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Research paper

Comparative mitochondrial genome analysis of *Pythium insidiosum* and related oomycete species provides new insights into genetic variation and phylogenetic relationships

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ABSTRACT

Oomycetes are eukaryotic microorganisms, which are phylogenetically distinct from the true-fungi, which they resemble morphologically. While many oomycetes are pathogenic to plants, *Pythium insidiosum* is capable of infecting humans and animals. Mitochondrial (mt) genomes are valuable genetic resources for exploring the evolution of eukaryotes. During the course of 454-based nuclear genome sequencing, we identified a complete 54.9 kb mt genome sequence, containing 2 large inverted repeats, from *P. insidiosum*. It contains 65 different genes (including 2 ribosomal RNA genes, 25 transfer RNA genes and 38 genes encoding NADH dehydrogenases, cytochrome b, cytochrome c oxidases, ATP synthases, and ribosomal proteins). Thirty-nine of the 65 genes have two copies, giving a total of 104 genes. A set of 30 conserved protein-coding genes from the mt genomes of *P. insidiosum*, 11 other oomycetes, and 2 diatoms (outgroup) were used for phylogenetic analyses. The oomycetes can be classified into 2 phylogenetic groups, in relation to their taxonomic lineages: Saprolegnialean and Peronosporalean. *P. insidiosum* is more closely related to *Pythium ultimum* than other oomycetes. In conclusion, the complete mt genome of *P. insidiosum* was successfully sequenced, assembled, and annotated, providing a useful genetic resource for exploring the biology and evolution of *P. insidiosum* and other oomycetes.

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1. Introduction

Oomycetes form a unique group of fungus-like, zoospore-producing, eukaryotic microorganisms (Beakes et al., 2012; Kamoun, 2003; Thines, 2014). The group can largely be divided into two Orders, Peronosporales and Saprolegniales. The Peronosporales include the Genera *Phytophthora* and *Pythium*, many of which are well known as plant pathogens. Those affecting agriculture, such as the devastating potato late blight pathogen, *Phytophthora infestans*, and natural ecosystems, for example the sudden oak death agent, *Phytophthora ramorum* are among the most studied. Members of the Genus *Pythium* often adopt saprotrophic or opportunistic pathogenic lifestyles. While many *Pythium* species are pathogenic to plants, *Pythium insidiosum*, in particular, is capable of infecting humans and animals (Gaastra et al., 2010; Kamoun, 2003; Krajaejun et al., 2006; Mendoza et al., 1996). *P. insidiosum* infection (known as pythiosis) is increasingly reported from tropical and subtropical areas around the world (Gaastra et al., 2010; Krajaejun et al., 2006; Mendoza et al., 1996). The infection leads to high morbidity and mortality in most affected patients (Gaastra et al., 2010; Krajaejun et al., 2006; Mendoza et al., 1996). Because the oomycetes, including *P. insidiosum*, have different genetic and biochemical characteristics to fungi, most antimicrobial drugs, designed to control human pathogenic fungi, are ineffective against these organisms (Beakes et al., 2012; Krajaejun et al., 2006). Surgical removal of infected organs (i.e., eye, leg) is an effective treatment for pythiosis

Abbreviations: A, adenine; ATP, adenosine triphosphate; *atp*, gene encoding ATP synthase; bp, base pair(s); C, cytosine; cDNA, DNA complementary to RNA; COB, mitochondrial cytochrome b; *cob*, gene encoding COB; COX, cytochrome c oxidase; *cox*, gene encoding COX; DDBJ, DNA Data Bank of Japan; DNA, Deoxyribonucleic acid; G, guanine; Gb, Giga base(s) or 1,000,000,000 bp; IR, inverted repeat; kb, kilobase(s) or 1000 bp; mt genome, mitochondrial genome; Mb, mega base pair(s) or 1,000,000 bp; *nad*, gene encoding NAD; NAD, NADH, nicotinamide adenine dinucleotide and its reduced from; nt, nucleotide; rDNA, DNA coding for rRNA; RNA, Ribonucleic acid; RPL, Ribosomal protein large subunit; *rpl*, gene encoding RPL; RPS, Ribosomal protein small subunit; *rps*, gene encoding RPS; rRNA, ribosomal RNA; T, thymine; *trn*, gene encoding transfer RNA; tRNA, transfer RNA; U, uracil; *ymf*, gene encoding conserved hypothetical protein.

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(Gaastra et al., 2010; Krajaejun et al., 2006; Mendoza et al., 1996). However, although intensive care is provided, many patients die from advanced infection. Basic information on biology and virulence of *P. insidiosum* is required to better understand the pathogen, and could lead to better methods for the control of pythiosis.

Genome sequences are now available for many oomycetes, including one animal pathogenic species, *Saprolegnia parasitica* (Jiang et al., 2013), and several plant pathogenic *Pythium* species (Adhikari et al., 2013; Lévesque et al., 2010). Genome sequencing of *P. insidiosum* and comparative genomics with other oomycetes is essential to fully understand the evolution and adaptation of *P. insidiosum* as a successful human pathogen. During the course of nuclear genome sequencing (Rujirawat et al., 2015), we identified a complete mitochondrial (mt) genome sequence from *P. insidiosum*. Mt genomes are valuable genetic resources and have been used to study evolution and gene expression in eukaryotes, such as, animals, plants, protists, and fungi (Lang et al., 1999; Paquin et al., 1997). Recently, the mt genomes of several plant-pathogenic and saprophytic oomycetes (i.e., *Phythophthora*, *Pythium*, *Achlya*, and *Thraustotheca* species) have been sequenced and made publicly available (Martin et al., 2007; Martin and Coffey, 2012; O'Brien et al., 2014). Comparative mt genome analysis of the human-pathogenic, plant-pathogenic, and saprophytic oomycetes could provide clues to the biology- and pathogenicity-related evolutionary history of *P. insidiosum*, the only member of the oomycetes that infects humans and animals.

In this study, we aimed to assemble and annotate the mt genome of *P. insidiosum*, analyze its structure and genetic contents, and study the phylogenetic relationship, between the mt genomes from *P. insidiosum* and other oomycete species, including the closely-related species, *Pythium ultimum*. The mt genome of *P. insidiosum* was successfully identified and characterized, providing a useful genetic resource for exploring the biology and evolution of this important pathogen. We herein present the results.

2. Materials and methods

2.1. Microorganisms and DNA extraction

P. insidiosum strain Pi-S, isolated from a Thai patient with vascular pythiosis, was recruited for this study. Identity of the organism was confirmed by zoospore induction and rDNA-sequence homology analysis (Badenoch et al., 2001; Chaiprasert et al., 1990). The organism was cultured, with shaking (150 rpm), in 100 ml Sabouraud dextrose broth, for 10 days at 37 °C. Hyphae were harvested and DNA was extracted using the conventional-extraction protocol described by Lohnoo et al. (2014). The resulting DNA was dissolved in TE buffer, and stored at 4 °C until use.

2.2. DNA sequencing and assembly

P. insidiosum DNA (10 μg) was used to prepare a 454-shotgun library and sequenced using the GS FLX Titanium platform (Margulies et al., 2005) at the in-house facility (National Center for Genetic Engineering and Biotechnology, Thailand). Raw sequencing reads were assembled using Newbler de novo sequence assembly software (Roche; quality score cutoff > 20, overlapping region > 40). Contigs with high sequencing coverage were considered as potentially derived from organellar genome sequences (Shearman et al., 2014; Tangphatsornruang et al., 2011). The mt genome sequence was constructed using the bb454Contig program (Iorizzo et al., 2012). Illumina HiSeq2000 paired-end sequence reads, generated from the same strain of *P. insidiosum* (Your Gene Bioscience, New Taipei City, Taiwan), were mapped to the draft mt genome to check for sequence accuracy, especially in the homopolymer regions, with Newbler Reference Mapper (seed = 12, identity > 90%). The structure of *P. insidiosum* mt genome sequence was compared to that of *Pythium ultimum* using Mauve software (Darling, 2004).

2.3. Genome analysis

Annotation of the complete mt genome sequence was performed using the DOGMA [Dual Organellar GenoMe Annotator (Wyman et al., 2004)] program and ORF finder (Wheeler et al., 2003). The predicted annotations of each gene were individually verified using BLAST similarity searches (Altschul et al., 1990). All genes, including protein coding genes, ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs) were identified and named using the organellar/bacterial genetic code classification system. Repetitive sequences were identified using REPuter (Kurtz and Schleiermacher, 1999) with the following criteria: cutoff n ≥ 30 bp, and a sequence identity ≥ 90%.

2.4. Phylogenetic analysis

A set of protein-coding genes, commonly present in mt genomes of 12 oomycete and two diatom (served as outgroup) species (Table 1) were identified and concatemerized for phylogenetic analysis. MUSCLE version 3.6 (Edgar, 2004) was used for alignment and clustering of nucleotide sequences, which were then manually edited to remove gaps. RAxML version 7.2.6 (Stamatakis, 2006) was used to perform maximum likelihood

Table 1
Comparison of mitochondrial gene contents from *P. insidiosum*, 11 other oomycetes, and 2 diatoms, included in this study.

Genome name	Accession number	Length (bp[a])	Inverted repeat	GC (%)[b]	Unique gene	Total genes	Protein-encoding gene	Transfer RNA gene	Ribosomal RNA gene
Phaeodactylum tricornutum[c]	NC_016739	77,356	No	35.0	59	61	33	26	2
Thalassiosira pseudonana[c]	NC_007405	43,827	Yes	30.1	61	64	35	27	2
Thraustotheca clavata[d]	NC_022179	47,382	Yes	23.5	68	79	41	34	4
Achlya hypogyna[d]	NC_022178	46,840	Yes	19.1	70	80	43	33	4
Saprolegnia ferax[d]	NC_005984	46,930	Yes	23.1	66	81	43	34	4
Phytophthora ramorum[e]	NC_009384	39,314	Yes	22.0	69	73	43	28	2
Phytophthora sojae[e]	NC_009385	42,977	No	19.7	74	76	47	27	2
Phytophthora phaseoli[e]	NC_015616	37,914	No	22.1	67	69	40	27	2
Phytophthora infestans[e]	NC_002387	37,957	No	22.3	67	69	40	27	2
Phytophthora andina[e]	NC_015619	37,874	No	22.1	67	69	40	27	2
Phytophthora mirabilis[e]	NC_015606	37,779	No	22.4	67	69	40	27	2
Phytophthora ipomoeae[e]	NC_015622	37,872	No	22.4	67	69	40	27	2
Pythium ultimum[e]	NC_014280	59,689	Yes	21.7	69	117	67	46	4
Pythium insidiosum[e]	AP014838	54,919	Yes	22.6	65	104	59	41	4

[a] Base pair.
[b] Percent frequency of guanine (G) and cytosine (C) in a mitochondrial genome.
[c] Diatom.
[d] Oomycete member of the Saprolegnialean lineage.
[e] Oomycete member of the Peronosporalean lineage.

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