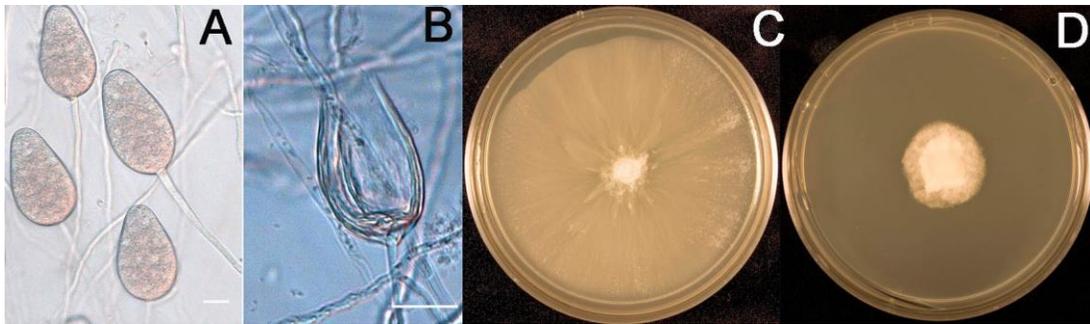
**Hyphal swellings.** Not present on agar media or when flooded with soil extract water.

**Chlamydospores.** No chlamydospores were produced.

**Colony growth pattern.** On PDA stoloniferous submerged, with no distinct forms, on V8A, stellate (fig).

**Temperature–growth relationship:** Optimum temperature for growth on clarified V8A was at  $30\text{ }^{\circ}\text{C}$  ( $4.5 \pm 2.9$  mm/d) and no growth at  $35\text{ }^{\circ}\text{C}$  (Table 3). After exposure to the upper temperature limit of  $35\text{ }^{\circ}\text{C}$ , isolates did not grow when returned to room temperature.

**Notes:** Phylogenetically *Phytophthora vulcanica* is closely related to the provisionally named taxon *P. gonapodyides* “chlamydospora”. Morphological features overlap considerably with *P. gonapodyides*. It can be differentiated by its sporangial size and shape and sympodial sporangiophore which is not reported with *P. gonapodyides*. While *vulcanica* produced large, elongated ovoid sporangia, *P. gonapodyides* produces smaller obpyriform sporangia. Growth pattern on cV8A and PDA were also different from *P. gonapodyides* (Erwin and Ribeiro 1996).



**Fig. 1** Morphological features and growth pattern of *Phytophthora vulcanica*.

A. Ovoid non papillate sporangia; B. internal, nested proliferation of sporangia when cV8 plugs were flooded with soil extract water; C. Stellate colony pattern on clarified V8 agar; D. Fluffy, undefined colony pattern on potato dextrose agar. Scale bar 20  $\mu\text{m}$ .

***Phytophthora aquatropicalis* Y. Balci & S. Wallace, sp. nov.**

-MycoBank no.: MBxxxxxx;

Etymology. “*aquatropicalis*” refers to its only occurrence in aquatic environments such as freshwater streams in the tropics.

Type: Costa Rica, Parque Nacional Manuel Antonio, 9°22'32"N 84°08'09"W. Isolate code CR 502 (site code CR7-LS-5A), ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx). In total five isolates were recovered at the same site. Isolation date January, 2013.

Ecology: All isolates were recovered from Lowland, dry tropical forest. All isolates including type species were isolated from leaves submerged in fresh water streams (LS).

**Gametangia.** *Phytophthora aquatropicalis* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** Absent in agar media, but majority of isolates including the type species produced small amounts of sporangia around 72 to 96 hours after flooding with soil extract water and multiple water changes. Sporangia were non-papillate and ovoid in shape with nested internal sporangiophores. Sporangial sizes (length x width) were; ovoid (most common)  $45.8 \pm 5.1$  (34.8 – 53.7)  $\mu\text{m}$  length x  $31.5 \pm 5.4$  (23.2– 41.7)  $\mu\text{m}$  width, and a length/ width ratio of  $1.4 \pm 0.3$  (Fig. 3).

**Hyphal swelling** were produced in liquid culture, none observed on agar media.

**Chlamydospores.** Thin walled chlamydospores were produced occasionally in liquid culture, averaging 30.1  $\mu\text{m}$  (21.3 - 42  $\mu\text{m}$ ) in diameter.

**Colony growth pattern.** No distinct form on PDA, and submerged, radiate on V8A

**Temperature –growth relationship:** Optimum temperature for growth on V8A was between 25 °C and 30 °C (4.3 mm/d), with the upper temperature limit of 35 °C, no growth was observed at 37.5 °C.

**Notes:** Phylogenetically *Phytophthora aquatropicalis* is closely related to the *P. irrigata* (Hong et al. 2008). However, it has smaller sporangia and larger length/width ratio (*P. irrigata*= 44x33 and l/b ratio of 1.3; *P. aquatropicalis*= 43x32 and l/b ratio of 1.4). In addition *P. aquatropicalis* is heterothallic and grows much slower with a 10 °C difference in optimum temperature (25 °C for *P. aquatropicalis* vs. 35°C for *P. irrigata*).



**Fig. 2** Morphological features and growth pattern of *Phytophthora aquatropicalis*.

A-B. Ovoid non-papillate sporangia with a nested proliferation of spores; C. typical branching of hyphae with inflated secondary hyphae; D. Submerged, radiating pattern on clarified V8 agar; E. Aerial, non-defined colony pattern on potato dextrose agar.

Scale bar 20  $\mu$ m.

***Phytophthora cararensis* Y. Balci & S. Wallace, sp. nov.**

Mycobank no.: MBxxxxxxx;

Etymology. “Carara” refers to the Carara National Park, Costa Rica where this species was first isolated.

**Type:** Costa Rica, Carara National Park, 9°47’08.2” N; 84°36’56.8” W. Isolate code CR 188 (site code CR5-LF-3A). Ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx); collection date: July 2012.

**Ecology:** Most isolates were recovered from lowland (humid/dry) tropical forest.

Type species was isolated from leaves collected from the forest floor (LF).

Additional isolates were recovered at Volcán Poás National Park (CR2-LS-3B= 1 isolate) from leaves submerged in fresh water stream, and Carara National Park from leaves collected from the forest floor (CR5-LF-3A1=1 isolate).

**Gametangia.** *Phytophthora cararensis* is homothallic and produced gametangia abundantly on cV8A within 30 days. Oogonia, terminal at the main hyphae, globose with rounded base. Mean oogonial diameter  $28.5 \pm 2.9$  ranging from 24 to  $33.7 \mu\text{m}$  on V8A. The oogonial stalk tapered and occasionally coiled (Fig. 4). Oospores always spherical, aplerotic on cV8A with an average diameter of  $24.2 \pm 3.2 \mu\text{m}$  ranging from 18 to  $29.9 \mu\text{m}$ . Average oospore wall thickness was  $2.3 \pm 0.5 \mu\text{m}$ . Paragynous antheridium, averaging  $7.5 \pm 1.4 \mu\text{m}$ , ranging from 4.9 to  $9.7 \mu\text{m}$  in length.

**Sporangia.** Seldom observed on cV8A, but abundantly formed 1 to 2 days after agar plugs were immersed in soil extract water. Sporangia was always terminal and papillate with ovoid, globose and obpyriform shapes. Occasionally two papilla are formed on a sporangia (Fig. 4). Sporangiphore was simple sympodial. Lateral displacement of papilla was common on ovoid-elongated sporangia. Sporangia size (length x width) were; obpyriform (most common)  $39.1 \pm 4.7$  (32-49  $\mu\text{m}$ ) length x  $27.1 \pm 4$  (17.8 – 35.6  $\mu\text{m}$ ) width and a length / width ratio of  $1.4 \pm 0.2$ .

**Chlamydospores.** Thin walled chlamydospores were produced in liquid culture averaging  $23.6 \mu\text{m}$  (range 18.1-28.6  $\mu\text{m}$ ) in diameter.

**Hyphal swelling.** Produced abundantly in liquid media, but none on solid agar.

**Colony growth pattern.** On PDA stoloniferous with no distinct form and cottony on cV8A.

**Temperature–growth relationship:** Optimum temperature for growth on V8A was at  $25^{\circ}\text{C}$  (3.4 mm/d), with the upper temperature limit of  $35^{\circ}\text{C}$  at which there were no

growth (Table 3). When isolates were returned to room temperature after exposure to 35°C it continued to grow.

**Notes:** Phylogenetically *Phytophthora cararaensis* is closely related to *P. quercetorum*. Morphological features overlap considerably with *P. quercetorum* (Balci et al. 2008). Oospores of *P. cararaensis* are smaller and lacks the common occurrence of markedly aplerotic, coiled and tapered oogonial stalk that is characteristic with *P. quercetorum*. In addition, *P. cararaensis* has a slower growth rate (3.4 mm/d at 25 °C) in contrast to *P. quercetorum* (5.7mm/day at 22°C). Hyphal morphology, colony pattern are also two other features that separates these species.

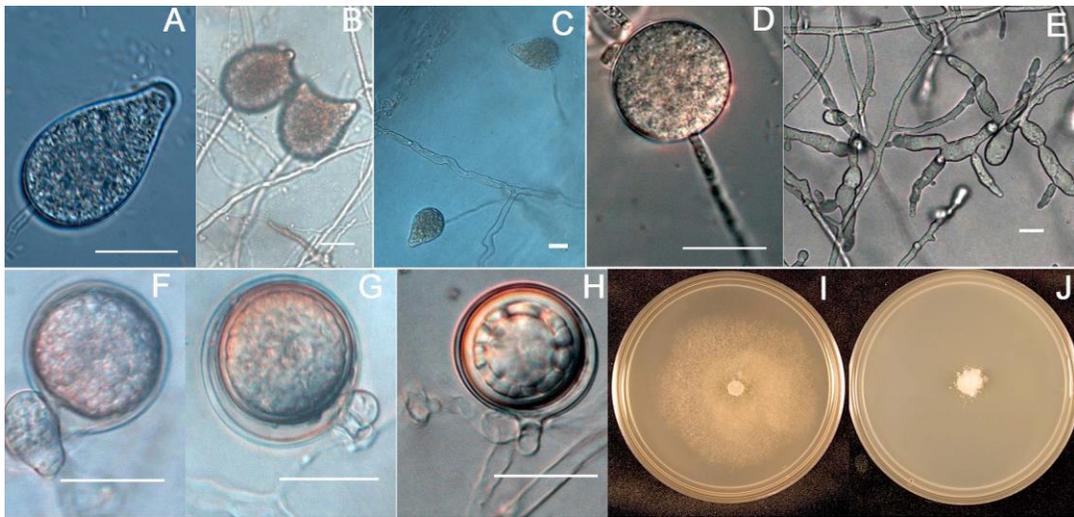


Fig. 3 Morphological features and growth pattern of *Phytophthora cararaensis*. A. ovoid–elongated papillate sporangia; B. Bipapillate sporangia; C. Ovoid elongated, laterally attached sporangia; D. Intercalarly produced chlamydospores; E. Swollen typical hyphae; F-H. Oogonia with paragynous antheridia; I. Cottony, slightly aerial colony pattern on clarified V8 agar; J. Stoliferous, colony pattern on potato dextrose agar after 10 days. Scale bar 20  $\mu$ m.

***Phytophthora lagoariana* Y. Balci, M. Coffey, S. Wallace sp. nov. –**

MycoBank no.: MBxxxxxxx

Etymology. “Lagoariana” refers to Ariana, the granddaughter of Mike Coffey who first discovered this species in Cuyabana reserve in Ecuador.

Type: Costa Rica, Carara National Park, 9°47'08.2” N; 84°36'56.8” W. Isolate code CR32 (Site code CR5-LS-13B) ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx); (40 isolates). Isolates were recovered between July 2012 and January 2013

**Ecology:** Isolates were recovered in all forest types in Costa Rica. Sites included; Volcán Poás National Park, (CR2-LS=1 isolate), Carara National Park (CR5-LS=3 isolates), (CR5-LF=3 isolates), Manuel Antonio National Park, (CR7-LS=10 isolates), CR7-LF=2 isolates, Corcovado National Park, (CR8-LS=4 isolates), CR8-LS=4 isolates), Barra Honda National Park, (CR9-LF=13 isolates) and Santa Rosa National Park (CR10-LS-2 isolates).

**Gametangia.** *Phytophthora lagoariana* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** The majority isolates including the type species produced sporangia 12 to 24 hours after flooding with soil extract water. Sporangia were non-papillate and obpyriform to ovoid in shape, with internal, extended sporangiophore and nested (2 to 3 sporangia) proliferation. Sporangial sizes (length x width) were ovoid elongated (most common)  $49.82 \pm 4.4$  (39.2 – 57.0)  $\mu\text{m}$  length x  $33.5 \pm 3.6$  (24.0 – 41.0)  $\mu\text{m}$  width and a length: width ratio of  $1.5 \pm 0.2$   $\mu\text{m}$  (Fig. 5). Sporangia was non-caducous.

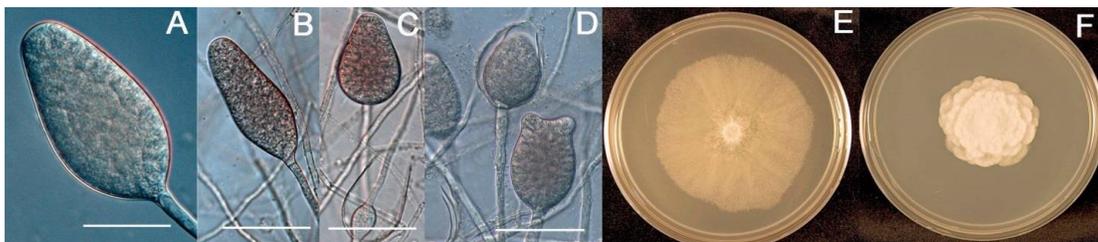
**Hyphal swelling.** Produced in liquid culture and in solid agar.

**Chlamydozoospores.** Thin walled chlamydozoospores were produced rarely in liquid culture, averaging 26.31  $\mu\text{m}$  (21.1 – 32.9  $\mu\text{m}$ ) in diameter.

**Colony growth pattern:** rosaceous pattern on PDA and V8A (Fig 5).

**Temperature –growth relationship.** Optimum temperature for growth on V8A was between at 30°C (5 mm/day), with an upper temperature limit of 35°C, at which growth stopped but resumed growing when placed at room temperature. No growth was observed at 37.5 °C (Table 3).

**Notes:** *P. lagoariana* is phylogenetically placed in clade 9 (Fig. 1) along many other *Phytophthora* species primarily isolated from aquatic environments. It is closest to *P. hydropathica* (Hong et al. 2010). Sporangia of *P. lagoariana* is narrower and smaller than *P. hydropathica* (*P. hydropathica* mean l/w= 55x40 l/w ratio 1.4; *P. lagoariana* l/w= 50x34, l/w ratio 1.5). *P. lagoariana* remained sterile while *P. hydropathica* produced oospores when paired with tested strains. *P. hydropathica* has also slower growth rate at the optimum temperature of 30 °C (5 mm/day vs. 13-14 mm/day). *P. hydropathica* grew also well at 35°C but with *P. lagoariana* scant growth was observed.



**Fig.4** Morphological features and growth pattern of *Phytophthora lagoariana*; A-B markedly ovoid-elongated non-papillate sporangia, C, D. internal, nested proliferation with an ovoid sporangia, and bipapillate sporangia; E. Non defined colony on

clarified V8 agar; F. Rosaceous colony pattern on potato dextrose agar. Scale bar 20  
μm.

***Phytophthora tapantiensis*** Y. Balci & S. Wallace, sp. nov.

-MycoBank no.: MBxxxxxx

**Etymology.** “Tapantí” refers to Tapantí National Park in Costa Rica where this species was first isolated.

**Type.** Costa Rica, Parque Nacional Tapantí, 9°44'58.6"N 83°46'54.4"W. Isolate code CR22 (site code CR1-LF-7A1). Ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx). Collection date: July 2012. In total 3 isolates that were recovered either at Parque Nacional Tapantí (CR1).

**Ecology.** All isolate were recovered from pre Montane humid tropical forests. Type species and an additional isolate was isolated from newly fallen leaves from tree canopy (LF) and one isolate from small necrosis on live plant (LP).

**Gametangia.** *Phytophthora tapantiensis* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** Sporangia of *Phytophthora tapantiensis* were formed around 24 to 36 hours after flooding with soil extract. Sporangia were semipapillate and lemoniform-ovoid in shape, with sympodial sporangiophore. Sporangial sizes (length x width) were (most common)  $50.0 \pm 5.9 \mu\text{m}$  (40.9 – 58.5  $\mu\text{m}$ ) length x  $32.3 \pm 3.55 \mu\text{m}$  (24.5 – 38.3 $\mu\text{m}$ ) width and a length/width ratio of  $1.5 \pm 0.1$ . Type species formed mainly lemoniform sporangia with a size of  $53.4 \pm 7.3 \mu\text{m}$  (44.6 – 59.4 $\mu\text{m}$ ) length x  $31.3 \pm 3.1 \mu\text{m}$  (25.1 – 36.2  $\mu\text{m}$ ) width.

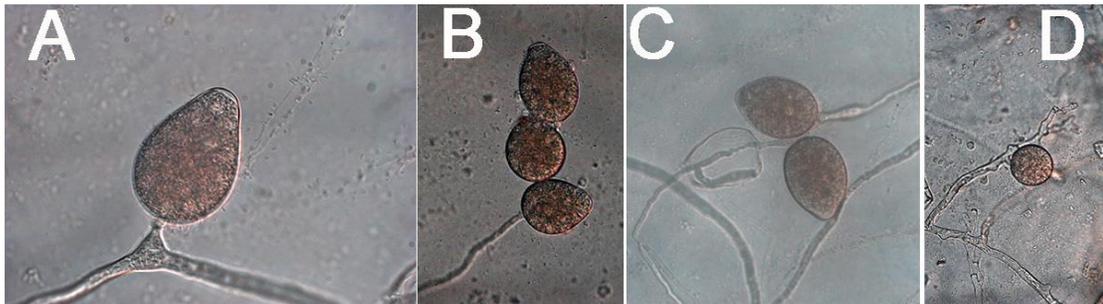
**Hyphal swelling.** Produced abundantly in liquid culture, none observed on solid agar

**Chlamydospores.** No chlamydospores were observed.

**Colony growth pattern.** On V8A cottony-petaloid and on PDA rosaceous, cottony and petaloid to rosaceous pattern.

**Temperature–growth relationship.** Optimum temperature for growth on V8A was between 20°C and 25°C (4.6 mm/d), with the upper temperature limit above of 30°C. No growth was observed at 35 °C but isolate continue to grow after been place at room temperature.

**Notes:** Phylogenetically *Phytophthora tapantiensis* is related to *P. multivesiculata* (Ilieva et al. 1998) in molecular clade 2c (Figure 2). Sporangia resembles *P. multivesiculata*, however, it can easily be differentiated because *P. tapantienses* does not produce oogonia.



**Fig. 5** Morphological features of *Phytophthora tapantiensis* A-C. Ovoid, semi papillate sporangia; D. thin walled chlamydospore.

*Phytophthora frondescens* Y. Balci & S. Wallace, sp. nov.

Mycobank no.: MBxxxxxx

**Etymology.** “frondes” (in Latin= foliage) refers to common incidence of this species in association with tree foliage.

**Type.** Costa Rica, Parque Nacional Tapantí, 9°44'58.6"N 83°46'54.4"W. Isolate code CR20 (Site code CR1-LF-6A-2) ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx). Collection date: July 2012. In total 6 isolates were recovered.

**Ecology.** Most isolates were recovered from pre-Montane (type isolate) and Montane humid tropical forests. Type species was isolated from newly fallen leaves from the canopy and collected from the forest floor (LF), but additional isolates were recovered from small necrosis on live plants (LP) and from leaves submerged in fresh water streams.

Additional isolates were recovered at Volcán Poás National Park (CR2-LS= 2 isolate) from leaves submerged in fresh water stream, and Guayabo National Monument, (CR3-LF= 1 isolate).

**Gametangia.** *Phytophthora frondescens* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** The majority of isolates including the type species produced sporangia around 24 hours after flooding with soil extract water. Sporangia were semipapillate, ellipsoid and lemoniform-ovoid shape, with terminal and sympodial sporangiophore (Fig. 7). Sporangial sizes (length x width) were lemoniform (most common)  $42.17 \pm$

5.6  $\mu\text{m}$  (31.64 - 52.04  $\mu\text{m}$ ) length x  $27.1 \pm 2.8 \mu\text{m}$  (21.8 – 33.03  $\mu\text{m}$ ) width and a length: width ratio of  $1.5 \pm 0.1$ . Sporangia was caducous with short pedicel.

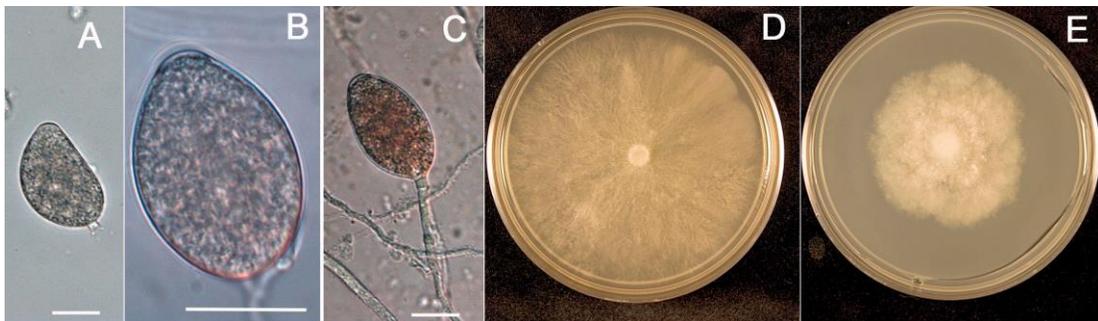
**Hyphal swellings.** Common in liquid culture.

**Chlamydospores.** None were observed.

**Colony growth pattern.** Various patterns on V8A included cottony, petalloid and stellate and on PDA primarily petalloid-rosaceous, rosaceous, and cottony rosaceous.

**Temperature –growth relationship.** Optimum temperature for growth on V8A was  $20^\circ\text{C}$  (5.2 mm/d), with the upper temperature limit of  $25^\circ\text{C}$  for all isolates. The maximum temperature for growth is  $30^\circ\text{C}$  neither where no growth occurred nor when returned to room temperature after exposure to this temperature.

**Notes** *Phytophthora frondescens* resides in molecular clade 8 and phylogenetically is closest to *P. foliorum* (Donahoo et al. 2006). Morphology also resembles *P. foliorum* but it can easily be distinguished from *P. foliorum* by being heterothallic; *P. foliorum* produces readily oogonia in growth media.



**Fig. 6** Morphological features and growth pattern of *Phytophthora frondescens*. A-C Caducous sporangia with short pedicel, ellipsoid semipapillate sporangia; D. Slightly petalloid colony pattern on clarified V8 agar; E slightly fluffy, non-defined colony on potato dextrose agar. Scale bar 20  $\mu\text{m}$ .

***Phytophthora montana*** Y. Balci & S. Wallace, sp. nov.

Mycobank no.: MBxxxxxx

Etymology. “montana” refers to the only finding of this species in Montane and pre Montane areas of Costa Rica that were above 1,000 m sea level.

**Type:** Costa Rica Parque Nacional Volcán Poás, 10°09'23.3"N; 84°13'17.9" W.

Isolate code CR 80 (side code CR 2- LS 24F). Ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx); collection date: July 2012.

**Ecology:** All isolates were recovered from Montane (type isolate) and pre Montane humid tropical forests. Type species was isolated from leaves submerged in freshwater streams (LS) but two isolates were recovered from foliage fallen from tree canopy at the site CR1 (LF). Additional isolates were recovered at other national parks. These sites include; Volcán Poás National Park (CR2-LS=2 isolates), Guayabo national Monument (CR3-LS=1 isolates) and Tapantí National Park (CR1-LS=1 isolate).

**Gametangia.** *Phytophthora montana* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** The majority isolates including the type species produced sporangia 12 to 24 hours after flooding with soil extract water. Sporangia were non-papillate and ellipsoid in shape, with sympodial sporangiophore and external proliferation.

Sporangial sizes (length x width) were ellipsoid (most common)  $46.1 \pm 5.6 \mu\text{m}$  (36.6 to  $58.8 \mu\text{m}$ ) length x  $31.2 \pm 4.8 \mu\text{m}$  (23.9 to  $41.3 \mu\text{m}$ ) width and a length/width ratio of  $1.5 \pm 0.1$ .

**Hyphal swelling.** Produced in liquid culture and solid growth media.

**Chlamydospores.** Thin walled chlamydospores were produced occasionally in liquid culture and averaged 32.1  $\mu\text{m}$  (22.6 – 40.4  $\mu\text{m}$ ) in diameter.

**Colony morphology.** Slight petalloid colony pattern on clarified V8 agar, petalloid colony pattern on potato dextrose agar.

**Temperature –growth relationship.** Optimum temperature for growth on V8A was between 25°C (6.6 mm/d), with a scant growth at 35°C for some isolates (Table 3).

**Notes:** Phylogenetically *P. montana* reside in molecular clade 8 (Fig 2) along with *P. foliorum* (Donahoo et al. 2006) and *P. frondescens* described in this study as the closest species. It can easily be separated by *P. foliorum* by being heterothallic and sterile and non-papillate caducous sporangia. It also produces chlamydospores in liquid culture a feature not observed in *P. foliorum*. It can be differentiated from *P. frondescens* by being non-papillate and production of chain-like hyphal swellings.



**Fig 7** Morphological features of *Phytophthora montana*. A,B Obturbinate, ovoid-elongated non- papillate sporangium; C; Internally extended sporangium, D; Caducous ovoid-elongated sporangium, E; catenulate hyphal swellings;. Scale bar 20  $\mu\text{m}$ .

***Phytophthora aerium***

Y. Balci & S. Wallace, sp. nov. -MycoBank no.: MBxxxxxxx

Etymology. “aerium” refers to the airborne biology of this species.

**Type:** Costa Rica, Parque Braulio Carrillo, 10°09'42.1”N 83°56'16.3”W. Isolate code CR274 (Site code CR4-LF-9A). Ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx). In total 6 isolates recovered. Collection date: July 2012

**Ecology:** Most isolates were recovered from lowland very humid, (type isolate) and lowland humid/dry tropical forests. Type species was isolated from newly fallen leaves from the canopy that were fallen to the forest floor (LF) (CR-LF= 3 isolates), from small necrosis on live plants (LP), and from leaves submerged in fresh water streams.

Additional isolates were recovered at Carara National Park (CR5-LP= 1 isolate) from leaves with small necrosis on live plants.

**Gametangia.** *P. aerium* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** Sporangia formed around 24 hours after flooding with soil extract. They were semi papillate and ovoid-elongate in shape, with sympodial sporangiophore. Internal or nested proliferation was not observed on any of the isolates. Sporangia frequently produced elongated neck and also occasionally misplaced. Sporangial sizes (length x width) were ovoid-elongated (most common)  $51.6 \pm 4.6 \mu\text{m}$  (44.2 – 58.0  $\mu\text{m}$ ) length x  $31.8 \pm 3.2 \mu\text{m}$  (24.1 – 36.2  $\mu\text{m}$ ) width and a length/width ratio of  $1.6 \pm 0.1$ .

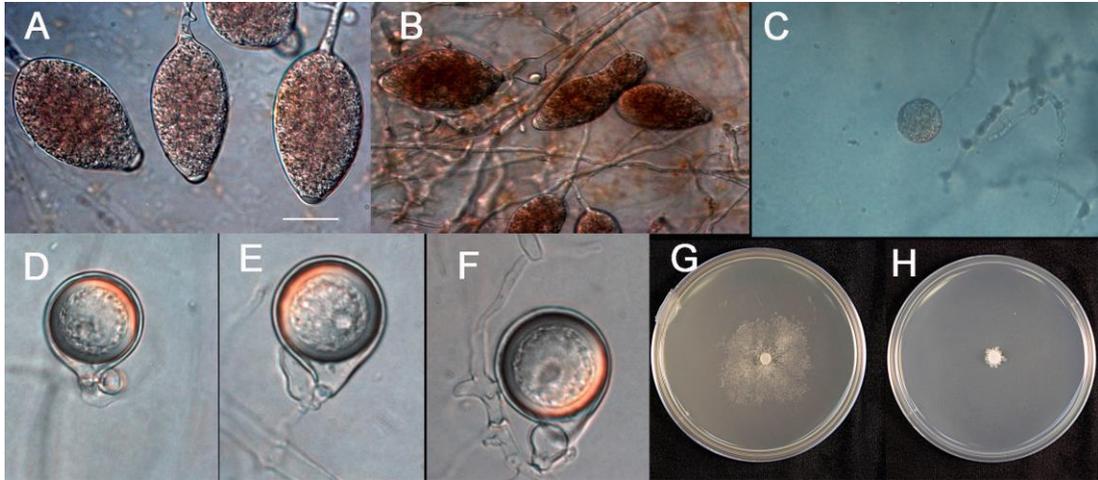
**Hyphal swelling.** None.

**Chlamydospores.** Thin walled chlamydospores were produced occasionally in liquid culture, averaging 26.6µm (21 – 33.7µm) in diameter.

**Colony morphology.** Submerged, non-defined colonies on cV8 and stoloniferous, very slow growth at PDA.

**Temperature –growth relationship:** Optimum temperature for growth on V8A was at 25°C (3.8 mm/d), with the upper temperature limit above 30°C.

**Notes:** Phylogenetically *P. aerium* was placed in cluster 2 and was closest to *P. bisheria* (Abad et al. 2008). It can be distinguished from *P. bisheria* easily on the basis of the sexual structures. *P. aerium* produced smooth walled and plerotic while *P. bisheria* has ornamentations, aplerotic and more than one antheridia is also found. Sporangium of *P. aerium* also differs by being mainly lemoniform and ovoid-elongate and taller (av. 52 µm) while *P. bisheria* they are mainly ovoid-obpyriform averaging a length of 34 µm. The formation of chlamydospores is another feature that separates the two species morphologically.



**Fig. 8** Morphological features and growth pattern of *Phytophthora aerium*. A Ellipsoid, semi-papillate sporangia; B. Elongated ovoid, peanut-shaped and ellipsoid sporangia; C. Thin walled terminal chlamydospores; D-E. Paragynous, plerotic gametangia; G. Non defined, submerged colony pattern on cV8A; H Stoloniferous colony pattern on potato dextrose agar. Scale bar 20  $\mu$ m

***Phytophthora myristicae***

Y. Balci & S. Wallace, sp. nov. -MycoBank no.: MBxxxxxxx

**Etymology.** “myristica” refers to the host *Myristica fregans* the nutmeg tree where it was first isolated from necrosis on stem in Grenada.

**Type:** Las Palmas, Braulio Carrillo National Park 10°09’42.1” N; 83°56’16.3” W.

Isolate code CR244 (site code CR4-LF-3A-2). Ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx). In total 18 isolates were recovered. Collection date July 2012.

**Ecology:** Most isolates were recovered from pre-Montane (type isolate) and Montane humid tropical forests. Type species was isolated from newly fallen leaves from the canopy and collected from the forest floor (LF), but additional isolates were recovered from small necrosis on live plants (LP) and from leaves submerged in fresh water streams. Additional isolates were recovered at Volcán Poás National Park (CR2-LS= 1 isolate) and CR2-LF=1 isolate, Braulio Carrillo National Park (CR4-LS=4C-2=1 isolate, Carara National Park CR5-LF=1 isolate, CR5-LS=1 isolate, and Corcovado National Park, CR4-LF=4 isolates

**Gametangia.** *P. myristicae* is homothallic and produces gametangia abundantly on V8A within 30 days. Oogonia, terminal at the main hyphae, globose with tapered base. Mean oogonial diameter was  $30.3 \pm 2.4 \mu\text{m}$  ranging from 23.6 to 33.5  $\mu\text{m}$  on cV8A. The oogonial stalk tapered and occasionally coiled (Fig.10). Oospores always spherical, markedly aplerotic on V8A with an average diameter of  $24.2 \pm 3.2 \mu\text{m}$

ranging from 18.0 to 29.9  $\mu\text{m}$ . Average oospore wall thickness was  $2.3 \pm 0.5 \mu\text{m}$ .

Amphigynous antheridium averaged  $7.5 \pm 1.4 \mu\text{m}$ .

**Sporangia.** Sporangia were produced around 24 hours after flooding with soil extract water. Sporangia were papillate and globose-ovoid in shape, with sympodial sporangiophore. Some isolates produced internal proliferation in liquid culture.

Sporangial sizes (length x width) were (most common)  $46.7 \pm 5.4 \mu\text{m}$  ( $37.6 - 54.5 \mu\text{m}$ ) length x  $30.6 \pm 3.8 \mu\text{m}$  ( $27.4 - 45.3 \mu\text{m}$ ) width and a length: width ratio of  $1.3 \pm 0.1$ . Type species (globose-ovoid)  $50.3 \pm 5.1$  ( $42.61 - 54.7 \mu\text{m}$ ) length x  $37.4 \pm 3.4 \mu\text{m}$  ( $30.8 - 42.4 \mu\text{m}$ ) width.

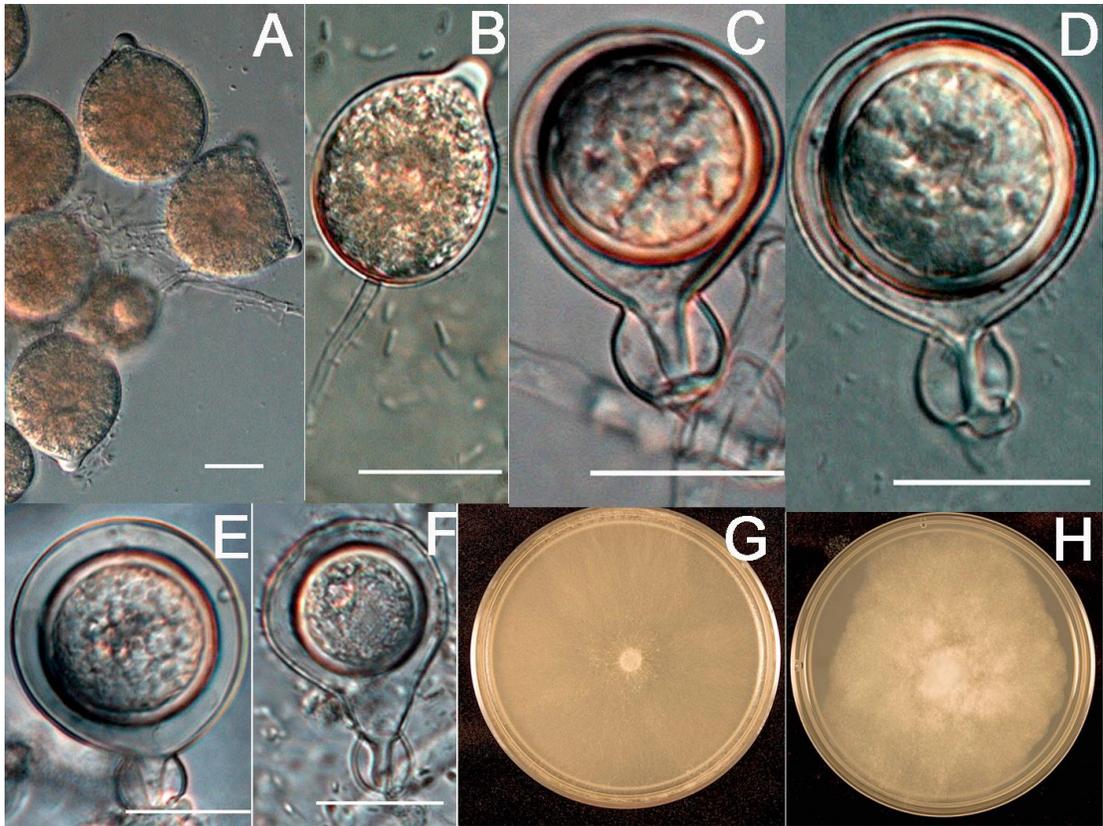
**Hyphal swelling.** Present both in liquid and solid media.

**Chlamydospores.** Thin walled chlamydospores were produced occasionally in liquid culture, averaging  $43.9 \mu\text{m}$  ( $27.4 - 36.3 \mu\text{m}$ ) in diameter.

**Colony growth pattern.** Various pattern on V8A including rosaceous, petalloid and stellate. On PDA irregular cottony to stoloniferous patterns were formed.

**Temperature–growth relationship.** Optimum temperature for growth on V8A was at  $25^\circ\text{C}$  ( $10.1 \text{ mm/d}$ ), with the upper temperature limit of  $35^\circ\text{C}$ . No growth occurred when isolates were returned to room temperature after exposure to  $30^\circ\text{C}$ .

**Notes:** Phylogenetically *P. myristicae* was placed in clade 5 (Figure 1). The closest species was *P. hevea* (Erwin and Ribeiro 1996).



**Fig. 9** Morphological features and growth pattern of *Phytophthora myristicae*. A, B. Globose, ovoid papillate terminal or laterally attached papillate sporangia; C-F. Amphigynous, markedly aplerotic oogonia; G. Radiate colony pattern on cV8 agar; H. Slightly fluffy colony pattern on PDA. Scale bar 20 μm.

***Phytophthora pteridophyta*** Y. Balci & S. Wallace, sp. nov. –

Mycobank no.: MBxxxxxxx

**Etymology.** “pteridophyta” refers to fern in Latin. The species was discovered on small lesions of a live fern species in Costa Rica.

**Type:** Costa Rica, Tapantí National Park 9°44'58.6" N: 83°46'54.4" W. Carara National Park, 9°47'08.2" N; 84°36'56.8" W. Isolate code CR1-LF-9B-1 (CR 7) ex-holotype at the American Type Culture collection (xxxx) and the World

*Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx); collection date: July 2012

**Ecology:** All isolates of this species were recovered from pre-Mountain (humid) tropical forest. Type species and other two isolate was collected from leaves fallen to the forest floor (LF) (CR-LF=2 isolates).

**Gametangia:** *P. pteridophyta* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** The majority isolates including the type species produced sporangia around 24 hours after flooding with soil extract water. Sporangia were semi-papillate and ovoid in shape, with sympodial sporangiophore and nested internal proliferation. Sporangial sizes (length x width) were ovoid (most common)  $38.1 \pm 2.7 \mu\text{m}$  (range 34.2 – 43  $\mu\text{m}$ ) length x  $25.9 \pm 3.4$  (range 22.2 – 31  $\mu\text{m}$ ) width, and a length: width ratio of  $1.4 \pm 0.2$ . Type species had similarly ovoid sporangial shapes with an average sporangial size of  $42.3 \pm 3.7 \mu\text{m}$  (36.3 – 48.2  $\mu\text{m}$ ) length x  $24.8 \pm 3.08 \mu\text{m}$  (20.4 – 31.1) width.

**Hyphal swelling.** Produced abundantly in liquid media, none observed on solid agar.

**Chlamydospores.** Thin walled chlamydospores were produced occasionally in liquid culture, averaging 21.7  $\mu\text{m}$  (18 – 26.6  $\mu\text{m}$ ) in diameter.

**Colony morphology.** Non defined colony pattern on V8A and stoloniferous on PDA (Fig).

**Temperature –growth relationship.** Optimum temperatures for growth on V8A was between 20°C and 25°C (3.4 mm/d), with the upper temperature limit of 35°C for some isolates, slow growth at 35°C and continue to grow after placing at room temperature.

**Notes:** Phylogenetically *P. pteridophyta* was placed in clade 9. The closest species was *P. cuyabensis* and *P. lagoriana* which are described in this study.



**Fig.10** Morphological features and growth pattern of *Phytophthora pteridophyta*. A Non-papillate ovoid sporangium; B. Internal, nested proliferation; C. Extensive, coralloid and swollen hyphae; D. non defined colony pattern on clarified V8 agar; E. Stoloniferous colony pattern on potato dextrose agar. Scale bar 20  $\mu\text{m}$ .

***Phytophthora costaricensis*** Y. Balci & S. Wallace, sp. nov.

Mycobank no.: MBxxxxxx

Etymology. “Costa Rica” refers to the country of Costa Rica where this species was discovered in several of the study sites throughout the country

**Type:** Costa Rica Tapantí National Park 9°44'58.6”N; 83°46'54.4” W. Isolate code CR1- LF-4B (CR 3); ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx); collection date: July 2012.

**Ecology:** Most isolates were recovered from Pre-Montane (type isolate) and the majority of isolates, two isolates were recovered at the Basal (very humid) forest type.

Type species was isolated from newly fallen leaves from tree canopy (LF). Other isolates from the same location (CR1-LF= 6 and CR1-LS= 3 isolates). Additional isolates were recovered at other two National Parks, these sites include; Guayabo National Monument (CR3-LF= 5 isolates), (CR3-LS= 1 isolate), (CR3-LP= 1 isolate) Las Palmas, Braulio Carrillo National Park (CR4-LS= 1 isolate), CR4-LF= 1 isolate.

**Gametangia:** *Phytophthora costaricensis* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** Sporangia formed around 24 hours after flooding with soil extract. They were semi papillate and with a variety of shapes including ovoid, ellipsoid, lemoniform, obpyriform and obturbinate and sympodial sporangiophore. Sporangial sizes (length x width) for the ovoid lemoniform (most common) shape was  $49.2 \pm 6.2$

$\mu\text{m}$  (37.8 – 58.1  $\mu\text{m}$ ) length x  $32.9 \pm 4. \mu\text{m}$  (23.5– 40.3  $\mu\text{m}$ ) width and a length/width ratio of  $1.5 \pm 0.2$ .

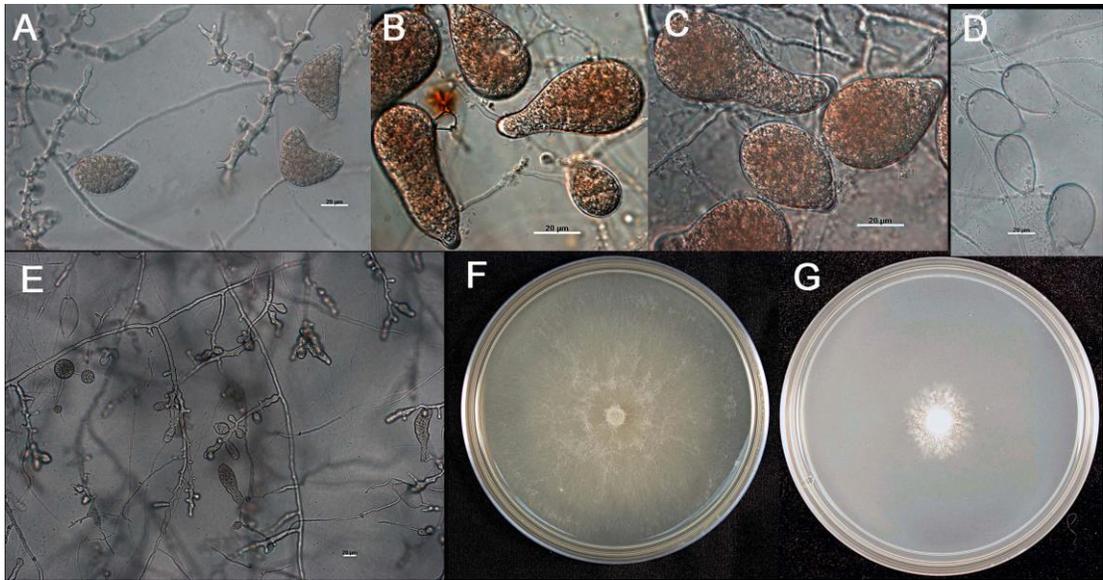
**Hyphal swelling.** Abundant in both liquid and solid media.

**Chlamydospores.** Thin walled chlamydospores were produced occasionally in liquid culture, averaging 28.6  $\mu\text{m}$  (22.6 – 39.1  $\mu\text{m}$ ) in diameter.

**Colony morphology.** Slightly stellate radiate colony pattern on v8A and petalloid on PDA (Fig 11).

**Temperature–growth relationship.** Optimum temperature for growth on V8A was at 25°C (7.7 mm/d), with an upper temperature of 35 °C for all isolates. Isolates continue to grow when placed at room temperature after exposure to 35 °C (Table 3).

**Notes:** Phylogenetically *P. costaricensis* is grouped within clade 2 (Fig. 1). It is most closely related to *P. capsici* and *P. tropicalis*. While its morphological features largely resembles both species it can be separated from *P. capsici* by its hyphal swellings in liquid media, the rounded base of sporangia and being not caducous. *P. costaricensis* also does not grow at 35°C while *P. capsici* grows readily at this temperature.



**Fig 11** Morphological features and growth pattern of *Phytophthora costaricensis* A-D Ovoid and distorted shapes and bipapillate sporangia; E, Hyphal morphology in soil extract water, F, Slightly stellate, radiate colony pattern on clarified V8 agar; and G Petalloid colony pattern on potato dextrose agar. Scale bar 20  $\mu\text{m}$ .

*Phytophthora cuyabensis* Y. Balci, M. Coffeey & S. Wallace, sp. nov. –

Mycobank no.: MBxxxxxx

**Etymology.** “cuyabensis” refers to the Cuyaba forest reserve of Ecuador where the species was first discovered.

**Type:** Costa Rica, Costa Rica, Las Palmas, Braulio Carrillo National Park

10°09'42.1” N; 83°56'16.3” W, Isolate code CR 105 (CR4-LS-3C). Ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx); Collection date: January, 2013

**Ecology.** There is only one isolate of this species and was recovered from lowland, very humid tropical forest. Type species was isolated from leaves submerged in fresh water streams (LS)

**Gametangia.** *Phytophthora cuyabensis* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** Sporangia were formed around 72 to 96 hours and re-flooding with soil extract at least three times. Sporangia were non-papillate and ovoid in shape with nested internal proliferation (Fig. 12). Sporangial sizes (length x width) for the most common shape (ovoid) was  $42.9 \pm 5.9 \mu\text{m}$  (34.3 – 55.5  $\mu\text{m}$ ) length x  $27.4 \pm 3.5 \mu\text{m}$  (20.6– 35.1 $\mu\text{m}$ ) width, and a length: width ratio of  $1.6 \pm 0.2$ .

**Hyphal swelling.** Abundant in liquid culture, none observed in solid media.

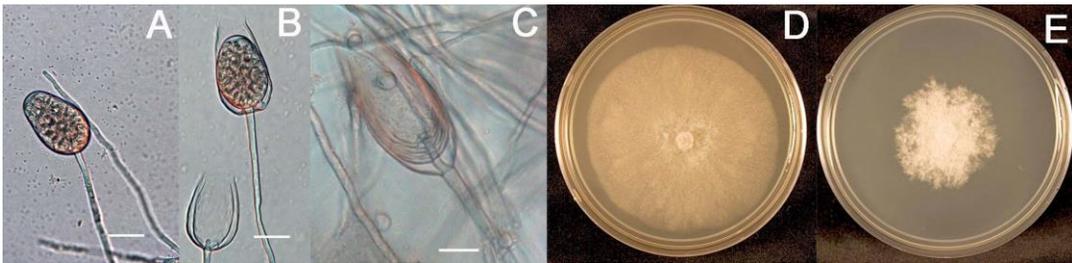
**Chlamydospores.** No chlamydospores were observed.

**Colony growth pattern.** Stellate pattern on V8A and stoloniferous on PDA.

**Temperature – growth relationship.** Optimum temperature for growth on V8A was 25 °C (4.7 mm/d), with the upper temperature limit of 35°C (Table 3). At 35°C

growth decrease almost to a stop but continue to grow after placing at room temperature.

**Notes:** Phylogenetically it was placed in clade 9 (Figure 1). The closest species were *P. lagoriana* and *P. pteridophyta*, which are also described in this study. For isolate comparison see *P. pteridophyta*.



**Fig. 12** Morphological features and growth pattern of *Phytophthora cuyabensis*. A Ovoid non papillate sporangium; B. Internally nested proliferation; C Empty nested proliferation of sporangium. D Slightly stellate, radiate colony pattern on clarified V8 agar; E Stoloniferous colony pattern on potato dextrose agar. Scale bar 20  $\mu\text{m}$ .

***Phytophthora rarus*** Y. Balci & S. Wallace, sp. nov.

Mycobank no.: MBxxxxxx

**Etymology.** “rarus” refers to rare in Latin. Only three isolates were discovered in Costa Rica.

**Type.** Costa Rica, Carara National Park, 9°47'08.2"N 84°36'56.8"W. Isolate code CR192 (CR5-LF-21A). Ex-holotype at the American Type Culture collection (xxxx) and the World *Phytophthora* Genetic Resources Collection at UC-Riverside (Pxxxx). In total 3 isolates were recovered. Isolation date July 2012.

**Ecology.** Isolates were collected at lowland, humid/dry tropical forests (type isolate) and lowland very humid tropical forests. Type species was isolated from newly fallen leaves from the canopy (LF) (CR-LF = 1 isolate) but additional isolates were recovered from leaves submerged in fresh water streams, (CR5-LS=1 isolate, and CR4-LS 19B=1 isolate).

**Gametangia:** *P. rarus* is heterothallic, gametangia was not produced in single culture or when paired with A1 and A2 tester strains of *P. cinnamomi*.

**Sporangia.** Slow producing sporangia... It takes 5 and up to 7 days after flooding with soil extract and several water changes before sporangia is produced. Sporangia mainly produces after chlamydospores germinate in liquid media. They were non-papillate and ovoid with an internal proliferation (Fig 13). Sporangial sizes (length x width) were ovoid (most common)  $46.8 \pm 5.4 \mu\text{m}$  (36.8 – 58.8  $\mu\text{m}$ ) length x  $33.7 \pm 5.7 \mu\text{m}$  (23.0 – 44.4  $\mu\text{m}$ ) width, and a l/w ratio of  $1.4 \pm 1$ . Type species with an average of  $46.8 \pm 5.4 \mu\text{m}$  (36.83 – 58.8) length x  $33.7 \pm 5.7 \mu\text{m}$  (23 – 44.3  $\mu\text{m}$ ) width, and a l/w ratio of  $1.4 \pm 1$ .

**Hyphal swelling.** Abundant in liquid culture, none observed on solid media

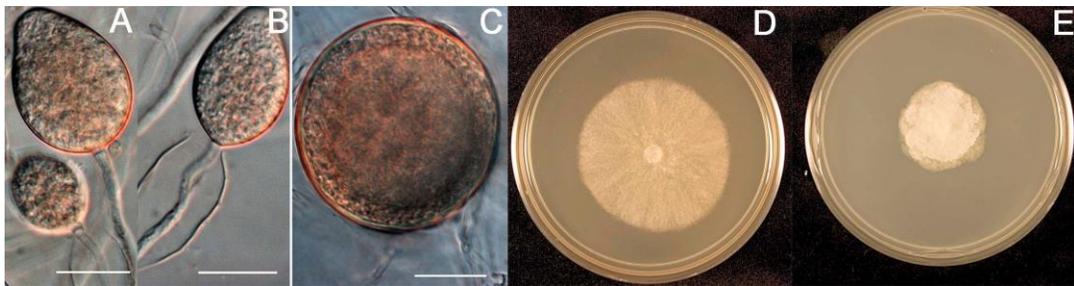
**Chlamydospores.** Large thin walled chlamydospores were produced abundantly in liquid and solid culture, averaging 58.3  $\mu\text{m}$  (range 54.12 – 64.4  $\mu\text{m}$ ) in diameter.

**Colony morphology.** Radiate pattern on V8A and slightly petalloid on PDA.

**Temperature – growth relationship.** Optimum temperature for growth on V8A was 30°C (4.2 mm/d), with the upper temperature limit above 35°C, but no growth was recorded at 37.5°C (Table 3).

**Notes:** Phylogenetically it was placed in clade 9 (Figure 1) with *P.*

*macrochlamydospora* being the most closely related species. It is very similar morphologically to *P. macrochlamydospora*, the most distinct character is the size of the chlamydospores (av. 58.3 $\mu\text{m}$  range 54 -64) for *P. rarus*, (55 $\mu\text{m}$ , range 24 – 84) for *P. macrochlamydospora*. *P. rarus* maximum temperature is above 35°C while the maximum temperature reported for *P. macrochlamydospora* was 34°C (Erwin and Ribeiro 1996).



**Fig. 13** Morphological features and growth pattern of *Phytophthora rarus*. A-B.

Ovoid non papillate sporangia and internal proliferation formed when cV8 plugs were flooded with soil extract water; C. Thin walled chlamydospores produced in cV8 agar; D. Slightly stellate, radiate colony pattern on clarified V8 agar; E. Petalloid colony pattern on potato dextrose agar. Scale bar 20  $\mu\text{m}$ .

## Discussion

This study represents the first systematic survey of non- agricultural environments in Costa Rica to determine the diversity and distribution of *Phytophthora* species. Species described in this study were all part of tropical ecosystems; however, a large variation exists among their morphological features. Similarly, they were placed in 6 different clades out of 10 clades that are identified in the genus *Phytophthora*.

While molecular clades do not support morphological delineation within a clade (Martin et al., 2014), some trends still exist. For example, all species placed in clade 9 were remarkably similar in morphology; all produce non-papillate sporangia, mainly heterothallic or sterile and produce hyphal swellings primarily. Similarly, when a species was placed in a particular clade the closest species had similar morphology, particular in sporangia type.

The most frequently occurring *Phytophthora* species was *P. taxon lagoariana*, followed by *P. taxon vulcanica*. The most widespread species was *P. capsici*, *P. taxon myristicae* and *P. taxon lagoariana*, which were found in half the sampling sites.

Despite the use of selective media, large numbers of isolates were recovered belonging to the genus *Pythium* and other Oomycetes. Clearly a close habitat preference and interactions exist among these organisms; the possibility of one genus

serving as a biological restraint to *Phytophthora* spp. is something that requires further investigation.

In Costa Rica there are eight known species of *Phytophthora*: *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. nicotianae*, *P. palmivora*, *P. Phaseoli*, and *P. porri*. All of these species were associated with disease problems in agricultural crops and trees especially in urban settings (Villalobos Calderon et al., 2009). Our study found eight species that are known to science (*P. capsici*, *P. cinnamomi*, *P. cryptogea*, *P. heveae*, *P. macrochlamydospora*, *P. palmivora*, *P. tropicalis* and *P. taxon PgChlamydo*). Of this assemblage only tree species, *P. capsici*, *P. palmivora* and *P. cinnamomi*, appear to cause damage in agricultural areas and other plant systems. However, it still needs to be investigated if this is true and whether misidentification is the reason why species like *P. heveae*, *P. macrochlamydospora*, *P. cryptogea*, and *PgChlamydo* were never reported in Costa Rica.

On the other hand, someone can ask the question if the discrepancy between the forest and agricultural is an indication that there is not much movement of species affecting agricultural crops into the forest or visa versa? or that in the forest setting these species are overshadowed by other more aggressive organism? These questions are worth of further research that can shed light into the biology and interaction of these organisms as well as emergence of infectious species of *Phytophthora* in a particular ecosystem.

Among the newly described species, *P. costaricensis*, *P. tapantiensis* and *P. aerium* are placed in clade 2b and clade 2c; same as *P. capsici* and *P. tropicalis*. One

species, *P. cararaensis* is placed in clade 4, and *P. taxon myristicae* in clade 5, these two clades are home of the very aggressive tropical plant pathogens *P. palmivora* and *P. heveae*. *P. vulcanica*, a species placed in clade 6 is one of the most widespread of all the newly described species, it is common in stream but was also recover from live plants and from leaves under the trees canopy. Species of this clade are considered non-aggressive pathogens but their biology and definite role in the environments where they are found are still unclear. The finding of a new *Phytophthora* species placed in clade 8 in four of the ten sites and on all but the soil source is especially interesting because its sequence aligns close to other important tree pathogens including *P. foliorum*, *P. lateralis* and, *P. ramorum* the causal agent of Sudden Oak Death. Nevertheless, these findings suggest that several of the important pathogens that currently threaten agricultural areas (such as *P. capsici*) and forest settings (such as *P. ramorum*) in temperate regions must have originated from the tropics.

This study was conducted as a preliminary survey that hopefully will lead others to look more closely into tropical areas that we believe harbors major *Phytophthora* taxa. Areas with diverse climatic differences should be the prime locations for species diversity and should be sampled more intensively.

### **Limitations and future directions**

This was a first attempt to survey protected areas in Costa Rica, with the ultimate goal to characterize and describe *Phytophthora* species. It is clear that different species have a preference for certain host material. The sampling methodology should be refined and more time allotted so that soil sampling and

stream baiting can be done in a more methodic way. Sampling size is another element that needs to be addressed, finding the adequate sampling size can be influential in our understanding of the complete diversity of species at a particular site. In addition, the composition of the selective media is another element that requires more attention, the possibility of missing out fast growing species or of others that are susceptible to hymexazol is always a possibility. One particular problem is bacterial contamination that requires readjusting the antimicrobial compounds added into the selective media.

## **Acknowledgements**

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Optimization of digital images was done by Selin Balci. Funding was obtained through the Department of Plant Science and Landscape Architecture, University of Maryland.

**Table 1 Summary of morphological features of tropical *Phytophthora* species described in this study.**

Character	<i>P. costarricensis</i>	<i>P. tapantiensis</i>	<i>P. aerium</i>	<i>P. cararaensis</i>	<i>P. myristicae</i>	<i>P. vulcanica</i>	<i>P. frondescens</i>
Clade	2b	2c	2c	4	5	6	8c
<b>Sporangia</b>							
Papilla	SP	SP	SP	P	P	NP	SP
Average length	49.2 ± 6.2	50.0 ± 5.9	51.6 ± 4.6	39.1 ± 4.7	46.7 ± 5.4	41.9 ± 4.7 <sup>1</sup>	42.2 ± 5.6
Range length	37.8 - 58.1	40.9 - 58.5	44.2 - 58	32 - 49.1	37.6 - 54.5	35.1 - 50.8 <sup>2</sup>	31.6 - 52
Average breadth	32.9 ± 4.2	32.3 ± 3.6	31.8 ± 3.2	27.1 ± 4	30.6 ± 3.8	28.2 ± 3.4	27.1 ± 2.8
Range breadth	23.5 - 40.3	24.5 - 38.3	24.1 - 36.2	17.8 - 35.6	27.4 - 45.3	21.9 - 35.3	21.8 - 33.0
Length/ breadth ratio	1.5 ± 0.2 : 1	1.5 ± 0.1 : 1	1.6 ± 0.1 : 1	1.4 ± 0.2 : 1	1.3 ± 0.1 : 1	1.5 ± 0.1 : 1	1.5 ± 0.1 : 1
Isolate averages							
Isolate length	53.5 ± 8	50.8 ± 6	44.3 ± 4.6	45.4 ± 5.9	50.3 ± 5.1	48.2 ± 4.5	40.0 ± 4
Isolate length range	36.2 - 64.8	38.7 - 59.9	38.3 - 53.7	33.9 - 59.3	42.6 - 54.7	40.3 - 56.9	31.8 - 48.9
Isolate breadth average	33.9 ± 5.5	37.2 ± 3.8	28.9 ± 3.2	30.3 ± 4.9	37.4 ± 3.4	29.4 ± 3	24.5 ± 1.7
Isolate breadth range	20.4 - 44.4	24 - 41	21.5 - 33	21 - 43.2	30.8 - 42.4	22.5 - 35	20.9 - 28.5
Isolate length/breadth ratio	1.6 ± 0.2 : 1	1.4 ± 0.1 : 1	1.5 ± 0.1 : 1	1.3 ± 0.2 : 1	1.0 ± 0.1 : 1	1.6 ± 0.2 : 1	1.6 ± 0.1 : 1
Shape(s)	Ov	Ellip - Ov	Ov	Ov - Obp	Gl	Ov	Ov
Distorted shapes	Y	N	N	Y	N	N	N
Base	R	R-T	T	R	R	R	R
Caducity							
Sporangiophore	Symp	Symp	Symp	Int	Symp	Int	Symp
Sexual system	Het	Hom	Het	Hom	Hom	Het	Het/Hom
Chlamydospores (y/n)	Y	N	Y	Y	Y	N	N
<b>Oogonia</b>							
Average diameter	n/a	26.7 ± 2.8	n/a	28.5 ± 2.9	30.3 ± 2.4	n/a	31.2 ± 2.2

**Table 1 continued.**

	<i>P. costarricensis</i>	<i>P. tapantiensis</i>	<i>P. aerium</i>	<i>P. cararaensis</i>	<i>P. myristicae</i>	<i>P. vulcanica</i>	<i>P. frondescens</i>
Character							
Range	n/a	21.8 -31.2	n/a	24 - 33.7	23.6 - 33.5	n/a	27.7 - 35.1
Isolate average	n/a	29.3 ± 3	n/a	25.2 ± 2.6	30.7 ± 2.2	n/a	31.2 ± 2.2
Isolate range	n/a	22.6 -34.1	n/a	20.4 - 30.9	32.2 - 34.0	n/a	27.7 - 35.1
Base (tapered= t/rounded=r)	n/a	r	n/a	R	r/t	n/a	T
<b>Oospores</b>							
Average diameter	n/a	27.7 ± 2.4	n/a	24.2 ± 3.2	25.2 ± 2.4	n/a	25.9 ± 2.2
Range	n/a	23.2 -32.6	n/a	18 - 29.9	19.1 -29.3	n/a	23.2 - 29.3
Isolate average	n/a	25.2 ± 2	n/a	21.4 ± 2.5	25.2 ± 2	n/a	25.9 ± 2.2
Isolate range	n/a	20.3 -30.9	n/a	16.9 - 26.4	19 - 30.2	n/a	23.2 - 29.3
Plerotic= P; Aplerotic = Apl	n/a	Apl	n/a	Apl	Apl	n/a	Apl
<b>Oospore wall</b>							
Average diameter	n/a	2.3 ± 0.4	n/a	2.3 ± 0.5	2.5 ± 0.5	n/a	2.3 ± 0.3
Range	n/a	1.8 - 2.7	n/a	1.5 - 3.3	1.9 - 3.4	n/a	2.1 -2.6
Isolate average		2.3 ± 0.3		2 ± 0.5	2.9 ± 0.5	n/a	2.3 ± 0.3
Isolate range	n/a	1.6 - 2.9	n/a	1.4 -3.0	2.2 -3.8	n/a	2.1 -2.6
<b>Antheridia</b>							
Average diameter	n/a	9.3 ± 1.1	n/a	7.5 ± 1.4	9 ± 1	n/a	13.8 ± 0.7
Range	n/a	7.7 - 11.4	n/a	4.9 - 9.7	7.2 - 10.3	n/a	12.4 - 14.6
Isolate average	n/a	8.1± 1.2	n/a	7.3 ± 1.3	8.3 ± 1	n/a	13.8 ± 0.7
Isolate range	n/a	6.5 -10.1	n/a	5.1 -10	6.9 - 9.7	n/a	12.4 - 14.6
<b>Hyphal swelling</b>							
In water Yes = Y / No = N	Y	Y	Y	Y	Y	N	Y
On agar	Y	N	N	N	Y	N	N
<b>Colony morphology</b>							
On V8A		Cot - Pet	Ros	Cot	Ste	Ste	Stel - Pet
On PDA	Ste	Pet - Ros	Ros	Sto	Ros	Sto	Cot - Ros

**Table 1 continued.**

Character	<i>P. montana</i>	<i>P. aquatropicalis</i>	<i>P. lagoriana</i>	<i>P. pteridophyta</i>	<i>P. cuyabensis</i>	<i>P. rarus</i>
Clade	8c	9	9	9	9	9
<b>Sporangia</b>						
Papilla	SP	NP	NP	NP	NP	NP
Average length	46.1 ± 5.6	45.8 ± 5.1	49.8 ± 4.4	38.1 ± 2.7	42.9 ± 5.9	46.8 ± 5.4 36.8 -
Range length	36.6 - 58.8	34.8 - 53.7	39.2 - 57.0	34.2 - 43	34.3 - 55.5	58.8
Average breadth	31.2 ± 4.8	31.6 ± 5.4	33.5 ± 3.6	25.9 ± 3.4	27.4 ± 3.5	33.7 ± 5.7 23.0 -
Range breadth	23.9 - 41.3	23.2 - 41.7	24 - 41	22.2 - 31.0	20.6 - 35.1	44.4
Length/ breadth ratio	1.5 ± 0.1 :	1.4 ± 0.3 : 1	1.5 ± 0.2 :	1.4 ± 0.2 : 1	1.6 ± 0.2 : 1	1.4 ± 0.1 :
Isolate averages						
Isolate length	44.7 ± 6.1	43.1 ± 4.8	53.9 ± 6	42.3 ± 3.7	42.9 ± 5.9	46.8 ± 5.4 36.8 -
Isolate length range	35 - 58.1	33.7 - 53.0	40.4 - 64.9	36.3 - 48.2	34.3 - 55.5	58.8
Isolate breadth average	27.4 ± 6	32.2 ± 5.8	35.7 ± 4.7	24.8 ± 3	27.4 ± 3.5	33.7 ± 5.7 23.0 -
Isolate breadth range	20.1 - 41.2	24.6 - 43.8	22.5 - 46.8	20.4 - 31.1	20.6 - 35.1	44.4
Isolate length/breadth ratio	1.6 ± 0.1 :	1.3 ± 0.2 : 1	1.5 ± 0.2 :	1.7 ± 0.1 : 1	1.6 ± 0.2 : 1	1.4 ± 0.1 :
Shape(s)	Ellip	Ov	Ellip	Obt	Ellip - Ov	Lim
Distorted shapes	N	N	Y	N	Y	N
Base	T	R	T	R	R	R
Caducity						
Sporangiophore	Symp	Int	Int	Int	Symp	Ext
Sexual system	Het	Het	Het	Het	Het	Het
Chlamydospores (y/n)	Y	Y	Y	Y	N	Y
<b>Oogonia</b>						
Average diameter	n/a	n/a	n/a	n/a	n/a	n/a

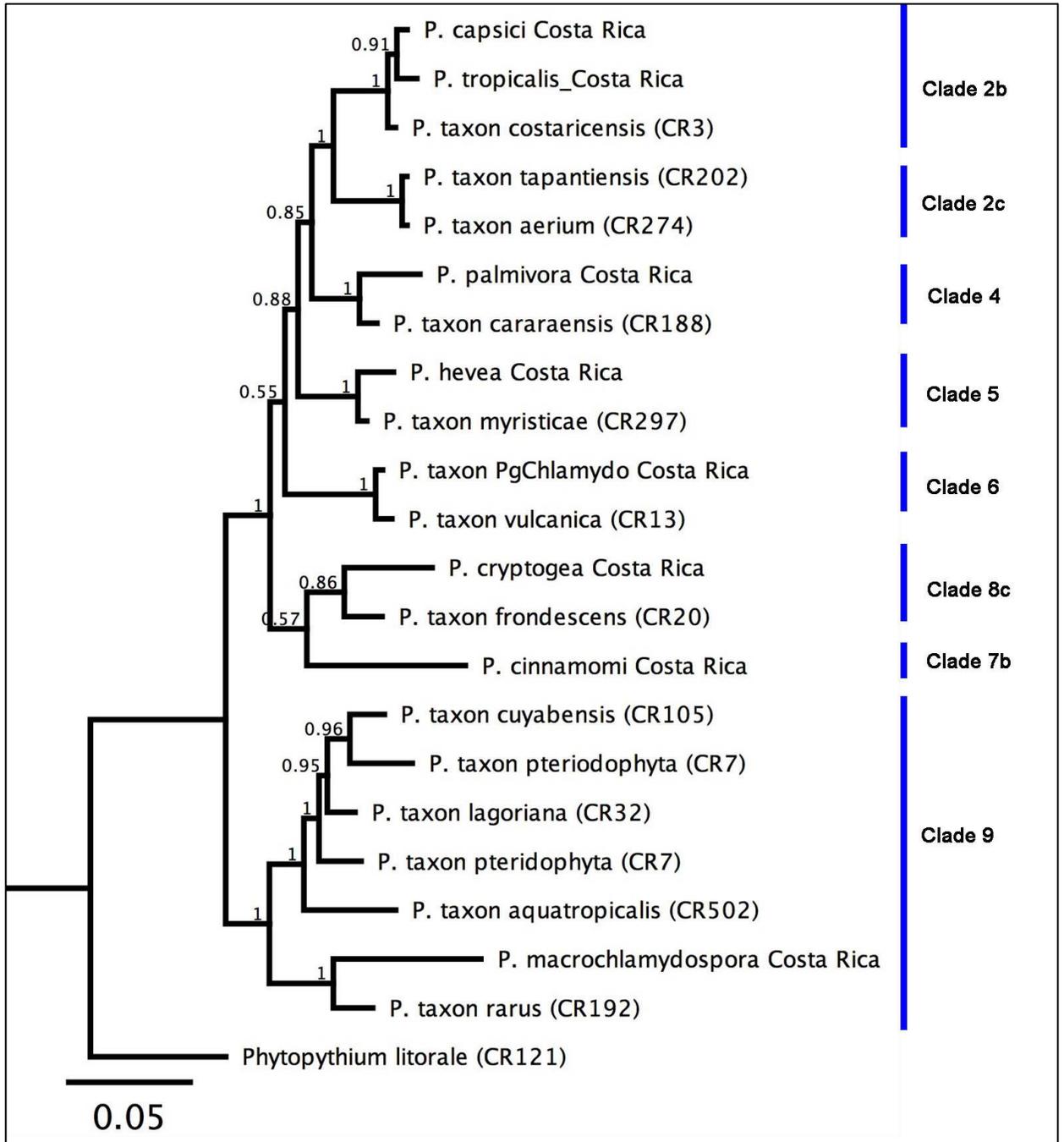
**Table 1 continued.**

	<i>P.</i> <i>montana</i>	<i>P.</i> <i>aquatropicalis</i>	<i>P.</i> <i>lagoriana</i>	<i>P.</i> <i>pteridophyta</i>	<i>P.</i> <i>cuyabensis</i>	<i>P.</i> <i>rarus</i>
Character						
Range	n/a	n/a	n/a	n/a	n/a	n/a
Isolate average	n/a	n/a	n/a	n/a	n/a	n/a
Isolate range	n/a	n/a	n/a	n/a	n/a	n/a
Base (tapered= t/rounded= r)	n/a	n/a	n/a	n/a	n/a	n/a
<b>Oospores</b>						
Average diameter	n/a	n/a	n/a	n/a	n/a	n/a
Range	n/a	n/a	n/a	n/a	n/a	n/a
Isolate average	n/a	n/a	n/a	n/a	n/a	n/a
Isolate range	n/a	n/a	n/a	n/a	n/a	n/a
Plerotic= pl; Aplerotic = apl	n/a	n/a	n/a	n/a	n/a	n/a
<b>Oospore wall</b>						
Average diameter	n/a	n/a	n/a	n/a	n/a	n/a
Range	n/a	n/a	n/a	n/a	n/a	n/a
Isolate average		n/a				
Isolate range	n/a	n/a	n/a	n/a	n/a	n/a
<b>Antheridia</b>						
Average diameter	n/a	n/a	n/a	n/a	n/a	n/a
Range	n/a	n/a	n/a	n/a	n/a	n/a
Isolate average	n/a	n/a	n/a	n/a	n/a	n/a
Isolate range	n/a	n/a	n/a	n/a	n/a	n/a
<b>Hyphal swelling</b>						
In water Yes = Y / No = N	Y	Y	Y	Y	Y	Y
On agar	N	N	N	N	Y	N
<b>Colony morphology</b>						
On V8A	Ros	Ste	Ros	Sto	Ste	Rad
On PDA	Ros	ND	Ros	Pet	Sto	Pet

**Table 2** Daily growth rate (mm/d) of *Phytophthora* spp. on clarified V8A at different temperatures. Growth rate at optimum temperature shown in bold.

<i>Phytophthora</i> spp.	Temperature ranges (°C)					
	10	15	20	25	30	35
<i>P. vulcanica</i>	1.3	1.7	<b>4</b>	<b>4.5</b>	4.4	0
isolate range (3)	-	-	<b>1.1-4.0</b>	<b>1.3-4.5</b>	-	-
<i>P. aquatropicalis</i>	0.7	2	<b>4</b>	<b>5.2</b>	<b>5.1</b>	1.9
isolate range (2)	-	-	<b>1.6-4</b>	<b>3-5.2</b>	-	-
<i>P. cararaensis</i>	0	0.9	<b>2.9</b>	<b>3.4</b>	1.4	0
isolate range (2)	-	-	<b>2 - 2.9</b>	<b>2.5 - 3.4</b>	-	-
<i>P. lagoriana</i>	0	1.1	<b>2.4</b>	<b>4.3</b>	<b>5</b>	1.6
isolate range (3)	-	-	<b>1.4 - 2.4</b>	<b>2.3 - 4.3</b>	-	-
<i>P. tapantiensis</i>	1.4	3.6	<b>6.0</b>	<b>7.7</b>	4.2	0
isolate range (3)	-	-	<b>3.9 - 6.0</b>	<b>4.5 - 7.7</b>	-	-
<i>P. frondescens</i>	2.1	4.3	<b>5.2</b>	<b>2.1</b>	0	0
isolate range (3)	-	-	<b>1.3 - 5.2</b>	<b>1.4 - 4.8</b>	-	-
<i>P. montana</i>	1.4	3.9	<b>5.6</b>	<b>6.6</b>	2.9	0.4
isolate range (3)	-	-	<b>3.4 - 5.6</b>	<b>6.4 - 6.6</b>	-	-
<i>P. aerium</i>	1.1	2.1	<b>3.1</b>	<b>3.8</b>	2.6	0
isolate range (2)	-	-	<b>2.5 - 3.1</b>	<b>3.8 - 4.5</b>	-	-
<i>P. myristicae</i>	0.8	4.3	<b>7.8</b>	<b>10.1</b>	1.3	0
isolate range (3)	-	-	<b>3.0 - 7.8</b>	<b>7.8 - 10.1</b>	-	-
<i>P. pteridophyta</i>	0.7	1.8	<b>3.4</b>	<b>6.4</b>	1.6	0.6
isolate range (2)	-	-	<b>1.9 - 3.4</b>	<b>1.7 - 6.4</b>	-	-
<i>P. costarriensis</i>	1.7	4	<b>5.9</b>	<b>7.7</b>	6.9	0
isolate range (3)			<b>2.9 - 5.9</b>	<b>4.1 - 7.7</b>	-	-
<i>P. cuyabensis</i>	1	1.3	<b>2.7</b>	<b>4.7</b>	2.9	0.8
isolate range(1)	-	-	-	-	-	-
<i>P. rarus</i>	0	0.9	<b>1.6</b>	<b>3.2</b>	4.2	3.2
isolate range (3)	-	-	<b>1.6 - 3.3</b>	<b>3.2 - 3.3</b>	-	-

**Figure 1** Bayesian inference tree based on four nuclear and four mitochondrial sequences showing phylogenetic relationship of *Phytophthora* species found in this survey. *Phytophthora litorale* from Maryland streams were used as outgroup. Number above the branches represent a bootstrap support >70% based on parsimony analysis. Molecular clades as shown in Martin et al., 2014 are shown.



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