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Molecular detection of *Mikrocytos mackini* in Pacific oysters using quantitative PCR

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ABSTRACT

Mikrocytos mackini is an internationally regulated pathogen and causative agent of Denman Island disease in Pacific oysters *Crassostrea gigas*. Recent phylogenetic breakthroughs have placed this parasite within a highly divergent and globally distributed eukaryotic lineage that has been designated a new taxonomic order, Mikrocytida. The discovery of this new radiation of parasites is accompanied by a heightened awareness of the many knowledge gaps that exist with respect to the general biology, epizootiology, and potential impact of mikrocytid parasites on hosts, ecosystems, and commercial fisheries. It has also highlighted current shortcomings regarding our ability to detect these organisms. In this study, we developed a species-specific, sensitive, and quantitative method for detecting *M. mackini* DNA from host tissues using probe-based real-time qPCR technology. A limit of sensitivity between 2 and 5 genome copy equivalents was achieved in a reaction matrix containing ≥40 ng/μL host gDNA without inhibition. This detection proved superior to existing methods based on conventional PCR, histology or gross pathology and is the first species-specific diagnostic test for *M. mackini*. Quantitative assessment of parasite DNA using this assay remained accurate to between 10 and 50 copies identifying that during infection, *M. mackini* DNA was significantly more prevalent in hemolymph, labial palp, and mid-body cross-sections compared to mantle or adductor muscle. DNA extracted from a mid-body cross-section also provided the highest likelihood for detection during diagnostic screening of infected oysters. Taken together, these findings provide strong analytical evidence for the adoption of qPCR as the new reference standard for detecting *M. mackini* and give preliminary insight into the distribution of the parasite within host tissues. Standardised operating methodologies for sample collection and qPCR testing are provided to aid in the international regulatory diagnosis of *M. mackini* and serve as a useful platform for the future development of multiplexed or alternate mikrocytid species detection.

1. Introduction

In 1960, mass mortalities were reported from farmed Pacific oysters *Crassostrea gigas* near Denman Island, Canada, and attributed to a newly emerging infectious disease characterised by focal green lesions within the mantle, body wall, labial palps and adductor muscle [1]. Heavy infections were observed to cause high levels of morbidity (17–53%) and the continued presence of lesions and scarring in non-lethal cases rendered many surviving oysters unmarketable. The disease has continued to manifest in cyclic annual patterns associated with long periods of low water temperature; however, another 28 years were required before the elusive microcell parasite *Mikrocytos mackini* was definitively identified as the causative agent of this disease [2]. Following discovery, *M. mackini* was shown to infect not only Pacific oysters but also at least three other oyster species through experimental infection and has been detected in coastal oyster beds throughout southern British Columbia as well as in some isolated locations along the Northwest coast of the United States (for recent review, see [3]). Still, in the subsequent decades following its identification, *M. mackini* has remained stubbornly refractive toward most efforts of scientific scrutiny. To date, *M. makini* (or any other *Mikrocytos* pathogen) has provided no morphological defining characteristics, remains uncultured in a laboratory setting, and has an unknown

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life cycle, host range, and global geographic distribution. This is not to say that *M. mackini* and Denman Island disease has been without import; on the contrary, *M. mackini* was listed by the World Organisation for Animal Health (OIE) until 2007, and continues to be a reportable and regulated pathogen in Canada, Europe, and Australia [3].

Two recent landmark phylogenetic studies provided a first step into cracking the enigmatic shell surrounding *M. mackini* by resolving its evolutionary origins and classifying it taxonomically. Burki et al. [4] used transcriptome sequencing to place *M. mackini* within Rhizaria and uncover it as one of the fastest-evolving eukaryotic lineages known to science. Hartikainen and colleagues [5] expanded upon these findings by discovering that *M. mackini* falls within a previously undetected and highly divergent radiation of Protists with a global distribution and broad host range. They resolved the phylogenetic position of this radiation as a sister to haplosporidians, formally described it as Mikrocytida ord. nov., and developed a lineage-specific PCR method to detect mikrocytids from organismal or environmental DNA samples. It is anticipated that uptake of this method by the international community will almost certainly uncover new and potentially substantial diversity within the order Mikrocytida and, by extension, also within *Mikrocytos* [6].

The continued international regulatory concern for *M. mackini* in conjunction with the unknown (and potentially high) species diversity within the genus creates an acute need for definitive species delineation and specific diagnostic assays for these parasites—tools which have hitherto remained conspicuously absent. Histopathology and PCR are the two diagnostic methods standardly employed for the detection of *M. mackini* and neither are species-specific [7]. *M. mackini* appears indistinguishable from *Bonamia* sp. during histological observation and relies on tissue tropism for presumptive diagnosis [7]. Conventional PCR targeting *M. mackini* 18S rDNA [8] is known to cross react with other *Mikrocytos* spp. [9] and thus requires sequencing for a confirmatory diagnosis. As such, a pressing and limiting factor regarding research advancement and management of mikrocytid parasites, specifically *M. mackini*, is the development of rapid and sensitive detection methods that can be internationally applied to ensure species-specific detection within this newly described and growing lineage of pathogenic organisms [3].

Real-time quantitative PCR (qPCR) has emerged as a powerful and rapid tool for detection of pathogens and presents a logical choice for molecular detection of *Mikrocytos*. Although a variety of other molecular diagnostic methods exist (e.g. Loop-mediated isothermal amplification, digital PCR, etc.), none have been implemented to such a pervasive extent in molecular genetics research as real-time qPCR [10]. Further, of the two main methods used for nucleic acid detection by qPCR, target-specific fluorescent-tagged hybridization (i.e. probe-based qPCR) [11] provides both an added layer of specificity and an ability to be applied in multiplexed analyses relative to fluorescent double-stranded DNA binding dyes such as SYBR® Green 1 [12]—two prospective benefits for mikrocytid detection given the high potential diversity within this order [5].

Previous work has identified the internal transcribed spacer (ITS) regions of rDNA for *M. mackini* to be highly conserved within individuals throughout its endemic range [9]. These regions have also shown substantial divergence in discrete species comparisons within the genus [13], and thus present a strong candidate target for species-specific diagnostic testing. In the present study, our aim was to develop a probe-based qPCR technique targeting the ITS-2 region of *M. mackini* rDNA for sensitive and specific detection of *M. mackini* and characterise its utility by applying it to various sample matrices. We demonstrate high analytical sensitivity, specificity, and repeatability for the quantitative ability of this assay as well as methodology for application in the international diagnosis of this parasite. We further contribute insight into the distribution of parasitic load within host tissues.

2. Materials and methods

2.1. Sample collection, histopathology, and DNA extraction

Between February 2010 and March 2011, more than 900 adult Pacific oysters were collected from locations around Vancouver Island, British Columbia, Canada, including Lemmens Inlet (N49 48.30; W125 51.27), Deep Bay (N49 27.83; W124 44.32) and near Nutchatlitz Island (N49 48.30; W126 57.67). All sampling locations were within the known endemic range for infection with *M. mackini* [9] and included wild, cultured, intertidal, and suspended oysters ranging from 7 to 23 cm shell length and 2 to >7 years of age. In each instance, oysters were collected and transported live to the Pacific Biological Station, Nanaimo, Canada, and stored live at 4 °C for 1 to 7 days until processing.

Each oyster was shucked and soft tissues transferred to a clean plastic weighing dish. Histology tissue samples consisted of a transverse cross section (3–4 mm thick) cut through the anterior region of the visceral mass which included mantle, gonad, labial palps, stomach and digestive gland as well as a small piece of heart and adductor muscle. Samples were preserved in Davidson's solution for 24 to 72 h and processed using routine histological techniques [14]. Deparaffinised 5 μm sections were stained with Harris' modified hematoxylin Solution (Sigma) and 0.5% eosin and examined via light microscopy for the presence of *M. mackini*. All sections were examined fully at low magnification (100×), with an additional detailed examination (minimum 50 fields of view at 1000× magnification) conducted on all sections where lesions or areas of haemocyte infiltration were observed.

For qPCR and conventional PCR analysis, small portions of mantle, gonad, digestive gland and gill (approx. 25 mg total) were excised from a transverse section cut from the middle region of the visceral mass and gills (hereafter referred to as the mid-body section), preserved in 95% ethanol, and stored at −20 °C. DNA was subsequently extracted using a DNEasy Tissue Kit (QIAGEN) as per manufacturer's instructions with the exception that elution buffer pre-heated to 70 °C was used to elute DNA and incubated at 55 °C for 3 min prior to the final centrifugal collection. For comparisons of assessing relative parasite DNA loads between localised samples within oysters, samples of 50–100 mg were collected. As this was greater than the maximum amount of tissue recommended for use in individual DNEasy columns (25 mg), samples were digested in 600 μL lysis buffer (Buffer ATL) and 20 U Proteinase K overnight at 55 °C and DNA was extracted from a 200 μL subsample following procedures described above.

Conventional PCR amplification of *Mikrocytos* DNA was performed using 18S primers (Mikrocytos-F/Mikrocytos-R) developed by Carnegie et al. to be *M. mackini* specific [8] but that are known to amplify other *Mikrocytos* sp. (reviewed by [3]). Reactions contained 5–500 ng total template DNA, 1× PCR Buffer, 2.5 mM of $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each forward and reverse primer, and 0.05 Units/μl Platinum Taq polymerase (Invitrogen) in a total volume of 25 μL.

2.2. qPCR assay design

The qPCR assay developed in this study targets species specific sequence within the ITS-2 *M. mackini* rDNA (GenBank accession no. HM563060). Alignment of *M. mackini* rDNA (HM563060) to rDNA of a second *Mikrocytos* species, *M. boweri* ([13]; GenBank accession no. HM563061), demonstrated substantial divergence between these sequences immediately adjacent to the 5.8 subunit