



Codon-based phylogenetics introduces novel flagellar gene markers to oomycete systematics



Gregg P. Robideau^{a,b,1}, Nicolas Rodrigue^{a,2}, C. André Lévesque^{a,b,*}

^a Agriculture and Agri-Food Canada, 960 Carling Ave., Ottawa, ON K1A 0C6, Canada

^b Department of Biology, Carleton University, 1125 Colonel By Dr., Ottawa, ON K1S 5B6, Canada

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ABSTRACT

Oomycete systematics has traditionally been reliant on ribosomal RNA and mitochondrial cytochrome oxidase sequences. Here we report the use of two single-copy protein-coding flagellar genes, PF16 and OCM1, in oomycete systematics, showing their utility in phylogenetic reconstruction and species identification. Applying a recently proposed mutation–selection model of codon substitution, the phylogenetic relationships inferred by flagellar genes are largely in agreement with the current views of oomycete evolution, whereas nucleotide- and amino acid-level models produce biologically implausible reconstructions. Interesting parallels exist between the phylogeny inferred from the flagellar genes and zoospore ontology, providing external support for the tree obtained using the codon model. The resolution achieved for species identification is ample using PF16, and quite robust using OCM1, and the described PCR primers are able to amplify both genes for a range of oomycete genera. Altogether, when analyzed with a rich codon substitution model, these flagellar genes provide useful markers for the oomycete molecular toolbox.

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1. Introduction

Oomycetes are ubiquitous fungus-like protists which can cause disease in a wide range of plants and animals. Many oomycetes are serious pathogens of agricultural, horticultural, and aquatic commodities. The oomycete lineage belongs to the heterokont phylum among brown algae and diatoms, and includes well-known genera such as *Phytophthora*, *Pythium*, and *Saprolegnia*. Oomycete evolution has been well studied without molecular data (Beakes, 1989; Dick, 2001; Sparrow, 1960), and continuing efforts are being made towards the most accurate reconstruction of phylogenetic relationships within this group (Beakes et al., 2012). Studies of molecular evolution and oomycete systematics have generally relied upon mitochondrial cytochrome oxidase subunit II (cox2) (Hakariya et al., 2007; Hudspeth et al., 2000; Sekimoto et al., 2008) and ribosomal RNA (rRNA) sequences from the 18S small

subunit (SSU), internal transcribed spacer (ITS), or the 28S large subunit (LSU) to provide phylogenies (Beakes et al., 2012; Cooke et al., 2000; Lévesque and de Cock, 2004; Petersen and Rosendahl, 2000; Riethmüller et al., 2002).

Prior to DNA sequencing, systematic studies were heavily reliant on ultrastructural characteristics to infer phylogenetic relationships. Some of the most influential work in this area came from studies of the flagellar apparatus (Barr, 1981; Barr and Allan, 1985; Beakes, 1987; Moestrup, 1982). Flagellar hairs, also known as mastigonemes, were consistently cited as an important morphological feature of heterokont flagella (Bouck, 1971; Leadbeater, 1989). In fact, the term “stramenopile” currently used to describe the entire heterokont phylum, is derived from the straw-like hairs present on the so-called “tinsel” flagellum (Patterson, 1989). Since flagellar ultrastructure has been regarded as such an important character in traditional heterokont systematics, it may be fruitful to continue this tradition on a molecular level using flagellar gene sequences, such as those from the axoneme central apparatus and tubular mastigoneme proteins.

The axoneme central apparatus is involved in flagellar dynein regulation and is a core component of the eukaryotic flagellum (Smith, 2002). It is comprised of several known proteins including PF16 (Smith and Lefebvre, 1996, 1997), which is highly conserved among very diverse eukaryotes (Straschil et al., 2010). Tubular tripartite mastigonemes are a defining feature of heterokont flagella,

* Corresponding author at: Agriculture and Agri-Food Canada, 960 Carling Ave., Ottawa, ON K1A 0C6, Canada. Fax: +1 (613) 759 1701.

E-mail addresses: gregg.robideau@inspection.gc.ca (G.P. Robideau), nicolas.rodrigue@ucalgary.ca (N. Rodrigue), andre.levesque@agr.gc.ca (C. André Lévesque).

¹ Current address: Canadian Food Inspection Agency, 3851 Fallowfield Rd., P.O. Box 11300, Ottawa, ON K2H 8P9, Canada.

² Current address: Department of Mathematics and Statistics, University of Calgary, 2500 University Drive NW, Calgary, AB T2N 1N4, Canada.

and their number, thickness, and length have been observed to be consistent at the family level (Hill and Outka, 1974; Moestrup, 1982). Most studies of mastigonemes have relied on the golden alga *Ochromonas*, which possesses only tinsel flagella rather than the combination of whiplash and tinsel flagella found in most heterokonts. To date, four distinct proteins named OCM1, OCM2, OCM3, and OCM4 have been isolated from the mastigonemes of *Ochromonas* (Yamagishi et al., 2007, 2009). Orthologs of PF16, OCM1, OCM2, and OCM4 proteins are present in the published genomes of *Pythium*, *Phytophthora*, and *Saprolegnia* (Adhikari et al., 2013; Haas et al., 2009; Jiang et al., 2013; Lamour et al., 2012; Lévesque et al., 2010; Tyler et al., 2006), and the OCM2 ortholog has been studied in detail in *Phytophthora nicotianae* (Blackman et al., 2011). Access to these flagellar gene sequences now makes the study of their evolution in oomycetes possible.

Since the advent of DNA sequencing, studies of molecular evolution have typically relied on nucleotide or amino acid substitution models, but the analysis of protein coding sequence evolution has significantly advanced since the introduction of codon substitution models (Goldman and Yang, 1994; Muse and Gaut, 1994). In contrast to nucleotide-based or amino acid-based models, codon-based substitution models use coding DNA triplets as character states. These models were originally developed to measure selective pressures, and their adoption in phylogenetic tree inference has been slow due to their high computational demands. Now, recent advances in computing technology combined with algorithmic refinements have created renewed interest in this approach and its potential for tree reconstruction (Gil et al., 2013; Ren et al., 2005) since the advantage of codon models over nucleotide and amino acid level models has been demonstrated in several contexts (Seo and Kishino, 2009).

Here we report DNA sequencing of novel markers PF16 and OCM1 from a variety of oomycetes using newly designed degenerate PCR primers. The coding DNA sequences are analyzed using a codon-based model of sequence evolution, built on the mutation–selection principle (Halpern and Bruno, 1998; Rodrigue et al., 2010; Yang and Nielsen, 2008), to produce a molecular phylogeny which is compared to the phylogenies produced by nucleotide and amino acid models. The evolution of PF16 and OCM1 among oomycetes is explored, and their utility in species identification is demonstrated.

2. Methods

2.1. DNA extraction

For cultures grown from the Centraalbureau voor Schimmelcultures (CBS isolates) and the Canadian Collection of Fungal Cultures (BR isolates), DNA extractions were performed as previously described (Robideau et al., 2011). DNA extraction from *Plasmopara halstedii* (Lev 5881 isolate) was identical to BR isolate extractions except that the starting material was ~150 µL of crushed dried *Helianthus annuus* leaves that were infected with *P. halstedii* race 2 (Rashid, 1993).

2.2. Reference genomes accessed

Available genome assemblies were accessed to obtain full length predicted PF16 and OCM1 sequences. The genomes from which sequences were obtained were: *Albugo candida* (Links et al., 2011), *Albugo laibachii* (Kemen et al., 2011), *Ectocarpus siliculosus* (Cock et al., 2010), *Phytophthora capsici* (Lamour et al., 2012), *Phytophthora infestans* (Haas et al., 2009), *Phytophthora ramorum*, *Phytophthora sojae* (Tyler et al., 2006), *Pythium aphanidermatum*

(Adhikari et al., 2013), *Pythium ultimum* (Lévesque et al., 2010), and *Saprolegnia parasitica* (Jiang et al., 2013).

2.3. DNA amplification

PCR primers were designed based on alignment of predicted PF16 and OCM1 sequences from assembled *Phytophthora*, *Pythium*, and *Saprolegnia* genomes (*Ph. infestans*, *Ph. ramorum*, *Ph. sojae*, *Py. aphanidermatum*, *Py. ultimum*, and *S. parasitica*). Predicted PF16 and OCM1 sequences were retrieved by performing tBLASTN on genomic DNA assemblies using *Chlamydomonas reinhardtii* PF16 protein (GenBank: AAC49169) and *Ochromonas danica* OCM1 protein (GenBank: BAF65668) as queries. Sequences of each primer used in this study are listed in Table S1. Standard primers designed for PF16 amplification were ACAup13 and ACAlo1475. Standard primers designed for OCM1 amplification were OCM1up130B and OCM1lo1842B. Alternative primers were designed which amplified species that did not amplify well with the standard primers. Alternative OCM1 primers were OCM1up124 and OCM1lo1853. *Pythium insidiosum* OCM1 sequences were amplified using OCM1oomup1 and OCM1oomlo1. *Pythium porphyrae* OCM1 sequences were amplified with OCM1up145B and OCM1lo1842B. *Apodachlya brachynema* OCM1 sequences were amplified with OCM1oomup2C and OCM1oomlo1E. *Dictyuchus monosporus* OCM1 sequences were amplified with OCM1oomup2D and OCM1oomlo1A. PCR reaction volume was 10 µL containing final concentrations of 1× Titanium Taq buffer (with 3.5 mM MgCl₂), 0.1 mM dNTPs, 0.08 µM each of forward and reverse primer, 0.5× Titanium Taq polymerase, and approximately 0.1–1.0 ng/µL of DNA. Reaction volume was brought up to 10 µL with sterile HPLC water. Thermocycler program for amplification of PF16 was: 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 69 °C for 45 s, 72 °C for 1 min 30 s. A final extension was made at 72 °C for 10 min. Program for amplification of OCM1 was: 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 2 min. A final extension was made at 72 °C for 10 min.

2.4. PCR product isolation

PCR products were run on 1.0% agarose gel in 0.5× TBE buffer at 60 V for 40 min to verify presence of a single band at desired length (~1.5 kb for PF16, ~1.8 kb for OCM1). In a few samples the desired band was not present, or multiple bands were present in the PCR product. In such cases, the PCR reaction was repeated using a more robust PCR mixture containing final concentrations of 1× Titanium Taq buffer (with 3.5 mM MgCl₂), 0.2 mM dNTPs, 0.5 µM each of forward and reverse primer, 1× Titanium Taq polymerase, and approximately 0.1–1.0 ng/µL of DNA. Reaction volume was brought up to 20 µL with sterile HPLC water. The desired band was then isolated from the PCR product using E-Gel® CloneWell™ 0.8% SYBR Safe™ gels run in the E-Gel® iBase™ Power System (Invitrogen).

2.5. DNA sequencing

PCR products were sequenced with ABI Big Dye Terminator v3.1 in a reaction volume of 10 µL, with Big Dye Seq Mix diluted 1:8 with Seq buffer. Final concentrations of each reagent were 0.875× Sequencing buffer, 5% trehalose, 0.125× Big Dye Seq Mix, and 0.16 µM primer. Reaction volume was brought to 10 µL with sterile HPLC water and either 1 µL of PCR product directly from initial PCR amplification or 1 µL of Clonewell™ isolated PCR product was added. Sequencing primers used for PF16 were ACAup13, ACAup109, ACAup156, ACAup829PP, ACAlo800, ACAlo854PP, ACAlo1445PP, ACAlo1453, ACAlo1472. Primers designed to sequence PF16 from *Aphanomyces cladogamus* and *A. cochlioides*

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