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A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences

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Abstract

Phytophthora species are devastating plant pathogens in both agricultural and natural environments. Due to their significant economic and environmental impact, there has been increasing interest in *Phytophthora* genetics and genomics, culminating in the recent release of three complete genome sequences (*P. ramorum*, *P. sojae*, and *P. infestans*). In this study, genome and other large sequence databases were used to identify over 225 potential genetic markers for phylogenetic analyses. Here, we present a genus-wide phylogeny for 82 *Phytophthora* species using seven of the most informative loci (approximately 8700 nucleotide sites). Our results support the division of the genus into 10 well-supported clades. The relationships among these clades were rigorously evaluated using a number of phylogenetic methods. This is the most comprehensive study of *Phytophthora* relationships to date, and many newly discovered species have been included. A more resolved phylogeny of *Phytophthora* species will allow for better interpretations of the overall evolutionary history of the genus.

Keywords: *Phytophthora ramorum*; *Phytophthora sojae*; *Phytophthora infestans*; Oomycetes; Genomes; Phylogeny; Molecular markers

1. Introduction

The genus *Phytophthora* contains a large diversity of devastating plant pathogens which occur in both natural and agricultural settings (Erwin and Ribeiro, 1996; Judelson and Blanco, 2005). Many species of *Phytophthora* are able to infect a broad range of hosts. For example, over 2000 plant species are thought to be susceptible to infection by *P. cinnamomi* in Australia, where this pathogen has severely altered native plant communities since its accidental introduction in the 1920s (Hardham, 2005). Other species show narrow host ranges; *P. sojae* has caused upwards of $2 billion (US) in agricultural losses on its primary host, soybean (Tyler, 2007). Emerging species (e.g., *P. ramorum*, Rizzo and Garbelotto, 2003) are inflicting immeasurable damage on forest ecosystems over extremely short timescales. In addition, the rapidly expanding global commodity trade will likely accelerate the introduction and establishment of invasive species. Due to their significant environmental and economic importance, there has been increasing interest in the molecular genetics and genomics of *Phytophthora* species (Govers and Gijzen, 2006; Kamoun, 2003), as well as in archiving phenotypic and genotypic data from across the genus (e.g., http://www.PhytophthoraDB.org, S.K. unpublished data).

While *Phytophthora* and related genera exhibit morphological features analogous to pathogens in Kingdom Fungi, they in fact reside in Kingdom Stramenopila with diatoms

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and brown algae (Dick, 2001; Förster et al., 1990; Gunderson et al., 1987; Sogin and Silberman, 1998). They are currently classified in the Peronosporomycetes within the Oomycota, although phylogenetic relationships within this group remain in question (Dick, 1990; Dick et al., 1999; Hudspeth et al., 2000; Peterson and Rosendahl, 2000; Riethmüller et al., 2002; Riethmüller et al., 1999). Unlike most members of Kingdom Fungi, oomycetes are predominantly diploid during their life cycles, producing a transient haploid phase prior to fertilization (Dick, 1990). *Phytophthora* species have traditionally been divided into six morphological groups based on features of the sporangium, antheridium, and reproductive behavior, although these characteristics are sometimes ambiguous (Newhook et al., 1978; Stamps et al., 1990; Waterhouse, 1963).

Previous molecular studies have explored the relationships among *Phytophthora* species using one or a few genetic loci, predominantly the ITS region of the nuclear ribosomal DNA (Cooke et al., 2000; Crawford et al., 1996; Förster et al., 2000; Lee and Taylor, 1992) and cytochrome oxidase I and II of the mitochondrion (Martin and Tooley, 2003a). More recent studies have used multiple loci from both the nuclear and mitochondrial genomes (Donahoo et al., 2006; Ivors et al., 2004; Kroon et al., 2004; Martin and Tooley, 2003b; Villa et al., 2006). While these studies have been successful in establishing a number of well-supported clades within the genus, they have been unable to resolve the deeper evolutionary relationships among the clades. In addition, some newly described *Phytophthora* species have been placed in an unresolved, basal group that appears to be outside the main radiation of the genus (Belbahri et al., 2006; Brasier et al., 2005; Dick et al., 2006). Establishing a well-resolved phylogeny of the genus *Phytophthora* is important not only for validating diagnostic methods of species identification (e.g., Kong et al., 2004; Lees et al., 2006; Martin et al., 2004; Schena et al., 2006; Tooley et al., 2006), but also for interpreting the evolutionary history of various genetic traits of interest, such as pathogenicity factors (Jiang et al., 2006; Liu et al., 2005; Shan et al., 2004; Whisson et al., 2004), transposable elements (Ah Fong and Judelson, 2004; Judelson, 2002), and mating types (Cvitanich et al., 2006; Qi et al., 2005).

The goal of this study was to utilize the available genome sequence data for *P. ramorum* and *P. sojae* (Tyler et al., 2006), along with the large numbers of expressed sequence tags (ESTs) from *P. infestans* (Randall et al., 2005; Win et al., 2006), *P. nicotianae* (Shan and Hardham, 2004), and others, to identify phylogenetically informative molecular markers. Loci were sought that would be informative across the genus and/or within clades or species complexes. Approximately 40 million bases (Mb) of genome sequence were analyzed, from which over 225 potential markers were identified. A subset of these markers was evaluated through PCR and sequence analyses. Here, we present a genus-wide phylogeny for 82 *Phytophthora* species using seven of the most informative loci, totaling approximately 8.7 kilobases (kb) of sequence data. Our results suggested the presence of 10 well-supported clades within the genus. We also addressed the relationships among the clades using an array of phylogenetic methods. The phylogenetic relationships recovered here are contrasted with those suggested by previous studies.

2. Materials and methods

2.1. Isolate selection and culturing

A total of 234 isolates from 82 species of *Phytophthora* and 2 species of *Pythium* were analyzed in this study. Ten new species currently being described were included, and their provisional names are used here. Most isolates (218) are maintained at the World *Phytophthora* Genetic Resources Collection (WPGRC, http://phytophthora.ucr.edu) at the University of California, Riverside, where accessions are preserved cryogenically under liquid nitrogen. Working cultures were maintained on either clarified or non-clarified V8 agar (Ribeiro, 1978) or ryeseed B agar (*P. infestans* and *P.* sp. "*andina*", Caten and Jinks, 1968). At all stages of growth, cultures were checked for bacterial contamination by incubation for 24 h in Luria broth. For DNA extraction, actively growing cultures were produced in either clarified (1:2) V8 broth or pea broth (for *P. infestans* and *P.* sp. "*andina*"), and harvested after 4–10 days. Approximately 200 mg of mycelium was rinsed with ultrapure water, placed in a 1.7 ml microcentrifuge tube and frozen by immersion in liquid nitrogen. DNA was extracted from frozen tissue using the FastDNA kit and FastPrep FP 120 instrument (MP Biomedicals Inc., Irvine, CA) according to the manufacturer's instructions, with modifications using 1 ml of CLS-VF cell lysis solution and omitting the PPS protein precipitation solution. DNA concentration was determined using a 260/280 ratio with a Beckman DU 64 UV spectrophotometer (Beckman Coulter Inc., Fullerton, CA). All DNA samples were stored in ultrapure water at −86 °C, and are available through the WPGRC DNA Bank upon request (to M.D.C.).

An additional 10 isolates were obtained from the Pennsylvania Department of Agriculture and six isolates from West Virginia University. Working cultures were maintained on either clarified or non-clarified V8 agar (Ribeiro, 1978) at 15–20 °C. For DNA extraction, a small piece of colonized agar was transferred to 50 ml sterile 10% V8 broth and incubated at room temperature for 3–7 days at 100 rpm. Mycelium was then collected, rinsed, and a small portion (10–20 mg) was lyophilized. Each sample was ground using a sterile pestle in 500 µl of extraction buffer (100 mM Tris–HCl, 10 mM EDTA, 1 M KCl, pH 8), and incubated at 75 °C for 15 min. Some samples were additionally treated with 300 µl phenol/chloroform (1:1). The aqueous phase was collected after centrifugation for 5 min (16,000*g*), and DNA was precipitated using 300 µl isopropanol. Samples were centrifuged for 10 min (16,000*g*) to pellet the DNA, which was then washed with 70% ethanol, dried, and resuspended in 100 µl TE buffer