AN ABSTRACT OF THE THESIS OF

Harold Amogan for the degree of Master of Science in Microbiology presented on February 26, 2004.
Title: Molecular Characterization of Crustacean Parasite Nadelspora canceri.

Abstract approved: ____________________________

Paul W. Reno

Investigations into the phylogeny, genome size, and karyotype of microsporidian Nadelspora canceri were initiated to further characterize the organism. Isolates of N. canceri spores were obtained from both Dungeness (Cancer magister) and red rock crabs (Cancer productus). Analysis of the ssu rDNA sequence from spore isolates of the two crab species showed 100% sequence identity among 1,081 nucleotide positions, indicating the same species of microsporidian is infecting both species of crabs. Phylogenetic studies based on the ssu rDNA sequences also showed N. canceri to be most closely related to another crustacean parasite, Ameson michaelis. Sequence comparison between the two microsporidian species showed 93% sequence identity (1,001/1081 nucleotide positions).
Pulsed field gel electrophoresis was used to estimate the genome size and karyotype of *N. canceri* isolates obtained from Dungeness and red rock crabs. Resolution of DNA bands on the pulsed field gels revealed both isolates to have a karyotype of ten chromosome-sized DNA bands. Estimation of the genome size revealed spore isolates from *C. magister* to have a total genome size of 7.44 Mb and spore isolates from *C. productus* to have a total genome size of 7.32 Mb. Variations detected in chromosome size culminated in a difference in the genome size between the two isolates. However, the variations in chromosome size were found not to be significant based on the Student’s t-test.
Molecular Characterization of Crustacean Parasite *Nadelspora canceri*

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Harold Amogan

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Dean of the Graduate School

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Sincere appreciation is expressed to Dr. Paul W. Reno for his guidance during this investigation and writing of the manuscript. I would like to thank Dr. Katharine G. Field for her help and generosity in providing laboratory space and materials during the investigation. Much overdue gratitude and sincere thank you to Weeratip Pongprasert, Anne Bernhard, the Giovannoni lab, the Ciuffetti lab, and the Ream lab for their support and advice during the investigation. I would also like to thank Dr. Robert Olson for his help in specimen collection and the OSU Central Services Lab for their help in acquiring data. This work was supported by the Hatfield Marine Science Center Mamie Markam Grant.

A warm-felt applause and hurrahs to friends and colleagues Ryan Mayfield (MS) and Dr. Carlos Bordador, for their help in revising the thesis manuscript. A warm-felt applause to my parents, whose quiet support has been a cornerstone of hope through the years. Finally, a warm-felt applause to childhood friends Cary Abayan and Patrick Young, for their perpetual upbeat attitude and funny remarks during the entire writing process. A long road has been traveled, a testimony to life never abiding to the road map we have scripted. I do hope, in time, a reflective smile will cross my face when I look back and ponder on this experience.
CONTRIBUTION OF AUTHORS

Dr. Paul W. Reno offered guidance and assistance in the collection of data, analysis of the results, and writing of the manuscript. Experiments to obtain sequences for inferring phylogeny were mostly conducted in the laboratory of Dr. Katharine G. Field, who also assisted in data interpretation and editing of the manuscript. Dr. Lynda Ciuffetti and Dr. Patrick Martinez assisted in developing conditions for resolving DNA band fragments by pulsed field gel electrophoresis and interpretation of the data.
# TABLE OF CONTENTS

1. INTRODUCTION 

2. PHYLOGENY OF CRUSTACEAN PARASITE *NADELSPORA CANCERI* BASED ON SSU rDNA SEQUENCE ANALYSIS
   - 2.1 Abstract
   - 2.2 Introduction
   - 2.3 Materials and Methods
   - 2.4 Results
   - 2.5 Discussion
   - 2.6 Literature Cited

3. ESTIMATING KARYOTYPE AND GENOME SIZE OF *NADELSPORA CANCERI* BY PULSED FIELD GEL ELECTROPHORESIS
   - 3.1 Abstract
   - 3.2 Introduction
   - 3.3 Materials and Methods
   - 3.4 Results
   - 3.5 Discussion
   - 3.6 Literature Cited

4. CONCLUSION

BIBLIOGRAPHY
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Distance and parsimony tree showing position of the <em>Nadelspora canceri</em> ssu rDNA sequences relative to other members of the phylum <em>Microspora</em>.</td>
<td>19</td>
</tr>
<tr>
<td>2.</td>
<td>Ethidium bromide stained gel of <em>Nadelspora canceri</em> DNA subjected to pulsed field gel electrophoresis.</td>
<td>41</td>
</tr>
<tr>
<td>3.</td>
<td>Pulsed field gel showing resolution of DNA bands in the 565 to 680 kb range.</td>
<td>43</td>
</tr>
<tr>
<td>4.</td>
<td>Pulsed field gel showing a difference in migration rate for band 10 of samples NC1 and NC2.</td>
<td>46</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Primers used in the amplification and sequencing of the ssu rDNA sequence of <em>Nadelspora canceri</em>.</td>
<td>16</td>
</tr>
<tr>
<td>2.</td>
<td>Comparison of biotic characteristics of <em>Nadelspora canceri</em> to members of the <em>Icthyosporidium</em> group as defined by Baker and colleagues (1995).</td>
<td>21</td>
</tr>
<tr>
<td>3A.</td>
<td>Estimates of number and size (in Kb) of DNA bands obtained from <em>Nadelspora canceri</em> using PFGE.</td>
<td>38</td>
</tr>
<tr>
<td>3B.</td>
<td>Student’s t-test results investigating the significance of the difference in corresponding mean band sizes between NC1 and NC2.</td>
<td>40</td>
</tr>
<tr>
<td>3C.</td>
<td>Coefficient of Variance (CV) values for NC1 and NC2.</td>
<td>40</td>
</tr>
<tr>
<td>4.</td>
<td>Estimates of chromosome number and genome size of microsporidians, with <em>Nadelspora canceri</em> shown in bold.</td>
<td>49</td>
</tr>
</tbody>
</table>
Microsporidians are obligate intracellular eukaryotic microorganisms parasitic to both vertebrates and invertebrates. Host species known to be infected by microsporidians range from insects (such as fire ants, bumblebees, and cabbage moths) to more complex organisms such as crustaceans, fish, and mammals. Microsporidians are often detected by the presence of mature spores in host tissue. Spore morphology within this phylum is highly diverse with spores appearing rod-shaped, spherical, ovoid, and even bell-shaped (Wittner and Weiss 1999). Investigations into the ultrastructure of the spores have yielded the following unifying salient characteristics: a polar filament, an anchoring disk, a polaroplast, and a posterior vacuole (Wittner and Weiss 1999). These four components work in conjunction to infect targeted host cells. Upon a change in external conditions (such as a change in pH) the polar filament is everted from the spore. This expulsion of the polar filament is made possible by the rapid expansion of the polaroplast and the posterior vacuole. Expansion of the polaroplast and posterior vacuole is instrumental in pushing the spore's cytoplasmic contents out of the spore and into the host cell. The anchoring disk holds the polar filament in place as the filament everts. Other structures present in a mature spore are an exospore (proteinaceous outer coat), endospore (chitinous inner coat), and a plasma membrane (Wittner and Weiss 1999).
The general life cycle of these organisms starts with the proliferative phase, continues with the sporogonic phase, and ends with the infective phase (the mature spore) (Wittner and Weiss 1999). The mature spore is the only stage in the microsporidian life cycle that can exist outside a host cell. The spore generally enters the host by ingestion. Carried to the host’s intestinal tract, the spore extrudes its polar filament due to a change in external conditions. The polar filament pierces a nearby host cell allowing the deposition of the spore’s cytoplasmic contents into the host. The cytoplasmic contents are referred to as a sporoplasm, which undergo multiple divisions and developmental stages to eventually become mature spores. In the proliferative phase, the sporoplasm undergoes cell growth and division (karyokinesis and cytokinesis). The division in the proliferative phase can occur by binary fission, multiple fission, or plasmotomy. The terminus of the proliferative phase and the initiation of the sporogonic phase is indicated by the collection of electron dense material on the developing spore’s plasmalemma. In the sporogonic phase, the sporont undergoes subsequent divisions and develops into sporoblasts. The sporoblasts are the final stage prior to forming mature spores. The mature spores in turn repeat the process of infection and spore development in adjacent host cells.

*Nadelspora canceri* is a crustacean-infecting microsporidian discovered off the Oregon Coast. Crab muscle tissue infected with *N. canceri* appears milky white and flaccid (Childers et al. 1996). In contrast, muscle tissue from uninfected crabs appears firm and translucent. The spores appear needle-shaped, a morphology unique among microsporidians. Spore size is approximately 10 μm long and 0.25 μm wide. From
research conducted by Olson and colleagues (1994), *N. canceri* spores were observed to have a polar filament, anchoring disk, and endospore, signature trademarks for the phylum Microspora. Intermediate stages in the life cycle were observed in infected crab muscle tissue, suggesting the spore did not require an alternate host to complete its life cycle. In laboratory experiments *N. canceri* was successfully transmitted to uninfected Dungeness crabs by feeding crabs a 1 g dose of infected crab muscle tissue (Childers 1993). The experiments suggest *N. canceri* spreads through a crab population by crabs feeding on dead or dying crustaceans infected with the parasite. Sampling of various Dungeness crab populations along the Pacific Coast of North America revealed *N. canceri* to be geographically distributed from Southern Washington to Northern California (Childers, Reno, and Olson 1996). The prevalence of crabs infected with the parasite was found to be higher in bays and estuaries compared to open ocean.

Research by Childers and colleagues (1996) had also shown *N. canceri* to have a prevalence of infection (in embayments) ranging from a low of 0.4% to a high of 41.4%. The values differed widely among adjacent bays and estuaries, suggesting no major movement by *N. canceri* or its host. Infection was most prevalent among 2-year old crabs, with males infected two and a half times as often as females. Crabs infected with the parasite invariably died, possibly from starvation due to *N. canceri* destroying the crab muscle tissue, thereby rendering the crab unable to feed itself.
From prior work investigating *N. canceri*, the microsporidian had been described in terms of its geographic distribution, prevalence of infection, and spore ultrastructure (Childer, Reno, and Olson 1996; Olson, Tiekotter, and Reno 1994). However, no work had been conducted to characterize the organism from a molecular perspective. Inquiries into the molecular biology of the organism are needed to determine where is *N. canceri* positioned in the phylum Microspora, to which microsporidian is *N. canceri* most closely related to in the phylum Microspora, and what is the genome size and chromosome number of *N. canceri*? Characterization from a molecular perspective could also help define what constitutes the species. Due to the absence of molecular information describing *N. canceri*, this project was initiated focusing on inferring the organism's phylogeny based on small subunit (ssu) rDNA sequence analysis, and estimating genome size and karyotype by use of pulsed field gel electrophoresis (PFGE). The needle-shaped spores have been observed in both Dungeness and red rock crabs. This study also investigated the degree of relatedness between spores infecting the two species of crabs.

Current understanding of microsporidian phylogeny has mainly been based on ssu rDNA sequences. The significance of the ssu rDNA sequence lies in its participation in translation, a mechanism that arose before the division of the universal ancestor into the three domains of life (Bacteria, Archaea, and Eukarya) (Woese 1996). Therefore, the ssu rDNA sequence is present and homologous among organisms. A second useful attribute of the molecule is its high degree of conservation. Since the molecule codes for RNA instead of protein, the ssu rDNA is excluded from the degeneracy of the genetic code. Despite being highly conserved, the ssu rDNA sequence does have variable regions
allowing the identification of unique sequences characteristic of a phylogenetic group. Based on ssu rDNA sequence analysis, a consistent branching pattern has been inferred within the phylum Microspora. Noting this consistency in branching pattern, Baker and colleagues (1995) identified 4 major groups in the phylum: *Nosema/Vairimorpha*, *Encephalitozoon*, *Endoreticulatus*, and *Icthyosporidium*. The *Nosema/Vairimorpha* group consists of insect-infecting microsporidians that are binucleate and have ovoid spores. Mammalian-infecting microsporidians are distributed in both the *Encephalitozoon* and *Endoreticulatus* groups. Research by Nilsen and colleagues (1998) has shown fish-infecting microsporidians to be in both the *Endoreticulatus* and *Icthyosporidium* groups. Crustacean-infecting microsporidians, of which *Ameson michaelis* was the only known sequenced representative, are positioned in the *Icthyosporidium* group. There is a lack of sequences in the gene database for crustacean-infecting microsporidians. Sequence data from *N. canceri*’s ssu rDNA may help elucidate whether crustacean-infecting microsporidians form a cluster in the *Icthyosporidium* group or are polyphyletic.

Inferring phylogeny based on molecular data is a dynamic process where relationships among organisms may change based on increasing information. Data based on ssu rDNA appears to be capable of resolving the phylogeny within the phylum Microspora. However, the phylogeny of the phylum as a whole is in question. Phylogeny based on ssu rDNA and several protein elongation factors (EF-1alpha and EF-2) positions the phylum at the base of the eukaryotic tree (Hirt et al. 1999). All microsporidians lack mitochondria, thereby supporting their position as primitive
eukaryotes. Being amitochondriate, Cavalier-Smith (1983) grouped the microsporidians into the kingdom Archezoa (Wittner and Weiss 1999). Archezoa consisted of basal amitochondriate eukaryotes believed to have diverged from the main eukaryotic lineage prior to the endosymbiotic event between eukaryotes and prokaryotes. Endosymbiosis between eukaryotes and prokaryotes led to the presence of mitochondria and chloroplasts observed in today’s eukaryotes. However, the discovery of heat shock gene sequences (HSP70) in microsporidians challenged the idea microsporidians had always been amitochondriate. HSP70 is of mitochondrial origin, suggesting microsporidians had participated in an endosymbiotic event with prokaryotes in the evolutionary past. Phylogenetic trees created based on HSP70, as well as other gene sequences (an RNA pol II subunit and a tubulin gene) position the phylum as a sister group to fungi (Hirt et al. 1999). The contradiction in sequence data has led researchers to review data positioning microsporidians as basal eukaryotes. Long branch attraction effects (in ssu rDNA sequence data) due to accelerated evolution may explain the positioning of microsporidians as basal eukaryotes (Morin 2000). Also, signature sequences found in the EF-1 alpha genes of fungi and metazoans (animals) have also been found in the EF-1 alpha gene of the microsporidian Glugea plecoglossi (Hirt et al. 1999).

In terms of taxonomy, most microsporidians can be divided into 3 major groups based upon spore morphology (Wittner and Weiss 1999). Microsporidians exhibiting short, thick polar filaments and lacking polaroplasts are referred to as “primitive” microsporidians. “Intermediate” microsporidians are described as having short, polar filaments with minimal development of the polaroplast and endospore. “Higher”
microsporidians exhibit a well-developed polar filament, polaroplast, and posterior vacuole. Various classification schemes are centered on these 3 groups, with major subdivisions suggested in the “higher” microsporidians. Tuzet and colleagues (1971) had separated microsporidians into 2 suborders based on the presence or absence of a membrane surrounding the sporoblast. Sprague and Vavra (1977) also used the presence or absence of a sporoblast membrane to separate the “higher” microsporidians.

Suborders in Sprague and Vavra’s classification scheme were further separated according to observed morphology and state of the nucleus in sporogony. Simultaneously, Weisser (1977) had also elucidated a separate classification scheme for grouping “higher” microsporidians. Similar to Sprague and Vavra’s classification scheme, Weisser’s classification scheme also emphasized the monokaryotic or diplokaryotic nature of the spores in sporogony. Issi (1986) used both spore morphology and observed stages of the life cycle to further separate the “higher” microsporidians (Baker et al. 1995). Sprague and colleagues (1992), in a revision of microsporidian taxonomy, emphasized the chromosome cycle of microsporidians as the cornerstone in classification. In summary, the classification of microsporidians is a dynamic process undergoing constant revision. However, there appears to be no clear agreement on which physical characteristics should be given more weight in taxonomy.

Phylogeny based on ssu rDNA may not always agree with the classification of microsporidians based on morphology. Molecular phylogeny (inferring evolution based on gene sequences) of microsporidians suggests many morphological traits used in taxonomy may have arisen multiple times. If the taxonomy of microsporidians is based
on homologous traits, then knowing which traits are homologous and which traits are analogous is crucial to the classification scheme. Unfortunately, no fossil record exists for microsporidians, thus obscuring which traits used in taxonomy are homologous. To further hinder resolving classification, the life cycle of most microsporidians has not been fully elucidated, giving an incomplete picture for many species.

In addition to the characterization of *N. canceri*’s ssu rDNA, the genome size and chromosome number of this species was also of interest. Hazard and colleagues (1979) used a lacto-aceto-orcein stain to approximate the karyotype of the microsporidian *Amblyospora* (Wittner and Weiss 1999). Observation under a light microscope of the stained specimen showed the specimen to have seven chromosomes. Pulsed field gel electrophoresis (PFGE) has provided an alternative to investigating the karyotype of microsporidians. Using an alternate switch time interval directing voltage in alternating directions, DNA of sizes greater than 50 kilobases (kb) can migrate through an agarose gel with little hindrance. The use of PFGE has led to the resolution of the karyotype and genome size of various microsporidians. *Vavraia oncoperae* samples (isolated from porina caterpillars (*Wiseana sp.*) and grass grubs (*Costelytra zealandica*)) were found to have two karyotypes (14 and 16) (Malone and McIvor 1993). *Encephalitozoon cuniculi* exhibited 6 different DNA band patterns on pulsed field gels suggesting chromosome size
polymorphism (Biderre et al. 1998). Investigations into the genome size of microsporidians have so far yielded a genome size range of 2.9 to 19.5 million bases (Mb) for the phylum (Wittner and Weiss 1999). Investigations into the karyotype of known microsporidians have also yielded a karyotype range of 7 to 18 chromosome-sized DNA bands for the phylum (Wittner and Weiss 1999).

At present, no information exists in literature describing the karyotype and genome size of crustacean-infecting microsporidians. The ssu rDNA of A. michaelis was sequenced in 1993 with no further inquiries into the molecular biology of the organism (Zhu et al. 1993). The karyotype and genome size was determined for N. canceri in this study and it will be interesting to see whether other crustacean-infecting microsporidians harbor the same chromosome number and genome size as N. canceri. If the crustacean-infecting microsporidians form a monophyletic group based on ssu rDNA sequence analysis, then they may also exhibit a similarity in electrophoretic band patterns on the pulsed field gels.
CHAPTER 2

PHYLOGENY OF CRUSTACEAN PARASITE NADELSPORA CANCERI BASED ON SSU rDNA SEQUENCE ANALYSIS

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Dr. Denis H. Lynn, Editor-in-Chief, Department of Zoology,
University of Guelph, Guelph, ON, CANADA N1G 2W1.
2.1 Abstract

*Nadelspora canceri* is a novel microsporidian pathogenic to Dungeness (*Cancer magister*) and red rock crabs (*C. productus*). The parasite has been described in terms of its geographic distribution, prevalence of infection, and spore ultrastructure (Childers, Reno, and Olson 1996; Olson, Tiekotter, and Reno 1994). To further characterize the organism, we have sequenced the ssu rDNA from spores infecting both Dungeness and red rock crabs. The ssu rDNA gene sequences were compared to each other and found to be 100% identical (1,081/1,081 identical nucleotide positions). The high sequence identity strongly suggests spores infecting Dungeness crabs are the same species infecting red rock crabs. The ssu rDNA sequences were also compared to 19 microsporidian sequences obtained from Genbank. Through distance and parsimony methods, our results show *N. canceri* to be most closely related to another crustacean-infecting microsporidian, *Ameson michaelis*.

**Keywords.** Distance methods, microsporidia, and parsimony.
2.2 Introduction

Microspora is a phylum of parasitic eukaryotes pathogenic to vertebrates and invertebrates. Microsporidian spores have been found in fish, arthropods, insects, crustaceans, and mammals (Wittner and Weiss 1999). The diversity of these eukaryotic microbes is wide and even today new species are being discovered. Classification of these organisms has traditionally been based on spore ultrastructure, observed life cycles, and target hosts (Wittner and Weiss 1999). However, taxonomy based on physical appearance raises the issue of determining which characters are homologs, and which characters have evolved independently. To help resolve classification within the phylum Microspora, the rDNA sequence has increasingly been used as a tool to determine relatedness (Baker et al. 1995). The use of rDNA to resolve classification also helps to infer the phylogenetic relationships these microbes have to one another and predict evolutionary trends.

* Nadelspora canceri* is a microsporidian parasitic to both Dungeness (*Cancer magister*) and red rock crabs (*C. productus*). The microsporidian is distributed from Bodega Bay, CA to Grays Harbor, WA (Childers et al. 1996). Crabs infected with the spore generally have muscle tissue appearing milky white or lightly yellow. Mortality of crustaceans infected with *N. canceri* approaches 100%. Under the microscope, the spores appear needle shaped and are approximately 10 μm long and 0.25 μm wide (Olson et al., 1994). Among sampled populations of Dungeness crabs inhabiting bays and estuaries along the United States Pacific Coast, the prevalence of infection ranged from as low as
0.4% to as high as 41.4% (Childers, Reno, and Olson 1996). The parasite has been described in terms of its geographic distribution, prevalence of infection, and observed spore morphology. No information exists on the phylogeny of *N. canceri* and its relation to other members of the phylum Microspora. We sequenced the small subunit (ssu) rDNA from needle-shaped spores isolated from Dungeness and red rock crabs. By sequencing the ssu rDNA of the parasite, we wanted to resolve *N. canceri*’s position in the phylum Microspora, and investigate whether the needle-shaped spores infecting Dungeness crabs are the same species infecting red rock crabs.

2.3 Materials and Methods

**Collection of Infected Crabs.** Crabs infected with *N. canceri* were caught using crab pots and rings in Yaquina Bay, Newport OR. The presence of infected crabs was determined by looking for the appearance of milky white or yellow colored muscle tissue at the carapace-leg junction. Observation of needle-shaped spores seen under a phase contrast microscope (1,000X) confirmed the presence of *N. canceri* in the crabs. In a total of 76 crabs examined, 1 Dungeness and 2 red rock crabs were infected with *N. canceri*. 
Spore Purification. Infected crab muscle tissue was minced using a razor blade, and homogenized in a Dounce homogenizer. The homogenized tissue was filtered through cheesecloth (2X) and the filtrate collected for spore purification. Spore purification was achieved by adding 10 ml of filtrate to 30 ml of sucrose gradient, consisting of 10 ml of 50%, 40%, and 30% v/v sucrose (10 ml of each concentration). The sample was then centrifuged for 1 hour at 1,000 g. The resulting band of spores was removed and placed into a new centrifuge tube. The spore sample was then diluted to 20% or 30% sucrose and centrifuged for another hour at 1,000 g. A pellet was recovered and suspended in 50 μl of dH2O. The process of recovering spores from the initial sucrose gradient was repeated 2 more times to obtain a total of 150 μl of purified spores. Approximately 1 X 10^6 to 1 X 10^7 spores/ml were obtained by this process.

Extraction of N. canceri DNA. Purified spore samples were subjected to sonication to release spore contents and obtain genomic DNA. The spore samples were sonicated for 3.5 to 5 minutes in six to eight 30-second bursts. The released N. canceri DNA was then purified by use of a QIAgen DNA minikit (QIAgen, Santa Clarita, CA). Approximation of DNA concentration was determined by taking the absorbance (OD_{260}) reading of the DNA samples. The amount of DNA recovered ranged from as low as 1 μg/μL to as high as 22.5 μg/μL.
Conditions for PCR. The ssu rDNA of *N. cancerti* was amplified using the primers listed in Table 1. Primers pmpl, 530f, and 530r are pan-microsporidian primers (Dowd et al. 1999; Weiss and Vossbrinck 1999), primer 1406r is a universal primer, and primers 918f and 918r were created based on an alignment of microsporidian sequences obtained from GenBank. Conditions for the thermocycler were: 90 °C for 1 min, 50 or 55 °C for 1 min, and 72 °C for 2 min. The thermocycler was programmed to run for 30 or 35 cycles.

Initial concentration of PCR reagents in a 20 μL reaction were as follows: 20 or 25 μM MgCl₂, 40 mM dNTP, 10 mM primer, 50 to 100 ng/μL DNA, and 1 U Taq polymerase.

PCR products were then purified using a QIAgen PCR Purification Kit (QIAgen, Santa Clarita, CA). Concentration of the purified samples was determined by running the samples on a 1% agarose gel alongside a low DNA mass ladder (Gibco BRL, Gaithersburg, MD). The purified samples were then submitted to Oregon State University’s Central Services Lab for sequencing. The PCR products were sequenced using an Applied Biosystems 377 DNA sequencer (Perkin-Elmer/ABI, Foster City, CA).
Table 1. Primers used in the amplification and sequencing of the ssu rDNA sequence of *Nadelspora canceri*. Primers 530f, 530r, and pmpl are pan-microsporidian primers (Dowd et al. 1999; Weiss and Vossbrinck 1999). Primers 918f and 918r were created based on an alignment of microsporidian ssu rDNA sequences available in Genbank. Primer 1406r is a universal primer obtained from S. Giovannoni (Oregon State University, Corvallis OR, USA; Vergin, K., pers. commun.).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence of primer (5’ to 3’)</th>
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<tr>
<td>Forward Primer</td>
<td></td>
</tr>
<tr>
<td>pmpl</td>
<td>CAC CAG GTT GAT TCT GCC TGA</td>
</tr>
<tr>
<td>918f</td>
<td>GCT TAA TTT GAC TCA ACG CGG</td>
</tr>
<tr>
<td>530f</td>
<td>GGT GCC AGC AGC CGC GGT AA</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td></td>
</tr>
<tr>
<td>1406r</td>
<td>ACG GGC GGT GTG TAC</td>
</tr>
<tr>
<td>918r</td>
<td>CCG CGT TGA GTC AAA TTA AGC</td>
</tr>
<tr>
<td>530r</td>
<td>TTA CCG CGG CTG CTG GCA CC</td>
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Phylogenetic Analysis. The *N. canceri* sequences were compared to 19 other microsporidian ssu rDNA sequences available in GenBank. The sequences were aligned using Pileup (default gap weight of 5) followed by manual editing. The final alignment was then subjected to distance methods (Kimura-2 parameter model for estimating nucleotide change with the Neighbor Joining algorithm) in the Wisconsin Package (GCG version 10, Madison, WI). The reliability of the distance tree was tested using bootstrap analysis. The alignment was also analyzed by parsimony methods using PAUP* (Swofford, 1998). A tree was created using a heuristic search and the result tested by bootstrap analysis.

2.4 Results

The ssu rDNA was sequenced from three spore samples. Sample NC1 was obtained from an infected Dungeness crab, and samples NC2 and NC3 were obtained from two separate red rock crabs. Sequence comparison between samples from both species of crabs showed the sequences to be 100% identical (1,081/1,081 identical nucleotide positions). The high sequence identity was reflected in Figure 1, where all three sequences clustered together with high bootstrap values of 97 (for parsimony) and 98 (for distance-based methods).

The phylogeny of the sequences relative to other microsporidians was also inferred using both distance and parsimony-based methods. Both methods showed the *N. canceri* sequences to be most closely related to *Ameson michaelis* (Figure 1). The
grouping of the *N. canceri* sequences to *A. michaelis* was supported by bootstrap values of 100. Sequence comparison between the *N. canceri* sequences and *A. michaelis* showed 93% sequence identity among 1,090 nucleotide positions (1,001/1,081). Baker and colleagues classified members of the phylum Microspora into 4 distinct groups: *Vairimorpha/Nosema, Encephalitozoon, Endoreticulatus,* and *Icthyosporidium* (Baker et al. 1995). Both trees generated in this study incorporating *N. canceri* show these four groups (Figure 1).

In order to determine whether spore shape might be a homologous trait, microsporidians with rod-shaped spores (*Culicosporella lunata* and *Bacillidium sp.*) were included in the trees (Figure 1). *Culicosporella lunata* was grouped with the *Amblyospora* sequences, and *Bacillidium sp.* was grouped with the *Thelohania* sequences. In contrast, the *N. canceri* sequences were positioned with members of the *Icthyosporidium* group, suggesting *N. canceri* developed its needle-shaped spore independently from the other rod-shaped microsporidians. Comparing the morphology of rod shaped spores to the morphology of the *Icthyosporidium* group also yielded no unifying trait (Table 2). However, the positioning of *N. canceri* in the *Icthyosporidium* group suggests the primary host of *N. canceri* is a crustacean.
Fig. 1. Distance and parsimony tree showing the position of the *Nadelspora canceri* ssu rDNA sequences relative to other members of the phylum Microspora. Samples in this study are shown in bold as NC1, NC2, and NC3. Microsporidians having rod-shaped spores are also shown in bold. *Giardia ardeae* was used as the outgroup. Analysis by Distance Methods used the Kimura-2 parameter model (for estimating nucleotide change) and the Neighbor-Joining algorithm. The most parsimonious tree was obtained using a heuristic search of the data set. Bootstrap values for both distance and parsimony trees were generated by 100 bootstrap resamplings of the data set. Bootstrap values from the distance tree are shown below the node, and bootstrap values from the parsimony tree are shown above the node. (Bootstrap values greater than 50 are shown on the tree.) I = *Nosema/Vairimorpha* group, II = *Encephalitozoon* group, III = *Endoreticulatus* group, and IV = *Icthyosporidium* group.
Figure 1.

NC1 (Dungeness crab)
NC2 (Red rock crab)
NC3 (Red rock crab)

Arnesonis michaelis

Icthyospordium sp.

Heterosporis anguillarum

Vairimorpha lymantriae

Vairimorpha necatrix

Nosema apis

Nosema bombycis

Encephalitozoon cuniculi

Encephalitozoon hellem

Enteroctozoon bieneusi

Nucleospora salmonis

Pleistophora sp.

Thelohanina solenopsae

Thelohanina sp.

Bacillidium sp.

Amblyospora californica

Amblyospora sp.

Culicosporella lunata

Vairimorpha sp. (S. richteri)

Giardia ardea
Table 2. Comparison of biotic characteristics of *Nadelspora canceri* to members of the *Icthyosporidium* group as defined by Baker and colleagues (1995). "Rod-shaped" microsporidians *Bacillidium* sp. and *Culicosporella lunata* were included in the table to detect for similarities to *N. canceri*. *Nosema bombycis* was included as an "outgroup" to the *Icthyosporidium* group. The table is a modification of a table created by Baker and colleagues (1995; Weiss and Vossbrinck 1999).

<table>
<thead>
<tr>
<th>Species</th>
<th>1ª</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ameson michaelis</em></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Icthyosporidium sp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Heterosporis anguillarum</em></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nosema bombycis</em></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Bacillidium sp.</em></td>
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<td></td>
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</tr>
<tr>
<td><em>Culicosporella lunata</em></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Needle-shaped crustacea</td>
<td></td>
<td></td>
<td>uninucleate</td>
<td></td>
<td>disporous</td>
</tr>
<tr>
<td>Oval crustacea</td>
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<td></td>
<td>uninucleate</td>
<td></td>
<td>octosporous</td>
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<tr>
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<td>diplokaryotic</td>
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<tr>
<td>Oval fish</td>
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<td>uninucleate</td>
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<td>polysporous</td>
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<tr>
<td>Oval insects</td>
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<td></td>
<td>diplokaryotic</td>
<td></td>
<td>disporous</td>
</tr>
<tr>
<td>Lanceolate insects</td>
<td></td>
<td></td>
<td>diplokaryotic</td>
<td></td>
<td>disporous</td>
</tr>
</tbody>
</table>

1ª: shape of spore  
2: host  
3: state of nucleus in sporogonic phase  
4: presence of sporophorous or parasitophorous vesicles in the life cycle  
5: sporogony
2.5 Discussion

Members of the phylum Microspora exhibit a diverse array of spore morphology. Some spores are bell-shaped, rod-shaped, and even bent to resemble a horseshoe (*Toxoglugea variabilis*) (Wittner and Wiess 1999). *Nadelspora canceri* is the only known microsporidian to have needle-shaped spores. These needle-shaped spores (10 µm X 0.25 µm) are rounded at the anterior end and taper to a point at the posterior end. This study was undertaken to determine the phylogeny of needle-shaped spores infecting Dungeness and red rock crabs. Through sequence comparison, this study also investigated the degree of relatedness between spores infecting Dungeness and red rock crabs.

In prior work conducted by Childers (1993), healthy Dungeness crabs were fed red rock crab muscle tissue containing *N. canceri*. The Dungeness crabs succumbed to infection, indicating *N. canceri* can infect both species of crabs. Dungeness crabs were also immersed in holding tanks containing free-floating *N. canceri* spores. Here, too, the crabs eventually succumbed to infection (Childers 1993). Both experiments imply *N. canceri* can infect both species of crabs and enter their host by ingestion. Sequencing the ssu rDNA of *N. canceri* samples from both Dungeness and red rock crabs revealed 100%
sequence identity among 1,081 nucleotide positions. The high sequence identity coupled with spores from red rock crabs being able to infect Dungeness crabs, as well as spores isolated from both species of crabs exhibiting identical spore morphology, would strongly suggest the same species of microsporidian is infecting both species of crabs. However, there have been no reports of needle-shaped spores infecting other crab species.

The high sequence identity among the 3 spore samples may also be due to the ssu rDNA sequence being highly conserved. To better resolve the degree of relatedness between spores infecting Dungeness and red rock crabs, more variable sequences (such as the internal transcribed spacer (ITS) region) and other informative sequences such as the lsu rDNA should be compared. DNA-DNA hybridization studies should also be investigated since microbiologists consider this technique important in defining a microbial species (Rossello-Mora and Amann, 2001).

Inferring phylogeny of *N. canceri* by both distance and parsimony-based methods showed the microsporidian to be most closely related to *A. michaelis*. Sequence comparison between *N. canceri* and *A. michaelis* showed the sequences to be 93% identical among 1,081 nucleotide positions (1,001/1,081). Both microsporidians infect crustaceans and lack parasitophorous and sporophorous vesicles in their life cycles (Table 2). However, *A. michaelis* is geographically isolated and morphologically different from *N. canceri*. *A. michaelis* infects blue crabs (*Callinectes sapidus*), a commercially important crab inhabiting the Gulf of Mexico and the United States Atlantic Coast. Unlike *N. canceri*, *A. michaelis* has spherical shaped spores with keratin filaments protruding from the exospore. Sequence data supports the two microsporidians being
related despite the two species being geographically isolated and morphologically
different. Each species will have to be described further in terms of life cycle and
physiology to allow for a more complete comparison. Sequence comparison using other
informative genes such as the lsu rDNA and DNA-DNA hybridization studies may be
used to investigate the degree of relatedness between the two species.

The positioning of *N. canceri* adjacent to *A. michaelis* also placed *N. canceri*
within the *Icthyosporidium* group. Most members of the *Icthyosporidium* group infect
either fish or crustaceans suggesting *N. canceri*'s primary host is a crustacean. Prior
work from Childers and colleagues (1996) had shown the parasite to be directly
transmitted by crabs participating in cannibalism or crabs ingesting free-floating spores in
the environment. Both modes of acquiring *N. canceri* suggest no other host is required to
transmit the parasite. The grouping of *N. canceri* with *A. michaelis* would also suggest
the crustacean-infecting microsporidians form a monophyletic group. However, in recent
research, several crustacean-infecting microsporidians were found not to group with *A.
michaelis*. A shrimp parasite characterized by Azevedo and colleagues (2000) was
grouped in the Thelohaniidae family (insect-infecting microsporidians). Not having its
ssu rDNA sequenced, the shrimp parasite was characterized and classified solely on
observed spore morphology. Cheney and colleagues (2000) inferred the phylogeny of
two crustacean-infecting *Pleistophora* species based on ssu rDNA sequence analysis.
One *Pleistophora* sequence grouped closely with *A. michaelis* (crustacean-infecting microsporidian) while the other sequence grouped closely with *Ichthysporidium* *sp.* (fish-infecting microsporidian). As new microsporidians continue to be discovered and described, the addition of these novel microsporidians’ ssu rDNA to the microsporidian gene tree will help determine if the crustacean-infecting microsporidians form a monophyletic group.

Sequences from *Bacillidium* *sp.* and *C. lunata* were included in the analysis to determine whether needle-shaped *N. canceri* spores would group closely with rod-shaped microsporidians. Both distance and parsimony trees positioned *Bacillidium* *sp.* with the *Thelohania* sequences, and *C. lunata* was positioned adjacent to the *Amblyospora* sequences. The rod-shaped microsporidians used in this study are grouped near the base of both distance and parsimony trees (Figure 1). In contrast, the *N. canceri* sequences are positioned in the *Ichthysporidium* group, suggesting *N. canceri* evolved its needle-shaped spore independently from *Bacillidium* *sp.* and *C. lunata*. However, the general question of whether rod-shaped microsporidians do form a monophyletic group still remains to be answered. A greater number of rod-shaped microsporidians will need to have their ssu rDNA sequenced, and their phylogeny inferred. So there still lies the possibility of the rod-shaped microsporidians forming a monophyletic group based on an ssu rDNA gene tree.
Based solely on spore ultrastructure, *N. canceri* has been grouped with other rod-shaped Microsporidian spores such as *Baculea* (Olson, Tiekotter, and Reno 1994). However, the issue of knowing which traits are due to homology and which traits are due to convergent evolution is a problem in classification. The absence of some traits in some spores as well as an incomplete description of many microsporidians’ life cycles further complicates classification. The use of genetic information from a highly conserved gene such as ssu rDNA is a helpful tool in resolving which traits are shared derived and which traits arose independently. The ssu rDNA sequence has universal conserved regions allowing comparison between domains, as well as regions of varying degrees of variability to register differences among related species (Woese 1996).

Based on ssu rDNA sequence analysis, the *N. canceri* spores were found to be most closely related to *A. michaelis*, an organism geographically isolated and morphologically different from *N. canceri*. Both parasites are pathogens to crustaceans and lack parasitophorous and sporophorous vesicles (Table 2). Sequence comparison between the *N. canceri* ssu rDNA sequences to each other suggests the spores infecting Dungeness crabs are the same species infecting red rock crabs.
2.6 Literature Cited


CHAPTER 3

ESTIMATING KARYOTYPE AND GENOME SIZE OF *NADELSPORA CANCERI* BY PULSED FIELD GEL ELECTROPHORESIS

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Submitted to The Journal of Eukaryotic Microbiology,
Dr. Denis H. Lynn, Editor-in-Chief, Department of Zoology,
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3.1 Abstract

Pulsed field gel electrophoresis (PFGE) was used to characterize the genome of the microsporidian *Nadelspora canceri*. *N. canceri* spore isolates obtained from Dungeness crab (*Cancer magister*) and red rock crab (*Cancer productus*) both yielded a karyotype of ten chromosome-sized DNA bands. Close inspection of the electrophoretic band patterns and band sizes showed a difference in genome size between the two isolates. Spore isolates from Dungeness crab had a genome size of 7.44 Mb while spore isolates from red rock crab had a genome size of 7.32 Mb. Previously characterized microsporidians have a genome size range of 2.9 to 19.5 Mb and a karyotype ranging from 7 to 18 chromosome-sized DNA bands. *N. canceri* falls well within the genome size range and karyotype number of known microsporidians. The difference in genome size observed between the two spore isolates suggests intraspecies chromosome-size polymorphism. However, analysis of the difference in band sizes using the Student’s t-test found the differences to not be significant.

**Key Words.** Chromosome-size polymorphism, microsporidia, protists.
3.2 Introduction

The microsporidian *Nadelspora canceri* is a pathogen of Dungeness (*Cancer magister*) and red rock crabs (*Cancer productus*). From previous research the organism has been described in terms of its geographic distribution, prevalence of infection, and spore ultrastructure (Childers et al. 1996; Olson et al. 1994). However, information describing *N. canceri*’s genome is lacking and warrants investigation. The phylogeny of *N. canceri* was recently investigated and based on small subunit (ssu) rDNA sequence analysis, the parasite was placed in Baker’s *Ichyosporidium* group (Amogan 2001; Baker et al. 1995). To further characterize the organism, we attempted to determine the karyotype and genome size of *N. canceri*.

The karyotype of other microsporidians has been determined with the number of chromosome-sized DNA bands ranging from 7 to 18 (Wittner and Weiss 1999). Intraspecies variations in karyotype, as well as chromosome size polymorphism have been detected in the phylum Microspora. Specifically, the microsporidian *Vavraia oncoperae* (isolated from porina caterpillar *Wiseana* spp. and grass grub *Costelytra zealandica*) was found to have karyotypes of 14 and 16 (Malone and McIvor 1993). The difference in band number may be due to failing to resolve certain DNA bands by pulsed field gel electrophoresis (PFGE), although three separate preparations of *V. oncoperae* samples were prepared and run on several pulsed field gels (Malone and McIvor 1993). The microsporidian *Encephalitozoon cuniculi*, also isolated from several different host organisms, exhibited variation in chromosome band patterns (Biderre et al. 1998).
Such variations (in karyotype and band patterns) are not limited to microsporidians, but have also been detected in both fungi and protists (Monaco 1995; Venegas et al. 1997). Variations in chromosome size and number may imply plasticity of the genomes and clonal reproduction of separate isolates.

In this study, pulsed field gel electrophoresis was used to estimate the genome size and karyotype of two *N. canceri* spore isolates. This study also looked into whether *N. canceri* exhibits plasticity in its genome.

3.3 Materials and Methods

**Collection of Infected Crabs.** A Dungeness crab containing *N. canceri* was donated for this study by Dr. Robert Olson (Coastal Oregon Marine Experiment Station, Oregon State University, Newport, OR). A red rock crab containing *N. canceri* was donated by a recreational fisherman in Yaquina Bay, Newport OR. Crabs were caught using baited crab pots and rings. Infected crabs were detected by examining for milky white or yellowish crab muscle tissue observed at the carapace-leg junction. Spore samples obtained from the Dungeness crab were identified as NC1 and spores obtained from the red rock crab were identified as NC2.
**Spore Purification.** To purify spores from crab muscle tissue, the infected tissue was minced with a razor blade and homogenized using a Dounce homogenizer. The homogenized sample was filtered through cheesecloth (2X) to remove large fragments of muscle tissue. The resulting filtrate was then centrifuged for 30 min at 1,000 g. A pellet was obtained and resuspended in 5 ml dH₂O. The sample was added to 30 ml of 70% Percoll and centrifuged for 1 hour at 1,000 g. Crab muscle tissue sedimented to the bottom of the centrifuge tube, and also formed a thin band above the 70% Percoll. Percoll lying between the thin band and pelleted crab muscle tissue was used for subsequent spore purification. Approximately 24 to 26 ml of Percoll was removed from the centrifuge tube and diluted to 40% Percoll using dH₂O. The diluted Percoll was centrifuged for 1 hour at 1,000 g. A pellet was obtained and resuspended in 250 µl of dH₂O. The 40% Percoll was centrifuged three more times to obtain a total of 1 ml of spores suspended in dH₂O. Spore concentration was then estimated using a hemacytometer. Sample NC1 had a spore count of $7.51 \times 10^7$ spores/ml and sample NC2 had a spore count of $8.64 \times 10^6$ spores/ml.

**Preparation of Plugs and DNA Extraction.** Prior to spore lysis, the spores were embedded in agarose plugs. The spore samples were first centrifuged for 30 min at 10,000 g. The pellets were then resuspended in 500 µl of 0.2 M KCl (pH 12) for 30 min.
at 30 °C. Approximately 500 µl of melted 1.6% low melting point agarose was added to the spore sample to give a final concentration of 0.8% agarose. The 0.8% agarose-spore mixture was immediately pipetted into several plug molds (Bio-Rad Inc., Hercules, CA) and allowed to solidify for 10 min at 4 °C.

To obtain spore DNA, the prepared agarose plugs were immersed in 2.5 ml of lysis buffer (1% lauryl sarcosine, 100 mM EDTA, and 10 mM Tris at pH 7.8). To this lysis buffer was added 125 µl of 20 mg/ml proteinase K to give a final dilution of 1 mg/ml proteinase K (Malone and McIvor 1993). The plugs were then incubated in a 50 °C water bath for 48 hours. After 48 hours the lysis buffer was replaced with 3 ml of 500 mM EDTA and incubated overnight at 4 °C. The 500 mM EDTA was then replaced with 50 mM EDTA and the plugs stored at 4 °C until ready to run pulsed field gel electrophoresis.

**Pulsed Field Gel Electrophoresis Conditions.** The following PFGE conditions were used to resolve the chromosome-sized DNA bands from samples NC1 and NC2 on 1% agarose gels (1 g agarose in 100 ml 0.5X TBE). Each PFGE condition was run at least twice.

<table>
<thead>
<tr>
<th></th>
<th>Condition 1</th>
<th>Condition 2</th>
<th>Condition 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Switch Time (Ramp)</td>
<td>45-100 sec</td>
<td>60 sec</td>
<td>85-100 sec</td>
</tr>
<tr>
<td>Voltage</td>
<td>200 V (6 V/cm)</td>
<td>200 V (6 V/cm)</td>
<td>200 V (6 V/cm)</td>
</tr>
<tr>
<td>Temperature</td>
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<td>14 °C</td>
<td>14 °C</td>
</tr>
<tr>
<td>Time</td>
<td>24 hours</td>
<td>24 hours</td>
<td>24 hours</td>
</tr>
</tbody>
</table>
To observe the PFGE results, the gels were stained with 0.5 µg/ml ethidium bromide (300 ml dH2O and 15 µl of 10 mg/ml ethidium bromide) for 30 minutes. The gels were rinsed in dH2O (2X) and results observed using a gel documentation device. To estimate the size of the chromosome-sized DNA bands, standard curves were created based on distance traveled by the *Saccharomyces cerevisiae* DNA markers (Bio-Rad Inc., Hercules, CA) run on each gel.

**Statistical Analysis.** Observed difference in mean band sizes between the two spore isolates were analyzed using the Student’s t-test (Sigma Stat 2000). Calculated P-values greater than 0.05 supported the observed differences in mean band size to not be significant. The standard deviation of each mean band size was calculated in Microsoft Excel. The coefficient of variance (CV) was calculated to compare the two spore isolates’ standard deviations. The CV was calculated using the equation: (SD/mean)100 (Daniel 1995).

**3.4 Results**

A total of ten chromosome-sized DNA bands were identified by pulsed field gel electrophoresis. Use of conditions appropriate for resolution of *Hansenula wingeii* chromosomes (Bio-Rad Inc., Hercules, CA) showed no additional chromosomes greater than 2,200 kb (kilobase) for either NC1 or NC2 (data not shown). Pulsed field conditions focusing on further separating the smallest DNA band (band 1, Table 3A.) also failed to
reveal any bands smaller than 225 kb (data not shown). Electrophoretic band patterns were fairly consistent for both samples under various PFGE conditions. Bands 1 and 2 were evenly spaced at the bottom of the gel, bands 3 to 7 were grouped closely together, and bands 8, 9, and 10 were well resolved near the top of the gel (Figure 2). The lack of resolution among bands 5 to 7 in Figure 2 implied another set of PFGE conditions was needed to separate DNA bands in the 565 to 680 kb range. Figure 3 shows improved resolution for bands 5 to 7 using Condition 2 (Materials and Methods). For sample NC1, bands 5 and 6 were still fairly close together and running the gel for an additional 4 hours did not improve resolution. The adjacent S. cerevisiae DNA marker showed good separation between bands that were 610 kb and 680 kb in size. Since there was good separation among DNA size markers in the 610 to 680 kb range, we felt we had optimized conditions for resolving bands 5 to 7.
Table 3A. Estimates of number and size (in Kb) of DNA bands obtained from *Nadelspora canceri* using PFGE. (NC1: spores isolated from Dungeness crab, NC2: spores isolated from red rock crab)

a.: only one measurement was taken.
b.: no band size variation.

<table>
<thead>
<tr>
<th>NC1</th>
<th>Condition 1</th>
<th>Condition 2</th>
<th>Condition 3</th>
<th>Average Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 1</td>
<td>Run 1 Run 2</td>
</tr>
<tr>
<td>1</td>
<td>225</td>
<td>230</td>
<td>268</td>
<td>335 340</td>
</tr>
<tr>
<td>2</td>
<td>260</td>
<td>265</td>
<td>340</td>
<td>385 385</td>
</tr>
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<td>3</td>
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<td>1915</td>
<td>1940</td>
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<td>1900 1760</td>
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</tbody>
</table>

Continues to next page.
Table 3A (Continued). Estimates of number and size (in Kb) of DNA bands obtained from *Nadelspora canceri* using PFGE.

(NC1: spores isolated from Dungeness crab, NC2: spores isolated from red rock crab)

a.: only one measurement was taken.
b.: no variation in band size.
Condition 3 Runs 2 and 2a gave identical results.

<table>
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<th>NC2</th>
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<th>Average Band Size</th>
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<td>Run 1</td>
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<tr>
<td>10</td>
<td>1860</td>
<td>1940</td>
<td>unresolved</td>
<td>unresolved</td>
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</table>

7315.71 Kb
Table 3B. Student's t-test results investigating the significance of the difference in corresponding mean band sizes between NC1 and NC2. P-values greater than 0.05 support the difference in corresponding mean band sizes to not be significant.

c.: no p-value obtained due to one measurement for sample NC1.

<table>
<thead>
<tr>
<th>Band#</th>
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<tbody>
<tr>
<td>1</td>
<td>0.74</td>
</tr>
<tr>
<td>2</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>0.85</td>
</tr>
<tr>
<td>4</td>
<td>0.46</td>
</tr>
<tr>
<td>5</td>
<td>c.</td>
</tr>
<tr>
<td>6</td>
<td>c.</td>
</tr>
<tr>
<td>7</td>
<td>c.</td>
</tr>
<tr>
<td>8</td>
<td>0.76</td>
</tr>
<tr>
<td>9</td>
<td>0.78</td>
</tr>
<tr>
<td>10</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 3C. Coefficient of Variance (CV) values for NC1 and NC2. Both samples gave similar CV values supporting similar distributions around the means.

NA: not applicable due to no standard deviation values.

<table>
<thead>
<tr>
<th>Band #</th>
<th>CV for NC1</th>
<th>CV for NC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.83</td>
<td>17.43</td>
</tr>
<tr>
<td>2</td>
<td>18.87</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>5.7</td>
<td>5.53</td>
</tr>
<tr>
<td>4</td>
<td>5.41</td>
<td>4.92</td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
<td>4.08</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
<td>1.55</td>
</tr>
<tr>
<td>8</td>
<td>17.73</td>
<td>17.21</td>
</tr>
<tr>
<td>9</td>
<td>21.81</td>
<td>22.45</td>
</tr>
<tr>
<td>10</td>
<td>4.3</td>
<td>6.78</td>
</tr>
</tbody>
</table>
Fig. 2. Ethidium bromide stained gel of *Nadelspora canceri* DNA subjected to pulsed field gel electrophoresis. Lanes identified from left to right as 1-8: (1) *Saccharomyces cerevisiae* DNA marker, (2) Uninfected Dungeness crab muscle tissue subjected to spore lysis buffer, (3) NC1; *N. canceri* spores obtained from Dungeness crab, (4) Uninfected Dungeness crab muscle tissue subjected to spore purification protocol and spore lysis buffer, (5) *S. cerevisiae* DNA marker, (6) Uninfected red rock crab muscle tissue subjected to spore lysis buffer, (7) NC2; *N. canceri* spores obtained from red rock crab, (8) Uninfected red rock crab muscle tissue subjected to spore purification protocol and spore lysis buffer. Result obtained using Condition 1 in Materials and Methods. Electrophoretic band patterns for NC1 and NC2 appear similar. Gel shows separate conditions are needed to resolve DNA bands in the 565 to 680 kb range, and band 8 for sample NC2 appears to be a doublet. Absence of DNA in lanes 2, 4, 6, and 8 indicates crab DNA did not contribute to the observed microsporidian DNA bands.
Figure 2. Initial Chromosome Band Patterns of *Nadelspora canceri* Spore Samples Obtained from Dungeness and Red Rock Crabs
Fig. 3. Pulsed field gel showing resolution of DNA bands in the 565 to 680 kb range. Result obtained using Condition 2 (Materials and Methods). From left to right the lanes are labeled 1-3: (1) *Saccharomyces cerevisiae* DNA marker, (2) NC1: *Nadelspora canceri* spores obtained from Dungeness crab, (3) NC2: *N. canceri* spores obtained from red rock crab. Under Condition 2 both samples (NC1 and NC2) appear to have five chromosome-sized DNA bands in the 565 to 680 kb range. Band patterns for both *N. canceri* samples in the 565 to 680 kb range appear different suggesting variations in chromosome size.
Figure 3. Resolution of DNA Bands in the 565 to 680 Kb Range
In various pulsed field results (data not shown) band 8 appeared brighter and wider than the other bands and called into question whether the band was a singlet or a doublet. Various switch times to improve resolution in the 945 to 1,125 kb range did not further alter band 8. However, under one of the pulsed field conditions (Condition 3) there was a discernable difference in size between band 10 of NC1 and band 10 of NC2 (Figure 4). The bands differed in size by approximately 80.8 kb, suggesting variation in chromosome size between the two spore samples (Table 3A). To determine whether the observed difference in band size was significant, corresponding mean band sizes between NC1 and NC2 were tested using the Student's t-test. As noted on Table 3B, P-values for all analyzed band sizes were greater than 0.05. A P-value greater than 0.05 suggests there is no significance to the observed difference in corresponding mean band sizes.

The estimated chromosome size for bands 1 to 5 appeared to increase from conditions 1 to 3 (Table 3A). In a reciprocal situation, chromosome size for bands 8 and 9 appear to decrease from conditions 1 to 3 (Table 3A). This observed pattern may be due to the standard curves used to estimate the sizes of NC1 and NC2's DNA bands. The coefficient of variance (CV) was calculated to better compare the standard deviations (SD) of NC1 to NC2 (Table 3C). Since the means of corresponding bands are not equal, what was needed for comparison are measures of relative variation rather than absolute variation (Daniel 1995). As noted on Table 3C, the CV values for both NC1 and NC2 were similar, with bands 1, 2, 8, and 9 exhibiting the highest values.
Fig. 4. Pulsed field gel showing a difference in migration rate for band 10 of samples NC1 and NC2. Result obtained using Condition 3 (Materials and Methods). From left to right the lanes are labeled 1-3: (1) *Saccharomyces cerevisiae* DNA marker, (2) NC1: *Nadelspora canceri* spores obtained from Dungeness crab, (3) NC2: *N. canceri* spores obtained from red rock crab. The arrow shows the band of interest (band 10) showing a difference in migration rate. In comparison, all other bands from both spore samples are parallel to each other.
Figure 4. Detection of Variation in Chromosome Size
Based on PFGE, the karyotype of microsporidians ranges between 7 to 18 chromosome-sized DNA bands. *N. canceri* is at the low end of the karyotype spectrum with ten chromosome-sized DNA bands. The size range of *N. canceri*’s DNA bands ranges from 279.6 to 1,878.75 kb for NC1, and 290.14 to 1,798 kb for NC2 (Table 4). The estimated genome and chromosome size range of *N. canceri* is most similar to the microsporidian *Nosema costelytrae* (Table 4). *N. costelytrae* is an insect-infecting microsporidian parasitic to the grass grub *Costelytra zealandica* (Malone and McIvor 1993). The estimated genome size of known microsporidians ranges from 2.9 to 19.5 Mb. *N. canceri*’s estimated genome size is at the lower end of the genome size range, 7.32 to 7.44 Mb (Table 4).
Table 4. Estimates of chromosome number and genome size of microsporidians, with *Nadelspora canceri* shown in bold.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Karyotype</th>
<th>Chromosome Size Range (kb)</th>
<th>Genome Size (Mb)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Viarimorpha sp.</em></td>
<td>Insects</td>
<td>8</td>
<td>720 to 1,790</td>
<td>9.2</td>
<td>Delarbre et al., 2001; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Nosema costelytrae</em></td>
<td>Insects</td>
<td>8</td>
<td>290 to 1,810</td>
<td>7.4</td>
<td>Delarbre et al., 2001; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Nadelspora canceri</em></td>
<td>Crustacea</td>
<td>10</td>
<td>280 to 1,879</td>
<td>7.44</td>
<td>Present Work</td>
</tr>
<tr>
<td><em>Nadelspora canceri</em></td>
<td>Crustacea</td>
<td>10</td>
<td>290 to 1,798</td>
<td>7.32</td>
<td>Present Work</td>
</tr>
<tr>
<td><em>Encephalitozoon cuniculi</em></td>
<td>Mammals</td>
<td>11</td>
<td>217 to 315</td>
<td>2.9</td>
<td>Biderre et al., 1995; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Encephalitozoon intestinalis</em></td>
<td>Mammals</td>
<td>11</td>
<td>190 to 280</td>
<td>2.3</td>
<td>Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Encephalitozoon hellem</em></td>
<td>Fish</td>
<td>12</td>
<td>175 to 315</td>
<td>6.2</td>
<td>Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Spragea lophii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Delarbre et al., 2001; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Nosema furnacalis</em></td>
<td>Insects</td>
<td>13</td>
<td>440 to 1,360</td>
<td>10.2</td>
<td>Munderloh et al., 1990; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Nosema pyrausta</em></td>
<td>Insects</td>
<td>13</td>
<td>440 to 1,390</td>
<td>10.6</td>
<td>Munderloh et al., 1990; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Vavraia oncoperae</em></td>
<td>Insects</td>
<td>14</td>
<td>130 to 1,930</td>
<td>8</td>
<td>Malone and McIvor, 1993; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Spragea lophii</em></td>
<td>Fish</td>
<td>15</td>
<td>266 to 1,076</td>
<td>7.3</td>
<td>Amigo et al., 2002</td>
</tr>
<tr>
<td><em>Spragea lophii</em></td>
<td>Fish</td>
<td>15</td>
<td>271 to 1,120</td>
<td>7.3</td>
<td>Amigo et al., 2002</td>
</tr>
<tr>
<td><em>Glugea stephani</em></td>
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<td>15</td>
<td>340 to 2,654</td>
<td>16.8</td>
<td>Amigo et al., 2002</td>
</tr>
<tr>
<td><em>Vavraia oncoperae</em></td>
<td>Insects</td>
<td>16</td>
<td>140 to 1,830</td>
<td>10.2</td>
<td>Malone and McIvor, 1993; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Glugea atherinae</em></td>
<td>Fish</td>
<td>16</td>
<td>420 to 2,700</td>
<td>19.5</td>
<td>Delarbre et al., 2001; Wittner and Weiss, 1999</td>
</tr>
<tr>
<td><em>Nosema locustae</em></td>
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<td>18</td>
<td>139 to 651</td>
<td>5.3</td>
<td>D.A. Street, 1994</td>
</tr>
<tr>
<td><em>Nosema bombycis</em></td>
<td>Insects</td>
<td>18</td>
<td>380 to 1,500</td>
<td>15.3</td>
<td>Kawakami et al., 1994</td>
</tr>
</tbody>
</table>
3.5 Discussion

Pulsed field gel electrophoresis was used to determine the karyotype and genome size of *N. canceri*. Under various PFGE conditions, the karyotype was estimated to consist of ten chromosome-sized DNA bands. Although electrophoresis was carried out under conditions which should permit resolution of DNA bands in the 220 kb to 2,200 kb size range, some DNA bands may yet be unresolved due to co-migration. In this study, band 8 may have consisted of several DNA bands. The use of densitometry may help determine whether band 8 is a singlet or a doublet (Amigo et al. 2002, Blunt et al. 1997).

By altering the switch time, a variation in chromosome size was detected for samples NC1 and NC2. Band 10 for both samples differed in migration rate when a switch time ramp of 85 to 100 seconds was used (Condition 3, Materials and Methods). Band 10 from sample NC1 appeared to be approximately 80.8 kb larger than the corresponding band in sample NC2 (Figure 4, Table 3A). Close inspection of Figure 3 also shows a slight difference in migration pattern for bands 5 and 6 between samples NC1 and NC2. The differences in migration pattern were reflected in the estimated mean DNA band sizes (Table 3A). To investigate on the significance of the difference in corresponding mean band sizes, the estimated values were subjected to the Student’s t-test. Calculated P-values for all compared band sizes were greater than 0.05, suggesting observed differences between corresponding bands are not significant (Table 3B). No significance suggests the observed difference in mean band sizes may be unique to the samples analyzed. Condition 3 was run twice on the spore samples and in both runs
band 10 showed a difference in band migration. To determine whether \textit{N. canceri}
exhibits plasticity in its genome, a greater number of spore samples will have to be
analyzed by PFGE. For now, the statistical results support the observed differences to be
unique to the samples and not a representation of a population of \textit{N. canceri}. Variations
in the numerical values of corresponding DNA bands may also be due to the process of
estimating the DNA band size. For example, the variability in measuring the distance
each band migrated on a gel and repeating the process on a subsequent repeat run.

Had the statistics supported the observed differences in mean band size to be
significant, the data would then suggest \textit{N. canceri} exhibits plasticity in its genome.

Genome plasticity and intraspecies variation in karyotype have been documented in other
microsporidians. Genome analysis of \textit{E. cuniculi} isolates from three different strains
gave six different electrophoretic band patterns (Biderre et al. 1998). \textit{V. oncoperae},
isolated from two different host species (the porina caterpillar \textit{Wiseana spp.} and the grass
grub \textit{Costelytra zealandica}), gave karyotype values of 14 and 16 (Table 4). \textit{Spragea
lophi} isolates gave karyotypes of 12 and 15 and a genome size of 6.2 Mb or 7.3 Mb
(Table 4). Intraspecies variation in electrophoretic band patterns has also been detected
for some protists and fungi (Monaco 1995; Venegas et al. 1997). The protist \textit{Leishmania}
displays variation in band patterns due to chromosomal rearrangement, \textit{Trypanosome
cruzi} isolates from separate geographic areas give different band patterns implying clonal
reproduction, and the yeast \textit{Candida albicans} exhibits variation in chromosome number
and size which may vary among individual hosts (Malone and McIvor 1993; Monaco
The mechanism for inducing variations in chromosome size or number among microsporidians is not well understood. Malone and McIvor (1993) suggested that variations in DNA band patterns might be common among microorganisms with high reproductive rates. The possibility of unequal crossover during mitosis (assuming the microsporidian is diploid) may account for the variability in chromosome size. If sexual reproduction is occurring, then there is also the possibility of unequal crossover in meiosis. Subtelomeric deletions or insertions of repeat sequences may also account for the variability in chromosome size (Biderre et al. 1998; Monaco 1995).

The stability of the electrophoretic band patterns is also an intriguing question. If the organism *N. canceri* exhibits plasticity in its genome, then the band pattern should change over time. In a study by Biderre et al. (1998) clonal cultures of *T. cruzi* exhibited variations in electrophoretic band pattern over a 5-year period. In contrast, cultures of *E. cuniculi* failed to show any significant variation within the same 5-year period (Biderre et al. 1998).

As noted in Table 3A, cross-wise comparison of DNA band sizes from conditions 1 to 3 shows bands 1 to 5 increasing in size and bands 8 and 9 decreasing in size. The standard curves used to estimate DNA band size may have contributed to the observed pattern (Data not shown). Standard curves for Condition 1 had a slope of -0.0005 and a y-intercept of 2.21 or 2.36. In comparison, standard curves for Condition 2 had a slope of -0.0008 and a y-intercept of 2.45. The steeper slope and higher y-intercept value for Condition 2 could account for the observed pattern in band size between conditions 1 and 2. Standard curves for Condition 3 had a slope of -0.0005 (similar to Condition 1) and y-
intercept values of either 2.4 or 2.5. The y-intercept value of 2.5 would suggest the standard curve was shifted upward in the graph relative to the standard curves created for conditions 1 and 2. So the position of Condition 3’s standard curve on the graph may have resulted in bands 1 to 5 having the largest estimated DNA band sizes. What caused the standard curves for each condition to be unique may be the PFGE conditions themselves, each condition focused on resolving a certain size range of DNA.

Comparison of the 2 spore samples’ coefficient of variance (CV) showed the CV values to be similar. The similarity in CV values would support DNA from both samples gave similar band patterns. Indeed, comparison of overall band patterns between NC1 and NC2 in Figures 2, 3, and 4 shows the patterns to be almost identical. The similarity in band patterns would support the phylogeny study in stating the spore isolates from Dungeness and red rock crabs are closely related and are probably the same species. Bands 1, 2, 8, and 9 gave the highest CV values for both samples NC1 and NC2. The high CV values may be a reflection of these bands being most affected by the conditions used in PFGE. Condition 1 was used to obtain a general overview of the karyotype of *N. canceri*. This condition may have also been optimal in resolving bands 1 and 2. Condition 2 was used to resolve DNA in the 565 to 680 Kb range. Condition 2 focused on resolving bands 5, 6, and 7, with less emphasis in resolving bands 1, 2, 8, 9, and 10. Condition 3 focused on resolving band 8, and the high CV values for bands 8 and 9 are probably due to the third condition shifting the positions of these bands. Band 10 for
both spore samples gave low CV values suggesting band migration was not affected to the degree of bands 1, 2, 8, and 9. The high CV values for bands 1, 2, 8, and 9 may also be due to the standard curves used to estimate the DNA band sizes. Similar to the spore samples, the DNA size markers used to create the standard curves were also affected by the various PFGE conditions.

The estimation of *N. canceri*’s genome size and karyotype serves as a platform to further understand the molecular biology of the parasite. Our study has shown *N. canceri* spore isolates (obtained from two separate crab species) to have a karyotype of ten chromosome-sized DNA bands. The total genome size for *N. canceri* is estimated to be 7.32 to 7.44 Mb. However, some DNA bands may yet be unresolved by the method of PFGE. Genome information for members of the phylum Microspora is limited with only 15 microsporidians listed in Table 4. A much greater number of microsporidians will need to have their genomes investigated if patterns are to emerge. From the information listed in Table 4, the insect-infecting microsporidians widely vary in their karyotype, chromosome size range, and genome size. In contrast, the mammal-infecting microsporidians have a karyotype of 11 to 12 DNA bands, and a genome size range of 2 to 3 Mb. *N. canceri* is the first crustacean-infecting microsporidian to have its genome characterized, and it will be interesting to see whether other crustacean-infecting microsporidians have a similar karyotype, chromosome size range, and genome size.
3.6 Literature Cited


Sequencing and analysis of *N. canceri*'s ssu rDNA revealed spore samples from Dungeness and red rock crabs to have 100% sequence identity among 1,081 nucleotide positions. The high sequence identity, coupled with a similarity in spore morphology and an ability to infect two species of crabs, suggests spores isolated from Dungeness and red rock crabs belong to the same species. The ability to infect both Dungeness and red rock crabs would imply *N. canceri* also infects other crab species. However, the parasite has not been detected in other species of crabs inhabiting the Pacific Coast. Efforts were made to detect for *N. canceri* in green crabs, but none were detected (Reno, P., pers. commun.).

The ssu rDNA gene is a highly conserved sequence useful in obtaining a big picture profile of an organism's degree of relatedness. At most, a high sequence identity concerning the ssu rDNA gene would support sequences being obtained from members of the same genus. To better address the issue of whether needle-shaped spores infecting Dungeness and red rock crabs are the same species, more variable sequences (such as the internal transcribed spacer (ITS) region) and a set of several other genes (such as the lso rDNA) should be compared. Other techniques such as DNA-DNA hybridization would also be helpful in this inquiry. But to have identical ssu rDNA sequences from two spore populations is supplemental information in supporting one species of microsporidian infects two species of crabs.
Both distance and parsimony analysis showed *N. canceri* to be most closely related to *A. michaelis*. Both crustacean parasites lack parasitophorous and sporophorous vesicles, and sequence comparison showed 93% sequence identity (1,001/1,081 identical nucleotide positions). Both distance and parsimony trees also showed no relationship between *N. canceri* and microsporidians with rod-shaped spores (Figures 1). The absence of clustering implies the character (rod shape) arose independently among separate lineages in the phylum Microspora. However, our sample size of rod-shaped microsporidians is rather small so a gene tree including a greater number of rod-shaped spores may better address this inquiry.

Pulsed field gel data complemented the sequence data by showing isolates from Dungeness and red rock crabs to have similar DNA band patterns (Figure 2). The karyotype of both isolates was estimated to be ten chromosome-sized DNA bands. The size range of *N. canceri*’s chromosome-sized DNA bands was most similar to *N. costelytrae*, an insect-infecting microsporidian (Table 4). Improved resolution of the pulsed field gels suggested chromosome-size variation between spore isolates from Dungeness and red rock crabs (Figures 3 and 4). A variation in band migration was detected for bands 5, 6, and 10 with band 10 showing the most pronounced difference in size. Analysis of the corresponding mean band sizes using the Student’s t-test did not support the size differences to be significant (Table 3B). So the difference in band migration observed for band 10 (in Figure 4) may be unique to the samples analyzed. To help determine whether *N. canceri* as a species exhibits variations in band profile, a greater number of spore samples will have to be analyzed by PFGE. For the samples
analyzed in this study, t-test analysis of the numerical difference in corresponding band size (as shown in Table 3B) would also suggest the difference arose from variability in estimating DNA size. Had t-test analysis supported the difference in band size to be significant, then the *N. canceri* samples would be stated to exhibit plasticity in their genomes. Variations in chromosome size and DNA band patterns have been detected in other microsporidians such as *S. lophii* and *E. cuniculi*. Events such as subtelomeric additions or deletions and unequal crossing-over during mitosis (assuming the organism is diploid), may explain the variations.

An investigation into *N. canceri*’s phylogeny, karyotype, and genome size provides a platform for further understanding the organism. Among further studies to be considered the following may be suggested. Based on *N. canceri*’s ssu rDNA sequence, probes can be created to help further characterize the organism. The organism may have an alternate host (such as a fish since *N. canceri* is clustered in the Icthyosporidium group), the organism may exhibit a different spore morphology if it infects tissue other than skeletal muscle tissue, and the organism may also reside in the freshwater tributaries feeding the bays and estuaries where they tend to be found. Understanding the life cycle of the parasite will help further describe *N. canceri* and allow for a better comparison to *A. michaelis*. 
Besides investigating on the degree of relatedness among spores infecting Dungeness and red rock crabs, sequencing of the ssu rDNA internal transcribed spacer (ITS) region should also be conducted to determine whether various strains of the parasite exist. PFGE results may complement the sequence data by showing a unique electrophoretic band profile for each *N. canceri* strain. Our investigation into the genome size and karyotype of *N. canceri* focused only on two spore samples. The karyotype and genome size from a greater sample of *N. canceri* spores from different geographic regions should be investigated. Isolates from different geographic areas may exhibit the same karyotype, or there may be a degree of variation in chromosome size between isolates from separate sampling areas.

The parasite-host interaction should be investigated as has been done with *A. michaelis*. In a study conducted by Findley et al. (1981) blue crabs infected with *A. michaelis* exhibited a change in blood ion composition. Infected crabs were found to have a decrease in chloride and sodium concentration, and an increase in the concentration of potassium and free amino acids. Infection by *A. michaelis* also increased the amount of lactic acid in the host’s blood and muscle. *N. canceri* may also alter the blood ion composition of hosts Dungeness and red rock crabs in a manner similar to *A. michaelis*.

The study of *N. canceri* is currently in a descriptive phase. The organism was previously described in terms of its prevalence of infection among Dungeness crabs, its geographic distribution, and observed spore morphology in the host. The microsporidian has now been described from a molecular perspective in terms of inferring the organism’s
phylogeny, estimating its karyotype, and estimating its genome size. The organism can be described further as to its interaction with the crab host and whether various strains of the parasite exist. Once the organism has been described extensively, the investigations to explain the observed descriptions (such as creating a model for the organism’s life cycle or explaining how the spores infect the skeletal muscle despite entering crabs through the mouth) may be initiated.
BIBLIOGRAPHY


