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Isolation, identification and genetic characterisation of a microsporidium isolated from the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)

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By

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Abstract

Carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae) is an economically important pest, yet its biology and pest status on citrus in South Africa was, until recently, poorly understood. A study was initiated to determine the cause of collapse of a laboratory carob moth colony that was established to investigate the biology of carob moth on citrus and to develop integrated management strategies for the pest. An organism was isolated from deceased larvae and was morphologically identified as a microsporidium, based on transmission electron microscopy. Microsporidia are obligate intracellular parasites that have been found to infect almost all eukaryotes. Several *Nosema* species have been isolated from economically important insect pests, yet little genetic information is available from online databases for identification. Mature spores were recovered and measured using transmission electron microscopy. Spores were ovocylindrical with a wrinkled exospore, and had a length of 2.8 ± 0.02 µm and a width of 1.6 ± 0.04 µm. The identity of the microsporidium was confirmed by PCR amplification, sequencing and analysis of the regions encoding the ribosomal RNA. BLAST analysis of the different rRNA regions amplified showed that the microsporidium shared a 96 – 99 % identity with *Nosema* sp. M-Pr, *Nosema carpocapsae*, *Nosema oulemae*, *Nosema* sp. CO1, Microsporidium 57864, and *Nosema bombi*. Phylogenetic analysis of the SSU and LSU rRNA genes showed that the microsporidium clustered with the *Nosema* / *Vairimorpha* clade, supported by a bootstrap value of 100. The organisation of the RNA cistron was determined by PCR amplification using the primer set 18f and L1328r to be 5'-SSU-ITS-LSU-IGS-5S-3', which confirms the placement of the microsporidium within the *Nosema* / *Vairimorpha* clade. Because the BLAST results showed a close relationship with *Nosema carpocapsae*, a microsporidium infecting codling moth, the pathogenicity of the microsporidium was tested against codling moth by inoculating artificial diet with a high spore concentration of 1.1 x 10^7 spores/ml and a low spore concentration of

1.1 x 10^4 spores/ml. DNA was extracted from deceased larvae inoculated with the high concentration, and PCR of the SSU rRNA gene and bacterial 16S region was performed. Mortality in the high concentration experiment was significant ($p = 0.05$), but the cause of infection was determined to be a bacterium, through sequencing and BLAST analysis of the bacterial 16S rDNA. The bacterium shared a 99 % identity with *Bacillus cereus*. Percentage mortality ($p = 0.09$), larval mass ($p = 0.09$) and instar ($p = 0.24$) did not differ significantly between treatments in the low concentration experiment. DNA was extracted from the larvae and PCR amplification of the SSU rRNA gene was performed to determine whether microsporidia were present. No SSU bands were observed in any of the treatments and percentage mortality was not significant, thus it was determined that no infection occurred. This is the first study to report the genetic characterisation of a microsporidium isolated from carob moth and provides important genetic information for classification of microsporidia within the *Nosema* / *Vairimorpha* clade. It is also one of few studies in which the complete rRNA cistron of a species within the *Nosema* / *Vairimorpha* clade has been sequenced. The identification of a microsporidium from a laboratory colony of carob moth is important as it provides information about pathogens infecting the carob moth and constraints to carob moth rearing, which is useful for further studies on rearing carob moth and for establishment of a clean colony for research purposes.

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List of abbreviations

General

Bt	–	*Bacillus thuringiensis*
BLAST	–	Basic Local Alignment Search Tool
CTAB	–	Cetyltrimethyl ammonium bromide
DMF	–	Dimethylformamide
DNA	–	deoxyribonucleic acid
ddH_2O	–	Double distilled water
IPM	–	Integrated pest management
IC_{50}	–	Inhibitory concentration (50 %)
ID	–	Infective dose
IGS	–	Intergenic spacer
IPTG	–	Isopropyl β-D-1-thiogalactopyranoside
ITS	–	Internal transcribed spacer
LD_{50}	–	Lethal dose (50 %)
LT_{50}	–	Lethal time (50 %)
Ltd	–	Limited
LSU	–	Large subunit
NaCl	–	Sodium chloride
Na_2CO_3	–	Sodium carbonate
NCBI	–	National Center for Biotechnology Information
PCR	–	Polymerase chain reaction
Pty	–	Propriety
RNA	–	Ribonucleic acid
rRNA	–	Ribosomal Ribonucleic acid
SDS	–	Sodium dodecyl sulphate
SE	–	Standard error
SSU	–	Small subunit
TEM	–	Transmission electron microscopy
USA	–	United States of America
X-Gal	–	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Units and symbols

bp	–	Base pairs
χ^2	–	Chi-square
°C	–	Degrees Celcius
df	–	Degrees of freedom
g	–	Gram
kb	–	Kilobase pairs
µl	–	Microlitres

μM	–	Micromolar
mM	–	Millimolar
mg	–	Milligrams
mm	–	Millimetre
ml	–	Millilitre
min	–	Minute
M	–	Molar
%	–	Percentage
p	–	Test level
x *g*	–	Times gravity
V	–	Volts
v/v	–	Volume per volume
w/v	–	Weight per volume

Research outputs

Publications

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Chapter 1: Literature review

1.1 Microsporidia

"Microsporidia" is a term used for organisms belonging to the phylum *Microspora*, which contains over 187 genera and 1500 species (Corradi, 2015). They are obligate intracellular parasites with no active metabolic stages of the life cycle occurring outside of the host cells (Franzen & Müller, 1999; Garcia, 2002).

1.1.1 Origins and taxonomy

The origins and classification of microsporidia is summarised by Capella-Gutiérrez *et al.* (2012). Originally microsporidia were described as yeast-like fungi, but later were reclassified as Sporozoa, which are spore-forming protozoa. They were then considered to be one of the most primitive eukaryotes because they contain eukaryotic traits such as a true membrane-bound nucleus and an intracytoplasmic membrane system; chromosome separation occurs on mitotic spindles and polyadenylation occurs on mRNA. However, they also exhibit prokaryotic characteristics such as a 70S ribosome consisting of a 50S and 30S subunit. They lack true mitochondria and peroxisomes, they possess a simple version of the Golgi apparatus, the ribosomal RNA (rRNA) has no separate 5.8S subunit, which is present in eukaryotes, and they have a small genome which is much less complex than those of most eukaryotes. They were thought to be direct descendants of primitive eukaryotes that occurred before mitochondrial endosymbiosis, which was supported by early phylogenies based on rRNA and elongation factor genes (Vossbrinck *et al.*, 1987; Kamaishi *et al.*, 1996a, 1996b). This was disproved upon discovery of a genomeless mitosome, which is a relic version of mitochondria in microsporidia (William *et al.*, 2002). The function of the mitosome is unclear, however it is believed that it may play a role in iron-sulphur biogenesis, which is required for the formation of some proteins (Goldberg *et al.*, 2008; Corradi, 2015). Later

phylogenetic studies suggested that microsporidia were in fact related to fungi and the erroneous basal position was due to long branch attraction, which commonly affects sequences that are highly divergent (Gribaldo & Philippe, 2002). The fungal relationship is supported by the fact that both microsporidia and fungi produce spores containing chitin and trehalose, and undergo closed mitosis (chromosomes divide within an intact nucleus). Although it is agreed that microsporidia are related to fungi, the exact position of the microsporidia relative to fungi is unclear.

It is now believed that microsporidia adapted to a parasitic lifestyle by evolving complex mechanisms to invade their hosts and evade host defences, and that they lost characteristics that were no longer required as their hosts provided the necessary resources (Nakjang *et al.*, 2013; Corradi, 2015). They have developed mechanisms to utilise host energy and proteins and have lost many genes for metabolic products, resulting in reduced genome size (Nakjang *et al.*, 2013; Corradi, 2015). The cellular and molecular consequences of gene loss have been reviewed by Corradi (2015). Microsporidia can utilise a number of biochemical pathways. Microsporidian genomes contain homologs of genes encoding products involved in core activities for synthesis of proteins (transcription, translation, protein folding and trafficking) and products involved in propagation (DNA replication and repair, and control of the cell cycle). The majority of microsporidia contain gene homologs encoding products involved in core carbon metabolism (glycolysis and the trehalose and pentose phosphate pathways), and products necessary for spore formation and invasion apparatus (*O*-mannosyl, spore wall proteins, chitin synthase). The products that cannot be synthesized are acquired from the host by molecular transporters. Twelve types of molecular transporters have been discovered in microsporidia, allowing them to steal their hosts' cellular compounds. The genome reduction is significant as it has been linked with their amazing ability to spread rapidly within host cells and with their substantial sequence divergence. It has been proposed that the loss of

many proteins involved in DNA repair has allowed for more rapid cell divisions, confounding the host's immune response. This has also allowed the microsporidia to accumulate many mutations in their genome, explaining their extreme sequence divergence (Nakjang *et al.*, 2013; Corradi, 2015).

1.1.2 Morphology

The morphology of microsporidia varies between species, and can vary within a single organism. Most microsporidia undergo three stages in the life cycle: merogony, sporogony and an environmental phase (Franzen & Müller, 1999; Ardila-Garcia & Fast, 2012; Cali & Takvorian, 2014). During merogony, meronts and merozoites are formed, and during sporogony, sporonts, sporoblasts and mature spores are formed. The structure of each developmental stage differs. The morphology of different life stages is discussed by Cali & Takvorian (2014).

1.1.2.1 Meronts and merozoites

Meronts and merozoites are rounded or slightly irregular cells surrounded by a cell membrane, and are identical in morphology. They may contain one or many nuclei occurring in isolation or in pairs known as diplokarya. Golgi vesicles cluster either in a single area or multiple areas. The endoplasmic reticulum is weakly developed or non-existent, and occurs in the cytoplasm, along with many free ribosomes. As merogony comes to an end, an electron-dense substance begins to be deposited on the outside of the plasma membrane. Once fully covered it is called a sporont.

1.1.2.2 Sporonts and sporoblasts

The presence of sporonts indicates that the microsporidium is in the initial stages of sporogony. Sporonts are similar in morphology to meronts and merozoites, however they are

covered fully by electron-dense material. Like meronts, sporonts can have isolated or diplokaryon nuclei. Sporoblasts are sporonts that have undergone nuclear divisions and have accumulated a dense continuous outer coat. The formation of the extrusion apparatus begins and the formation of most of the organelles specific to spores is nearing completion.

1.1.2.3 Mature spores

Sporoblasts mature into spores that range from 1 to 40 μm in length. Spore size and morphology is generally conserved within a species, however spore polymorphism in different hosts or geographic regions can occur, and some species produce spores belonging to two distinct size classes (Vávra & Larsson, 2014). Most spores are oval to pyriform in shape, although rod-like, circular or horseshoe-shaped spores have been identified. The spore wall consists of two layers: an electron-dense, proteinaceous exospore, and an electron transparent endospore consisting of α-chitin. A plasma membrane lines the internal surface of the endospore. The outer layers enclose cytoplasm, nucleus, a posterior vacuole, polaroplast membranes and an extrusion apparatus (**Fig.1.1**) (Franzen & Müller, 1999). One to two spherical to ovoid shaped nuclei are present in the middle of the spore. The extrusion apparatus is made up of a coiled polar filament and its anchoring disc (Franzen & Müller, 1999). The number and arrangement of coils can be a diagnostic feature as this varies between genera and species (Franzen & Müller, 1999). When conditions inside the host are appropriate, the polar filament discharges through the thin anterior part of the cell, penetrates the host's cell and inoculates it with the infective sporoplasm (Franzen & Müller, 1999).

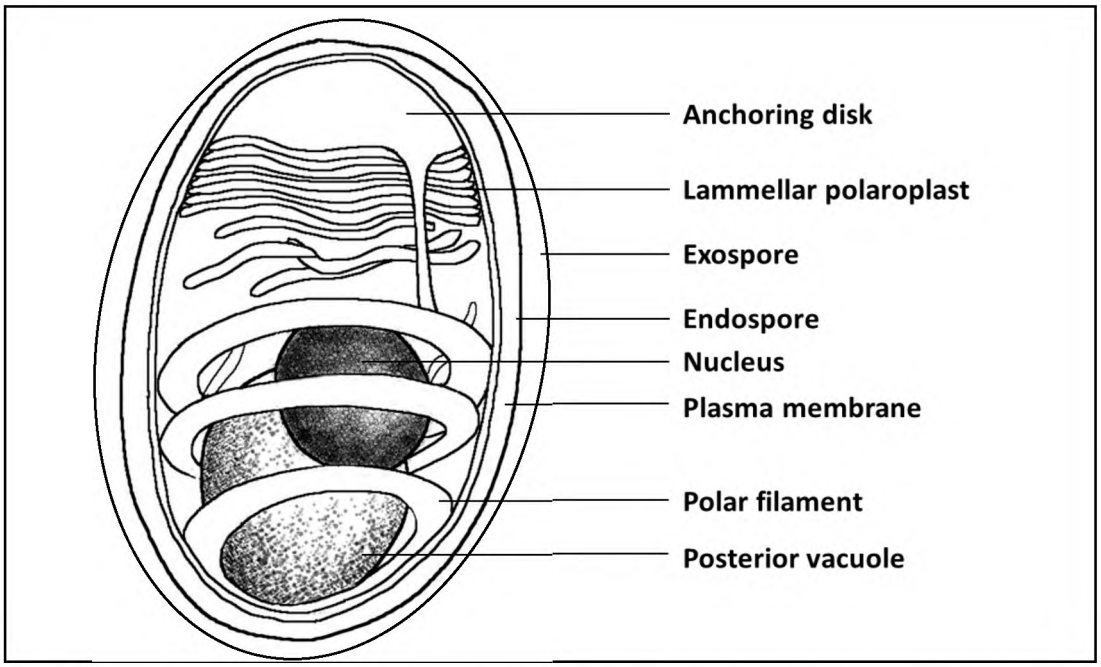


Fig.1. 1 General spore morphology of microsporidia (adapted from Franzen & Müller (1999)).

1.1.3 Life cycle and transmission

1.1.3.1 Life cycle

The life cycle of microsporidia is highly variable, but consists of at least three phases: the environmental phase, the proliferative merogonic phase, and the sporogonic phase (**Fig.1.2**) (Franzen & Müller, 1999; Ardila-Garcia & Fast, 2012; Cali & Takvorian, 2014). These processes may differ between species. The life cycle has been reviewed by Cali & Takvorian (2014) and is summarised below.

1.1.3.1.1 Environmental or infective phase

The first phase is the environmental or infective phase (**Fig.1.2.1**). This phase is the only phase occurring outside of the host. Mature environmental spores are taken up by the host, usually by ingestion. If conditions are appropriate, the spore germinates and is activated, causing the polar tubule to evert.

1.1.3.1.2 Merogony

Phase two, the merogonic phase, begins if the polar tubule pierces a host cell and injects its sporoplasm into it (**Fig.1.2.2**). During merogony, which occurs inside the host cell, meronts are formed. Merogony is repeated divisions by binary or multiple fissions. If nuclei divide without cell division, plasmodial forms with multiple nuclei are formed.

1.1.3.1.3 Sporogony

Phase three results in the development of meronts into sporonts that have a dense surface coat, which becomes the exospore (**Fig.1.2.3**). Sporonts multiply by binary or multiple fission and divide to form sporoblasts which will develop into mature spores once spore organelles are synthesised. Some divide directly into sporoblasts by binary fission, while others become multinucleated plasmodial forms. Sporogony results in the formation of spores containing a polar tubule, which is used to inject the infectious contents of the spore into the host cell. The spores have the ability to withstand harsh environmental conditions, and can remain infectious for several years. The spores are released into the environment, and the infectious cycle continues.

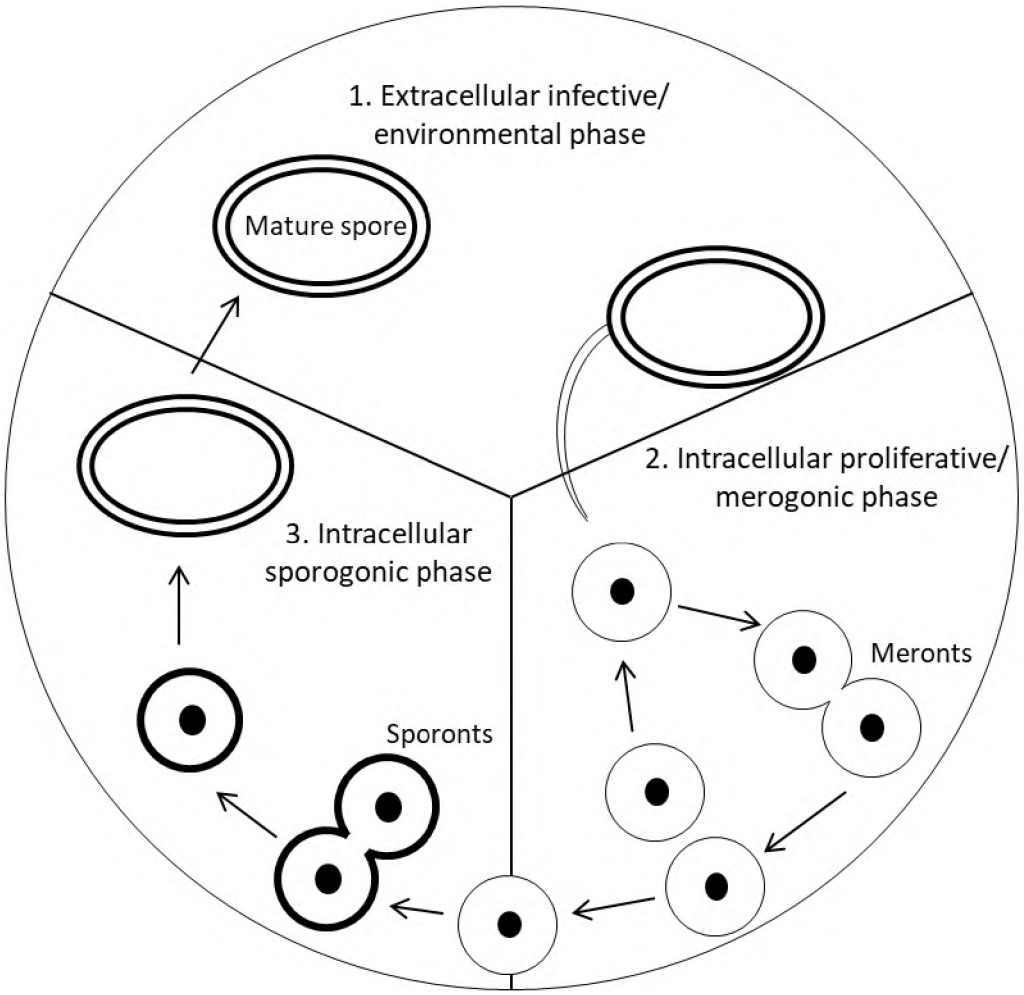


Fig.1. 2 A representative diagram of a generalised life cycle of microsporidia (adapted from Cali & Takvorian (2014)).

1.1.3.2 Transmission in invertebrates

Modes of transmission have been reviewed by Cali & Takvorian (2014). Both horizontal and vertical modes of transmission have been reported in microsporidia. Horizontal transmission is the most common mode in microsporidia infecting invertebrates, occurring either directly from one host to another or from an infected host to a new host via the environment. Spores from an infected host can be released into the environment via body exudates such as faeces and silk, through degradation of an infected host cadaver or through cannibalism. Some species of microsporidia are transmitted vertically from infected females to their offspring.

Vertical transmission is the direct transfer of a parasite from the parents to the offspring, and is an important pathway of transmission in many species of microsporidia infecting insects. Two routes of vertical transmission are transovum and transovarial transmission. Transovum transmission occurs when the microsporidium is transmitted from the mother to the offspring on the egg surface. Transovarial transmission is an important vertical transmission route whereby microsporidia in the cytoplasm of the host's ova are transmitted to the offspring during reproduction (Dunn *et al.*, 2001; Becnel & Andreadis, 2014). Transmission usually occurs via the maternal host because sperm cells are much smaller than ova, and rarely contribute to the zygote's cytoplasm (Dunn *et al.*, 2001).

1.1.4 Identification and classification methods

Ghosh *et al.* (2014) describes methods of identifying and classifying microsporidia. Traditional methods of species classification of microsporidia are based mainly on biological characters, microsporidium-host relationships and life cycle characteristics viewed using a light microscope or transmission electron microscope (TEM). Biological characters include spore morphology and development, and presence, length and number of coils of the polar tubule. Relying on morphological classification methods can be problematic and unreliable because often all stages of the life cycle are not present in samples, the microsporidium is present at very low concentrations that go undetected by TEM or there is variation in the number of coils made by the polar tubule.

Molecular methods of identification and classification of microsporidia tend to be more beneficial because they are more sensitive, specific and unbiased than traditional microscopy-based methods. Polymerase Chain Reaction (PCR)–based methods for identification and classification usually focus on the small subunit (SSU) rRNA gene, internal transcribed spacer (ITS) and large subunit (LSU) rRNA gene, which make up part of the region encoding

the ribosomal RNA cistron. These regions contain both conserved and variable regions. Sequence information on the microsporidian rRNA regions of certain groups is abundant in online sequence databases such as GenBank, allowing for the development of universal primers that work on numerous species of microsporidia, enabling identification of previously uncharacterised species. Other genomic markers have been proposed and successfully used as potential DNA barcodes in microsporidia research e.g. the largest subunit of RNA polymerase II (RPB1) (Hirt *et al.*, 1999), alpha- and beta-tubulin (Keeling, 2003), polar tubule proteins (Delbac *et al.*, 2001), and elongation factors EF-1α (Kamaishi *et al.*, 1996a) and EF-2 (Kamaishi *et al.*, 1996b).

1.1.5 Genus *Nosema*

Approximately 187 genera of microsporidia have been discovered consisting of more than 1500 species. The genus *Nosema* is one of the most important microsporidia in arthropods, especially in lepidopteran hosts. Most species within the genus *Nosema* are parasites of invertebrates, and are commonly found to infect Lepidoptera (Franzen & Müller, 1999). The development of the majority of *Nosema* species occurs in direct contact with the host cell cytoplasm, and nuclei are paired throughout the life cycle (Franzen & Müller, 1999). The infection cycle of *Nosema* species has been described by Maddox *et al.* (1998) (**Fig.1.3**). The genus *Nosema* is characterised by formation of two non-enveloped spores from each sporont. Only one type of infectious spore is produced by most *Nosema* species, and this spore contains two nuclei. Most of the host tissues are infected (namely, the midgut, Malpighian tubules, fat body, salivary glands, muscles and gonads), and tissues are usually infected sequentially. Spores are released from infected living hosts through frass or silk, or through deceased hosts by decomposition. *Nosema* species are moderately pathogenic as relatively few spores are required to initiate infection, but higher doses are usually required to induce larval mortality. Infected larvae may develop into infected adults, and infected females

usually transmit infection to their offspring. Transmission can be both horizontal and vertical. Transovarially infected larvae result from infection of the embryo within the egg, and often have higher mortality rates. Infected larvae develop more slowly and their behaviour, such as movement and responses to stimuli, may be affected. The mating efficiency is often reduced due to decreased pheromone production, sperm production and transfer. *Nosema* species can infect all developmental stages of their hosts.

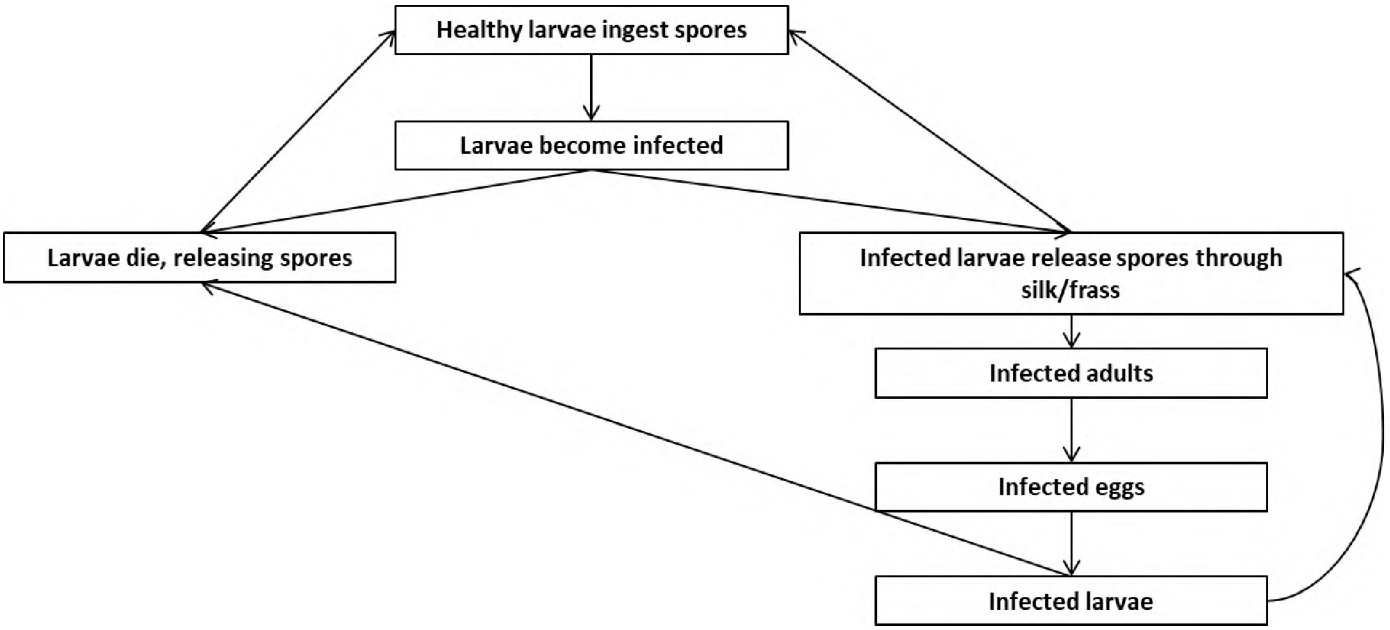


Fig.1. 3 Diagrammatic representation of the infection cycle of *Nosema*-type microsporidia (adapted from Maddox *et al.* (1998)).

1.1.6 Hosts

Microsporidia are parasitic in all major groups of animals, both vertebrates and invertebrates (Franzen & Müller, 1999; Stentiford *et al.*, 2016). Microsporidia were first recognised as pathogens in silkworms by Nageli (1857), and now have been found in numerous hosts such as insects, fish, crustaceans, reptiles, amphibians, arachnids and mammals (Franzen & Müller, 1999; Stentiford *et al.*, 2016). Symptoms of microsporidiosis differ depending on the species of the microsporidium and the host (Ardila-Garcia & Fast, 2012). Some infections

can be acute or chronic, can infect single or multiple tissues, can result in mild symptoms or can lead to death (Ardila-Garcia & Fast, 2012). Some species of microsporidia cause different symptoms and infect different tissue types in different hosts (Ghani *et al.*, 2013).

The first case of a human infection was reported in 1959. Since then cases have been reported globally, and microsporidia are considered to be the most common pathogens in HIV-infected patients (Franzen & Müller, 1999; Garcia, 2002). Microsporidia cause a wide range of infections in humans, including sinus, ocular, renal, intestinal and muscular infections (Reddy *et al.*, 2011). They appear to be opportunistic pathogens, causing disease mainly in immunosuppressed patients such as those infected by HIV or transplant patients receiving immunosuppressant drugs (Fedorko *et al.*, 1995; Franzen & Müller, 1999; Garcia, 2002; Reddy *et al.*, 2011). However, they have been found in immunocompetent individuals (Fedorko *et al.*, 1995; Reddy *et al.*, 2011; Corradi, 2015). Seven genera are known to infect humans, namely *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Pleistophora*, *Vittaforma*, *Trachipleistophora* and *Brachiola* (Franzen & Müller, 1999; Garcia, 2002).

Microsporidia are ubiquitous, and are hosted by all major groups of animals (Franzen & Müller, 1999; Stentiford *et al.*, 2016). It is impossible to create an exhaustive list of the diverse hosts of microsporidia, however examples of economically important hosts have been collated (**Table 1.1**).

1.1.6.1 Lepidopteran hosts

Microsporidia have been found in nearly all insect groups and play an important role in regulating insect population densities (Becnel & Andreadis, 2014). Microsporidia are especially common in lepidopteran hosts. The microsporidium in this study was isolated from carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). Microsporidia with a lepidopteran as the type host are found in the genera *Cystosporogenes*, *Nosema*,

Orthosomella and *Vairimorpha*, however the most commonly encountered entomopathogenic microsporidia belong to the genus *Nosema* or to the genus *Vairimorpha* (Becnel & Andreadis, 2014).

Table 1. 1 Collated list of species of microsporidia and the hosts in which they are found

Host	Microsporidium	Reference
Agriculture		
Broiler chicken, *Gallus gallus domesticus*	*Enterocytozoon bieneusi*	(Reetz *et al.*, 2002)
Cattle, *Bos Taurus*	*Encephalitozoon cuniculi*	(Halánová *et al.*, 1999)
	Enterocytozoon bieneusi	(Fayer *et al.*, 2012)
Goat, *Capra aegagrus hircus*	*Encephalitozoon cuniculi*	(Khanna & Iyer, 1971)
Horse, *Equus caballus*	*Encephalitozoon cuniculi*	(Patterson-Kane *et al.*, 2003)
	Enterocytozoon bieneusi	(Santín *et al.*, 2010)
Pig, *Sus scrofa*	*Enterocytozoon bieneusi*	(Reetz *et al.*, 2009)
	Encephalitozoon cuniculi	
	Encephalitozoon intestinalis	(Valenčáková *et al.*, 2006)
Aquaculture		
Angelfish, *Pterophyllum scalare*	*Heterosporis finki*	(Schubert, 1969)
Anglerfish, *Lophius* spp.	*Spraguea lophii*	(Lom, 2002)
	Spraguea gastrophysus	
Antarctic king crab, *Lithodes sentolla*	*Areospora rohanae*	(Stentiford *et al.*, 2014)
Black tiger shrimp, *Penaeus monodon*	*Enterocytozoon hepatopenaeii*	(Tourtip *et al.*, 2009)
Chinese mitten crab, *Eriocheir sinensis*	*Hepatospora eriocheir*	(Stentiford *et al.*, 2011)
Crangonyx pseudogracilis	*Fibrillanosema crangonycis*	(Slothouber Galbreath *et al.*, 2004)
Deep-water lobster, *Metanephrops challenger*	*Myospora metanephrops*	(Stentiford *et al.*, 2010)
Dungeness crab, *Cancer magister*	*Nadelspora canceri*	(Olson *et al.*, 1994)
Edible crabs, *Cancer pagurus*	*Enterospora canceri*	(Stentiford *et al.*, 2007)
Eel, *Anguilla japonica*	*Heterosporis anguillarum*	(Seong *et al.*, 2007)
European shore crab, *Carcinus maenas*	*Ameson pulvis*	(Sprague, 1977)
Flatfishes, *Pleuronectes* spp.	*Glugea stephani*	(Olson, 1976)
Gammarus duebeni	*Nosema granulosis*	(Ironside *et al.*, 2003)
Gizzard shad, *Dorosoma cepedianum*	*Glugea cepedianae*	(Price, 1982; Canning & Lom, 1986)
Pike perch, *Stizostedion lucioperca*	*Glugea luciopercae*	(Canning & Lom, 1986)
Plaice, *Hippoglossoides platessoides*	*Pleistophora hippoglossoideos*	(Canning & Lom, 1986)
Rainbow smelt, *Osmerus mordax*	*Glugea hertwigi*	(Nepszy & Dechtiar, 1972)
Red sea bream, *Pagrus major*	*Kabatana seriolae*	(Lom & Dyková, 1992)
Salmonids	*Nucleospora salmonis*	(Kent *et al.*, 2014)
Salmonids in genus *Oncorhynchus*	*Loma salmonae*	(Kent *et al.*, 1995; Shaw *et al.*, 2000)
Sand smelt, *Atherina boyeri*	*Glugea atherinae*	(Canning & Lom, 1986)

Seabream, *Sparus aurata*	*Enterospora nucleophila*	(Stentiford *et al.*, 2016)
Sticklebacks, *Gasterosteus aculeatus*	*Glugea anomala*	(Dyková & Lom, 1978)
Turbot, *Scophthalmus maximus*	*Tetramicra brevifilum*	(Figueras *et al.*, 1992)
White-clawed crayfish, *Austropotamobius pallipes*	*Thelohania contejeani*	
Whiteleg shrimp, *Penaeus vannamei*	*Enterocytozoon hepatopenaeii*	(Tangprasittipap *et al.*, 2013)
Wolffish, *Anarhichas* spp.	*Pleistophora ehrenbaumi*	(Meyer, 1952)
Yellowtail, *Seriola quinqueradiata*	*Kabatana seriolae*	(Egusa, 1982)
Apiculture		
Bumblebees, *Bombus* spp.	*Nosema bombi*	(Fantham & Porter, 1914)
Honeybees, *Apis* spp.	*Nosema apis*	(Zander, 1909)
	Nosema ceranae	(Fries *et al.*, 1996)
Sericulture		
Eri silkworm, *Philosomia ricini*	*Nosema ricini*	(Chakrabarty *et al.*, 2012)
Muga silkworm, *Antheraea assama*	*Nosema assamensis*	(Chakrabarty *et al.*, 2012)
Mulberry silkworm, *Bombyx mori*	*Nosema bombycis*	(Nageli, 1857)
Tasar silkworm, *Antheraea mylitta*	*Nosema mylitta*	(Chakrabarty *et al.*, 2012)
Research and biological control		
Anaitis efformata	*Pleistophora schubergi*	(Briese & Milner, 1986)
Boll weevil, *Anthonomus grandis*	*Nosema* sp.	(McLaughlin, 1966)
Carob moth, *Ectomyelois ceratoniae*	Identified in this study	(Thackeray, 2016)
Eastern spruce budworm, *Choristoneura fumiferana*	*Cystosporogenes* sp.	(Van Frankenhuyzen *et al.*, 2004)
European corn borer, *Ostrinia nubilalis*	*Nosema pyrausta*	(Cossentine & Lewis, 1988)
Gypsy moth, *Lymantria dispar*	*Vairimorpha disparis*	(Solter & Hajek, 2009)
	Nosema lymantriae	
Mouse, *Mus musculus*	*Encephalitozoon cuniculi*	(Sak *et al.*, 2011)
	Encephalitozoon hellem	(Sebek & Weiser, 1989)
	Enterocytozoon bieneusi	
	Thelohania apodemi	
Muscidifurax raptor	*Nosema muscidifuracis*	(Geden *et al.*, 1995)
Oak processionary moth, *Thaumetopoea processionea*	*Endoreticulatus* sp.	(Hoch *et al.*, 2008)
Ocinara lida	*Endoreticulatus* sp.	(Wang *et al.*, 2005)
Rangeland grasshoppers	*Paranosema locustae*	(Lockwood *et al.*, 1999)
Spotted stem borer, *Chilo partellus*	*Nosema partelli*	(Kfir, 1997)
	Nosema bordati	(Bordat *et al.*, 1993)
Zebrafish, *Danio rerio*	*Pseudoloma neurophilia*	(Matthews *et al.*, 2001)

1.1.7 Importance of microsporidia in mass-reared organisms

The mass-rearing of organisms under controlled conditions is essential for scientific research and for many industries including agriculture, aquaculture, apiculture and sericulture. Microsporidia infect most major animal groups, making them a significant threat. Although infections can be sub-lethal, they can be detrimental as infection can decrease food consumption, prolong development, reduce fecundity, and damage tissues that are potentially important for human consumption (Stentiford *et al.*, 2016).

1.1.7.1 Agriculture

Species of microsporidia have been isolated from a number of livestock species, including cows, pigs, horses, goats and chickens (Snowden, 2014; Stentiford *et al.*, 2016). In the majority of cases no clinical impact on the host was observed, however some of the strains of microsporidia isolated were pathogenic to humans (Snowden, 2014; Stentiford *et al.*, 2016). The main concern relating to microsporidia in livestock is the potential for zoonotic transfer of pathogens to humans. Few studies have been conducted to determine the prevalence of zoonotic transfer from animals to humans, therefore the possibility needs to be investigated more comprehensively (Snowden, 2014; Stentiford *et al.*, 2016).

1.1.7.2 Aquaculture

The aquaculture industry has grown steadily over the years, and as of 2013 production values surpassed that of captured organisms (Food and Agriculture Organization of the United Nations (FAO), 2016). Approximately 73.8 million tonnes of fish were harvested from aquaculture worldwide, with an estimated value of US$162.2 billion (Food and Agriculture Organization of the United Nations (FAO), 2016). Most of the harvest is destined for human consumption.

The effect of microsporidia on aquaculture has been reviewed by Stentiford *et al.* (2016). Microsporidia tend to impact animals that are destined for human consumption directly, or indirectly by altering prey populations on which some organisms rely. Microsporidian infection in aquaculture is common, negatively affecting production and causing collapse of some commercial fisheries. The association between suboptimal environmental conditions in farms, relative immune suppression by pathogens or inbreeding, and the close proximity in which the animals are reared encourages microsporidiosis in aquaculture. *Enterospora nucleophila* infecting farmed seabream (*Sparus aurata*) suppresses the host's immune system, making it more susceptible to infections by other pathogens, and results in a disease known as emaciative syndrome (**Fig.1.4**). Decapod crustaceans such as shrimp, crab and lobster are an important part of aquaculture. Microsporidiosis in these organisms can cause the meat to be inedible or can make high value hosts visually unpleasing. *Enterocytozoon hepatopenaeii* intensely infects the shrimp species *Penaeus monodon* and *P. vannemei*, and is highly prevalent in mass-reared colonies. The microsporidium is associated with early mortality syndrome in shrimps.

Fig.1. 4 *Sparus aurata* **A**) Healthy individual. **B**) Individual experiencing reduced growth due to infection with *Enterospora nucleophila* (Palenzuela *et al.*, 2014).

1.1.7.3 Apiculture

Apiculture provides important products produced by bees such as honey and beeswax, as well as providing essential pollination services, without which crop yields would decrease dramatically (Stentiford *et al.*, 2016). Three species of microsporidia threaten the industry: *Nosema apis*, *N. ceranae* and *N. bombi*.

1.1.7.3.1 *Nosema apis* and *Nosema ceranae*

Nosema species in honeybees has been reviewed by Fries (2014). The honeybees, *Apis mellifera* and *A. cerana*, are the species mass-reared for honey production. *Nosema apis* and *N. ceranae* are the pathogens that cause nosemosis, a disease of honeybees. All life stages are infected, with symptoms that include dysentery (**Fig.1.5**), shortened lifespan and decreased colony sizes. *Nosema apis* was first discovered in the European honeybee, *A. mellifera* in 1909 (Zander, 1909). It is believed to have a narrow host range. *Nosema ceranae* was first discovered in the Asian honeybee, *A. cerana* (Fries *et al.*, 1996). *Nosema ceranae* is now considered to be the dominant cause of nosemosis. It has a broader host range than *N. apis*, infecting members of the genus *Apis* as well as some species within the genus *Bombus*, and is more virulent.

Fig.1. 5 Dysentery caused by infection with *Nosema apis* (Oliver, 2009).

1.1.7.3.2 *Nosema bombi*

Bumblebees (*Bombus* spp.) are mass-reared to provide pollination services. *Nosema bombi* was first isolated from bumblebees by Fantham & Porter (1914). van der Steen (2008) found that infection of *Bombus terrestris* by *N. bombi* had a significant negative impact on colony development and rearing. *Nosema bombi* infection decreased colony foundation, disturbed the metabolism of diseased queens and was transmitted to successive generations (van der Steen, 2008).

1.1.7.4 Sericulture

According to the International Sericulture Commission (2015), the major silk producing countries are China, India, Uzbekistan, Brazil, Japan, Republic of Korea, Thailand, Vietnam and Iran. In 2015, China and India produced 170 000 and 28 523 metric tonnes respectively, out of a total of 202 072.83 metric tonnes produced worldwide. A number of diseases threaten the industry, causing decreases in production. One such disease is pebrine, the disease caused by various *Nosema* species (Chakrabarty *et al.*, 2012). Species include *N. bombycis* isolated from the mulberry silkworm (*Bombyx mori*), *N. mylitta* isolated from the tasar silkworm (*Antheraea mylitta*), *N. ricini* isolated from the eri silkworm (*Philosomia ricini*) and *N. assamensis* isolated from the muga silkworm (*Antheraea assama*) (Chakrabarty *et al.*, 2012). Some of the *Nosema* species can develop in multiple hosts (Chakrabarty *et al.*, 2012). One of the symptoms of pebrine is melanisation (**Fig.1.6**).

Fig.1. 6 Melanisation caused by infection of *Bombyx mori* with *Nosema bombycis* (Pan *et al.*, 2013).

1.1.7.5 Role of microsporidia in rearing of animals for research

Healthy laboratory populations of species to be studied are required for research. Any abnormalities in the colony can influence the validity of the findings, and diseases can cause failure of colony establishment. In the 1960s and 1970s encephalitozoonosis was common in rodent test subjects, significantly interfering with experimental results (Snowden, 2014). The problem has diminished since greater precautions have been taken to prevent microsporidian infections (Snowden, 2014). Recently zebrafish, *Danio rerio*, have become important in biomedical research as biological models (Sanders *et al.*, 2012; Kent *et al.*, 2014). The microsporidium *Pseudoloma neurophilia* has been found to infect zebrafish reared in numerous research facilities (Sanders *et al.*, 2012). Insects are mass-reared for use in laboratory studies on genetics, physiology and behaviour, and for biological control (Knipling, 1966). Microsporidia can be a significant constraint in laboratory insect cultures, and often go undetected due to sub-lethal effects on the insects (Kfir & Walters, 1997; Rebelo, 2002). These cultures are often established for use in bioassays and mass-production of biological control agents, and contamination thereof may lead to distorted experimental results, biological control agents with limited fitness and introduction of novel microsporidia to release sites (Kfir & Walters, 1997; Rebelo, 2002).

1.1.8 Microsporidia in carob moth

Carob moth is one of the species from which a microsporidium has been isolated. Based on morphological evidence, the microsporidium isolated from carob moth by Lange (1991) is thought to belong to the genus *Nosema*, which is the genus that has been found to be the most common infecting Lepidoptera. Lange (1991) reported that infected larvae appeared to be more sluggish and stunted than healthy larvae, however no external symptoms could be relied upon to unequivocally distinguish between the infected and uninfected larvae. The larvae had

to be dissected and their tissues examined, with infected larvae having pale, hypertrophied Malpighian tubules. A laboratory colony of carob moth was initiated in South Africa in order to study the pest and develop integrated management strategies (Thackeray *et al.*, 2017). Individuals within the colony began to show symptoms of disease, and the colony collapsed (Lloyd *et al.*, 2017; Thackeray *et al.*, 2017). The cause of mortality was investigated in this study.

1.2 Carob moth, *Ectomyelois ceratoniae*

1.2.1 Distribution and host range

Carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is an economically important phytophagous pest with a wide distribution including the Mediterranean regions, southern Russia, Central and South America, Western Australia and Africa (Millar, 1990; Honiball & Catling, 1998). It infests a variety of fruit and nut crops in the field, as well as stored dried fruits, nuts and seeds (Millar, 1990; Blumberg, 2008). Economically important hosts include almonds, carobs, citrus, dates, figs, macadamias, pistachios, pecans, pomegranates and walnuts (Gothilf, 1969; Millar, 1990; Honiball & Catling, 1998; Mediouni & Dhouibi, 2007; Mozaffarian *et al.*, 2007; Blumberg, 2008; Norouzi *et al.*, 2008). Carob moth causes primary damage to its main hosts when larvae burrow into the fruit (**Fig.1.7**), as well as accentuating losses on crops such as peaches, apples, pears and apricots, where the primary damage is caused by another pest (Millar, 1990).

Fig.1. 7 Damage to a citrus fruit caused by *Ectomyelois ceratoniae* larvae (Thackeray, 2016).

1.2.2 Pest status and economic importance

The pest status of carob moth varies between hosts and regions. It is the most important and destructive pest of dates in Tunisia and Algeria, resulting in 10 to 40 % damage annually (Mediouni *et al.*, 2004; Mediouni & Dhouibi, 2007; Norouzi *et al.*, 2008). Carob moth is a serious pest of pomegranates in Tunisia, resulting in significant economic losses (Mediouni *et al.*, 2004; Mediouni & Dhouibi, 2007). Infestation rates in pomegranates can range from 20 to 80 % (Mediouni & Dhouibi, 2007). Carob moth is a major pest of dates in the United States of America, specifically the Coachella Valley in California, with infestation rates ranging from 2 to 10 % (Nay & Perring, 2005). In South Africa, carob moth is considered a minor pest of pecans, citrus, pomegranates and macadamias (Moore, 2012; Moore & Hattingh, 2017).

Carob moth was first recorded in South Africa in 1974, inhabiting damaged fruit from the Clanwilliam-Citrusdal region of the Western Cape (Honiball & Catling, 1998). Since then its distribution has spread to the Lowveld region of Mpumalanga Province and Limpopo Province, the Vaalharts region of the Northern Cape Province, the Eastern Cape Province and Western Cape Province (Honiball & Catling, 1998; Morland, 2015; Moore & Hattingh,

2017). Although carob moth is considered to be a minor pest in South Africa, research suggests that it can become a major pest if environmental conditions are favourable for rapid growth and reproduction (Mehrnejad, 2001; Morland, 2015). The potential impact of the pest on the South African citrus industry is especially of concern because South Africa is the second largest exporter of citrus worldwide, valued at approximately R11.5 billion per annum (CGA, 2016). The pest status and economic importance of carob moth in the South African citrus industry may require further research (Thackeray, 2016). Carob moth larvae are morphologically similar to the larvae of false codling moth (FCM), *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), which is a major pest of citrus in South Africa, and the two species can be confused (Honiball & Catling, 1998). Such misidentification can lead to confusion when determining levels of infestation.

1.2.3 Life history

Carob moth adults are small with wings that are creamy white to grey, brownish or dark brown in colour (**Fig.1.8.D**) (Catling, 1970; Blumberg, 2008). The wingspan is approximately 22 to 24 mm and the wings are marked with two oblique stripes (Blumberg, 2008). The body of the adult is 8 to 10 mm in length. Adults live for approximately 5 to 15 days (Gothilf, 1969). Adult moths are active at night and are inactive during daylight hours (Cox, 1979). Females exhibit calling behaviour which is initiated earlier as they age (Soofbaf *et al.*, 2007). After mating, females oviposit eggs during the twilight to dark hours, and the number of eggs oviposited varies with diet, temperature and host moisture content (Gothilf, 1969; Al-Izzi *et al.*, 1987; Nay & Perring, 2006).

Eggs are oviposited individually or in small clusters (Morland, 2015). They are approximately 0.7 mm in length and 0.05 mm in width, ovoid in shape and are white in the early stages after oviposition (**Fig.1.8.A**) (Morland, 2015). If the eggs are fertilised they turn

pink (Morland, 2015). The developmental time of eggs is temperature-dependent, ranging from 1 to 3 days at 30 °C to 8 days with a decline in temperature (Gothilf, 1969; Mediouni & Dhouibi, 2007).

Larvae are cream white to light pink in colour, with a brown head and front dorsum (**Fig.1.8.B**) (Blumberg, 2008). They usually undergo 5 instars that differ in length according to variations in light, temperature and nutritional quality of the diet (Gothilf, 1969; Mediouni & Dhouibi, 2007). Larvae do not tend to wander, entering the host where they hatch (Cox, 1979). The larval stage is the most detrimental to economically important hosts.

Pupation occurs at the feeding site of the larvae (Blumberg, 2008). Pupae are approximately 11 mm in length, are yellow to red-brown in colour and have darker ventral marking on the abdomen (**Fig.1.8.C**) (Morland, 2015). The pupal stage lasts approximately 7 days when reared at 30 °C and is not influenced by characteristics of the host (Nay & Perring, 2006; Mediouni & Dhouibi, 2007).

Carob moth can have as many as three to four generations annually (Blumberg, 2008). The life cycle of a single moth from egg to adult can range from 45 to 62 days, depending on the host and environmental conditions (Blumberg, 2008).

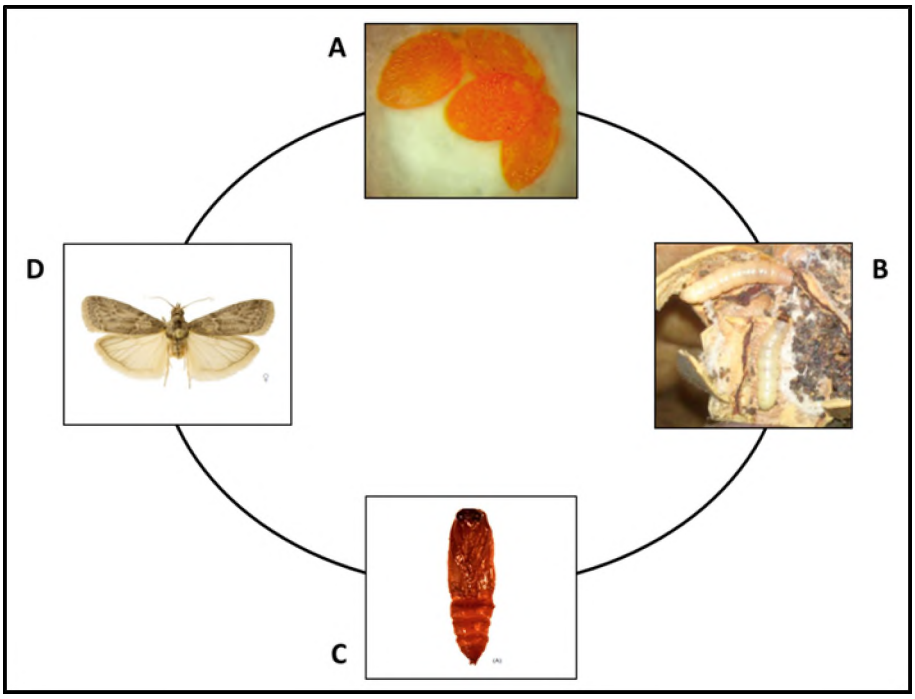


Fig.1. 8 Life cycle of the carob moth, *Ectomyelois ceratoniae*. **A)** Eggs are oviposited individually or in small clusters; **B)** Larvae undergo 5 instars before pupating at the feeding site, **C)** Pupation occurs, **D)** Adult moths emerge, mate and the cycle is repeated (photo credits: S. Thackeray and G. Morland).

1.2.4 Management

1.2.4.1 Population monitoring

Populations of carob moth can be monitored using pheromone trapping systems. Males are attracted to dispensers containing a female pheromone lure, 7, 9, 11-dodecatrien-1-formate, (7Z, 9E), and are consequently trapped, allowing for monitoring of male flight activity. Two pheromone dispensers are available for carob moth detection in South Africa: Chempac Carob moth lure (Paarl, South Africa) and Insect Science Carob lure (Tzaneen, South Africa) (Thackeray, 2016). No treatment thresholds are available for the carob moth as it is considered to be a pest of minor importance (Moore, 2012; Thackeray, 2016).

1.2.4.2 Chemical control

No chemical sprays targeting carob moth are registered in South Africa. Pesticides used for the control of FCM may affect carob moth populations inadvertently (Honiball & Catling, 1998). Thackeray (2016) found that two products registered for the control of FCM, Delegate® and Runner®, were able to reduce carob moth infestation in citrus orchards.

1.2.4.3 Biological control

Two species of parasitoids have been recorded in South Africa: *Phanerotoma ornatulopsis* De Seager (Hymenoptera: Braconidae) reared from acorns in the Citrusdal region, and *Phanerotoma carobivora* van Achterberg and Thackeray (Hymenoptera: Braconidae) found on pecan and citrus fruits (Honiball & Catling, 1998; Van Achterberg *et al.*, 2017).

1.2.4.4 Cultural practices

The recommended method of carob moth control on citrus in South Africa is orchard sanitation, which is the process whereby dropped fruit and infested fruit on the tree are removed and unharvested fruit remaining on the tree are removed before the next flowering season begins (Moore & Hattingh, 2017). This eliminates over-wintering sites, however other hosts in the vicinity can act as a reservoir (Mozaffarian *et al.*, 2007). The removal of alternative hosts of carob moth from areas in close proximity to orchards is also recommended (Moore, 2012).

1.2.4.5 Post-harvest control

Carob moth is on the list of phytosanitary pests when exporting citrus to China, which is one of the largest export markets for South Africa. Any indication of carob moth infestation results in suspension of acceptance of fruit from the infested orchard for the rest of the season (Thackeray, 2016). No post-harvest control protocols exist in South Africa, however

Thackeray (2016) found that compulsory post-harvest cold treatments for FCM were effective against carob moth larvae and suggested the phytosanitary status of the carob moth in citrus when exporting to China should be revised.

1.3 Problem statement

Carob moth is a pest of citrus, pomegranates and certain nuts in South Africa. The pest status of carob moth and its biology on citrus in South Africa needs to be evaluated. Consequently, a laboratory colony of carob moth was initiated in order to study its biology on citrus and to develop integrated management strategies for the pest, however the colony collapsed. The collapse was attributed to microbial infection as individuals within the carob moth culture started to exhibit symptoms such as discolouration, hypertrophied tissues and stunted growth. The cause of mortality needed to be identified before a new laboratory colony of carob moth could be initiated in order to implement strategies to prevent the infection from inhibiting colony establishment.

1.3.1 Overall aim

The aim of this research was to isolate, identify and genetically characterise the causal agent of the disease found in the culture of carob moth. This information will be invaluable for understanding the organism in question, and can aid the development and application of protocols to eradicate it from cultures and prevent further contamination.

1.3.2 Specific objectives

1. The first objective was to develop a method for extracting and purifying the organism infecting the laboratory culture of carob moth.
2. The second objective was to examine and determine the organism(s) and to characterize them morphologically

3. The third objective was to develop a method of extracting DNA from the organism to be used in downstream applications such as polymerase chain reaction (PCR)
4. The fourth objective was to synthesise and optimise specific primers for identification of the organism based on the primers for the small subunit (SSU) rRNA gene, internal transcribed spacer (ITS), large subunit (LSU) rRNA gene, intergenic spacer (IGS) and 5S rRNA gene
5. The fifth objective was to determine the organisation of the ribosomal RNA cistron
6. The sixth objective was to determine the susceptibility of codling moth to the microsporidium

Chapter 2 describes the initial isolation and detection of an organism from laboratory reared carob moth larvae in South Africa. The organism was believed to be a microsporidium based on preliminary detection and morphological analysis using transmission electron microscopy.

The initial diagnosis of microsporidian infection was confirmed in Chapter 3 by PCR amplification and sequencing of the ribosomal RNA cistron using microsporidia-specific primers. Furthermore, phylogenetic analysis showed that the microsporidium belonged in the *Nosema* / *Vairimorpha* group and that its closest relative was a *Nosema* species. The organisation of the rRNA cistron was established and determined to be the same as the rRNA organisation of most species of microsporidia.

In Chapter 4, the pathogenicity of the isolated microsporidium towards *Cydia pomonella*, the host of the microsporidium's closest available relative, was determined.

Chapter 5 is a general discussion of the results focusing on microsporidia in laboratory cultures. Recommendations are made for the improvements of the results obtained in this study and for future work.

Chapter 2: Initial microscopic identification of a pathogen isolated from carob moth, *Ectomyelois ceratoniae*

2.1 Introduction

Insects are reared for a variety of purposes. These include mass-rearing of insects for food and feed for humans and animals, apiculture, sericulture, for biological control and for laboratory studies on genetics, physiology and behaviour (Bjørnson & Oi, 2014; Stentiford *et al.*, 2016). One of the most significant problems encountered when rearing insects is microbial contamination (Sikorowski & Lawrence, 1994). The pathogen groups most often responsible for microbial contamination of insect cultures are viruses, bacteria, protists, fungi and nematodes. Microbial contamination can result in acute infections that cause significant mortality within cultures, or may produce sub-lethal effects within the host that lead to reduction in fitness (Eilenberg *et al.*, 2015). This chapter describes the initial identification of an organism isolated from carob moth, *Ectomyelois ceratoniae*.

A carob moth colony was initiated by Thackeray *et al.* (2017) at Citrus Research International (CRI), Port Elizabeth, South Africa, in order to study the basic biology and possible monitoring and control methods for the pest. Larvae for the colony were obtained from the Vaalharts region in the Northern Cape Province of South Africa. Individuals within the carob moth culture exhibited symptoms of entomopathogen infection, eventually leading to the collapse of the culture. The colony collapsed due to increased larval mortality. The cause of the increased larval mortality was unknown, but a viral infection was suspected.

Handling and diagnosis of diseased invertebrates is discussed by Lacey & Solter (2012) and is summarised below. Diseased organisms usually exhibit characteristic symptoms that are useful for initial diagnosis of disease. Some symptoms are overt and can be seen easily, while others go undetected due to their subtle nature. Symptoms that cannot be identified easily are

referred to as sub-lethal effects. Some common signs of disease are colour change, physical signs of an entomopathogen, abnormal changes in behaviour, changes in form and texture, and odour. A colour change is often one of the first symptoms of disease. Sometimes physical signs of infection can be observed, such as fungal growth on the host, lysis of the host, or hypertrophied tissue. Behavioural changes such as unusual dispersion or aggregation, or cessation of feeding are indicative of disease. Common changes in form and texture caused by microbial infection include mummification, liquefaction, and cadavers that are dry and leathery. A strong odour is usually associated with liquefied body tissues.

The procedure for extraction and purification of entomopathogens such as viruses, microsporidia and other protists is discussed by Eberle *et al.* (2012) and Solter *et al.* (2012) and is discussed in some detail below. Extraction and purification of entomopathogens such as viruses, microsporidia and other protists often involves maceration of symptomatic or dead insects in detergent to release the causal agent of disease from host cells. The resulting homogenate is filtered through cheese cloth to remove residues such as head capsules and pieces of integument. Various centrifugation and washing steps are undertaken to remove fat bodies and other contaminating particles. Further, more specific purification methods such as sucrose, glycerol or Percoll® gradients are utilised once the potential causal agent has been identified.

Transmission electron microscopy (TEM) is an important step in the identification of pathogens, based on ultrastructural characteristics (Curry *et al.*, 2006). It provides high resolution images and can be used to visualise organisms that are too small to be detected using traditional light microscopy techniques (Jahanzad *et al.*, 2011). An important advantage of using EM for diagnosis is that no prior knowledge of the organism to be viewed is required, and it does not require organism-specific reagents (Jahanzad *et al.*, 2011). Once the

organism has been morphologically identified using TEM, other more specific methods can be used to confirm the identity.

The aim of this chapter was to make an initial identification of the pathogen causing mortality in a carob moth colony (See Chapter 1). The specific objectives were to develop a crude extraction protocol and to observe the causal agent using transmission electron microscopy.

2.2 Materials and methods

2.2.1 Symptomatic larvae

Thackeray *et al.* (2017) initiated a carob moth colony at Citrus Research International (CRI), Port Elizabeth, South Africa, in order to determine appropriate rearing conditions and to study the basic biology and possible monitoring and control methods for the pest in South Africa. Larvae were reared either individually or in threes in 30 ml Polytop vials containing sterilised artificial diet including the anti-microbial agents Nipagin and sorbic acid, and sealed with a sterilised cotton wool plug. Larvae were reared at 25 ± 2 °C, 30 % relative humidity and 16:8 light:dark. Larvae that displayed signs of microbial infection were collected from the carob moth laboratory colony and frozen for analysis. Symptoms included swelling, liquefaction, colour changes and stunted growth (Thackeray, 2016).

2.2.2 Crude pathogen extraction

The method was modified from Hunter-Fujita *et al.* (1998). Symptomatic larvae were homogenised in 1 ml 0.1 % (w/v) SDS in a 2 ml plastic tube and were vortexed for 1 to 2 minutes. The tube was centrifuged at 100 x *g* for 5 to 10 seconds to pellet large debris. The supernatant was decanted into a new 2 ml tube. The pellet was resuspended in 1 ml 0.1 % SDS and the 100 x *g* spin was repeated. The supernatants from each 100 x *g* spin were combined and centrifuged at 10 000 x *g* for 20 minutes. The supernatant was discarded and

the pellet was resuspended in 1 ml double distilled water (ddH_2O). The resuspension was centrifuged at 10 000 x *g* for 20 minutes. The resultant supernatant was discarded and the pellet was finally resuspended in 150 µl ddH_2O.

2.2.3 Transmission electron microscopy

Spores were stained using a negative staining method modified from Fries *et al.* (1996). Approximately 5 µl of spores was added to a carbon grid for 1 minute. The excess was removed using filter paper and the grid was left to dry. A drop of 1 % (w/v) uranyl acetate was added to the grid and was left for 15 seconds. The excess was removed using filter paper and the grid was left to dry. The purpose of the uranyl acetate was to allow for viewing of the spore surface. Spores were viewed and measured using a Libra 120 (Zeiss, Germany) transmission electron microscope using Mega view (G2) Olympus analysis software.

2.3 Results

2.3.1 Symptomatic larvae

Infected larvae displayed a variety of symptoms included swelling, liquefaction, colour changes and stunted growth (**Fig.2.1**).

Fig.2. 1 Symptomatic carob moth larvae (photo credit: S. Thackeray).

2.3.2 Transmission electron microscopy of homogenate extracted from diseased carob moth larvae

Spore-like organisms were extracted from symptomatic carob moth larvae, and were analysed using TEM. Two types of organism were observed. The first type of organism was ovocylindrical with a wrinkled outer surface, and had a length of 2.8 ± 0.02 µm and a width of 1.6 ± 0.04 µm (mean ± standard error) (**Fig.2.2**). These organisms tended to be found on the grid in clumps.

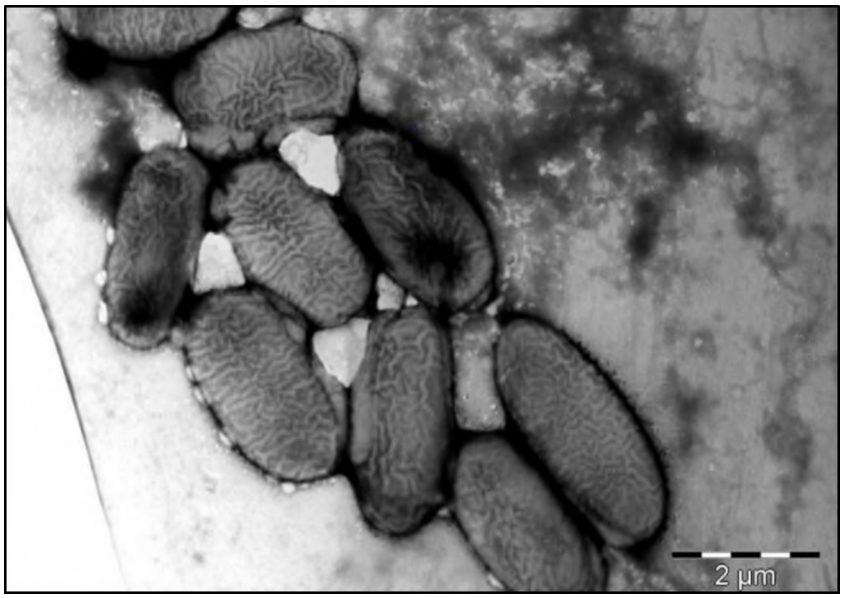


Fig.2. 2 Transmission electron micrograph of wrinkled, ovocylindrical organisms isolated from carob moth, *Ectomyelois ceratoniae*. Scale bar = 2 µm.

The second type of organism was similar in shape to the first type, however they were smaller (2.05 ± 0.04 µm by 1.04 ± 0.02 µm) and did not possess a wrinkled outer surface (**Fig.2.3**).

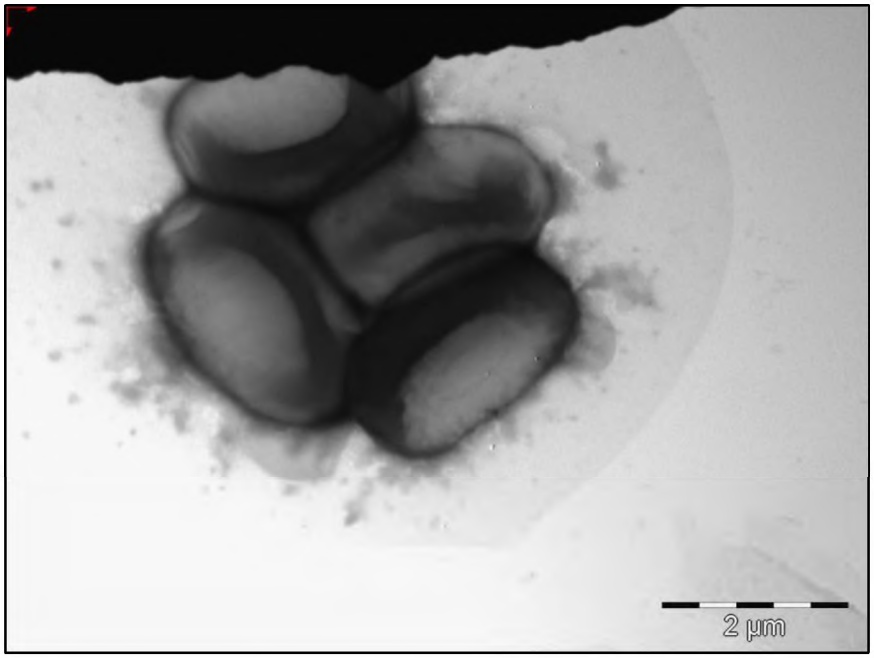
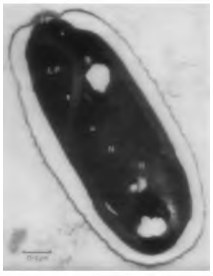
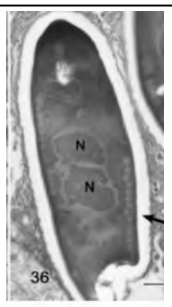
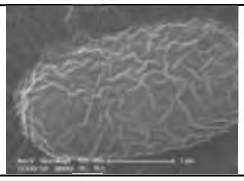
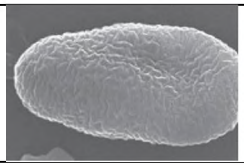


Fig.2. 3 Transmission electron micrograph of smaller ovocylindrical organisms isolated from carob moth, *Ectomyelois ceratoniae*. Scale bar = 2 µm.

The organism isolated from carob moth is similar in morphology to other species of microsporidia (**Table 2.1**). Specifically, the shape and wrinkled surface of the microsporidia is similar to other microsporidia, and the size of the spores is in a similar range.

Table 2. 1 Comparison of mature spore morphology of different species of microsporidia

Microsporidium species	Host	Mature spore size (µm)	Spore description	Image of spore	Reference
Species from this study	Carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)	2.8 ± 0.02 x 1.6 ± 0.04	Wrinkled exospore, ovocylindrical		
Nosema carpocapsae	Codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae)	3.16 x 1.7	Dense rippled exospore		(Malone & Wigley, 1981b)
Nosema sp.	Carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)	3.7 ± 0.01 x 1.3 ± 0.006	Ovocylindrical with wrinkled exospore		(Lange, 1991)
Vairimorpha disparis	Gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae)	4.6 ± 0.28 x 2.8 ± 0.26	Slightly wrinkled exospore		(Vavra *et al.*, 2006)
Nosema bombi	*Bombus* spp. (Hymenoptera: Apidae)	4.42 – 5.85 x 1.69 -2.60	Crumpled and wrinkled exospore		(Kwak *et al.*, 2013)
Nosema apis	Honeybees (Hymenoptera: Apidae)	6 x 3	Thick wrinkled exospore		(Fries *et al.*, 2013; Ptaszyńska *et al.*, 2014)
Nosema ceranae	Honeybees (Hymenoptera: Apidae)	4.4 x 2.2	Thick wrinkled exospore		(Chen *et al.*, 2009; Ptaszyńska *et al.*, 2014)

2.4 Discussion

The development of clean and healthy laboratory cultures of insect pests for use in experimentation is an important step in the process of developing strategies to mitigate their impact on the environment (Walters & Kfir, 1993; Sørensen *et al.*, 2012). Microbial contamination of the insect colony is a significant hindrance, potentially leading to reduction of fitness, or even failure of the colony to establish (Eilenberg *et al.*, 2015). Diagnosis of the causal agent of the infection is essential to implementing strategies to remove the pathogen from the colony. Spore-like particles isolated from deceased carob moth larvae reared under laboratory conditions were imaged and analysed with a transmission electron microscope. Two sizes of ovocylindrical spore-like structures were observed: one with a smooth surface measured 2.05 ± 0.04 µm by 1.04 ± 0.02 µm, and the other with a wrinkled surface measured 2.8 ± 0.02 by 1.6 ± 0.04 µm.

The larger, wrinkled organisms were similar in morphology to microsporidian spores observed in studies by Malone & Wigley (1981b), Lange (1991), Vavra *et al.* (2006), Kwak *et al.* (2013) and Ptaszyńska *et al.* (2014). The species of microsporidium, the host, the morphology of the spore exterior and the size of the spores is summarised in **Table 2.1**. The microsporidium shares similar spore morphology with *Nosema carpocapsae, Vairimorpha disparis*, *Nosema* sp. from carob moth, *N. bombi*, *N. apis* and *N. ceranae*. Based on comparisons of the morphology of the spores analysed in this study with other studies on microsporidia, and the fact that a microsporidium with spores of a similar morphology has already been discovered in the carob moth and various other species in the order Lepidoptera, microsporidian infection was suspected.

Microsporidia with a lepidopteran as the type host are found in the genera *Cystosporogenes*, *Nosema*, *Orthosomella* and *Vairimorpha* (Becnel & Andreadis, 2014). Members of the

genera *Nosema* and *Vairimorpha* are the most commonly encountered (Becnel & Andreadis, 2014). Microsporidia have been isolated from a number of lepidopteran laboratory colonies. *Nosema fumiferanae* was discovered in a laboratory colony of *Choristoneura fumiferana* by Grisdale (1970), who believed the microsporidium was introduced from wild populations of *C. fumiferana*. Another microsporidium, a *Cystosporogenes* sp., was isolated from *C. fumiferana* kept at a multi-species rearing facility for forest entomology research (Van Frankenhuyzen *et al.*, 2004). The infection led to a drastic drop in colony production and nearly caused the colony to collapse (Van Frankenhuyzen *et al.*, 2004). Habib *et al.* (1983) isolated a *Vairimorpha* sp. from *Spodoptera latifascia*. They believed that the microsporidium was latent in the hosts, and was only activated after the hosts were fed a nutrient deficient diet. *Helicoverpa zea* reared under laboratory conditions was discovered to be infected by *Nosema heliothidis*, which was probably introduced in field-collected insects (Waldbauer *et al.*, 1984). Briese & Milner (1986) discovered that a colony of *Anaitis efformata*, being reared for evaluation as a biological control agent, was infected by *Pleistophora schubergi*, resulting in 27 to 90 % mortality in larvae.

Microsporidia have also been isolated from laboratory and mass-reared colonies of South African Lepidoptera. *Pleistophora reciprocaria* spec. nov. was isolated from mass-reared citrus loopers, *Ascotis selenaria reciprocaria* (Buitendag, 1965). *Nosema partelli* sp. n. was isolated from a laboratory colony of the spotted stem borer, *Chilo partellus*, an important pest of maize and sorghum (Walters & Kfir, 1993). The pathogen caused high mortality rates when the moths were stressed.

The isolation of bacteria from diseased insects is not uncommon, however they are seldom the sole causative agent of disease (Weiser, 1963). A notable exception is the biological control agent *Bacillus thuringiensis* (Weiser, 1963). Usually other factors such as physical damage or damage caused by other pathogens are necessary to initiate bacterial infection

(Weiser, 1963). Pilley (1976) discovered bacterial infections in all instars of *Spodoptera exempta* infected by the microsporidium *Vairimorpha necatrix*. The cause of the bacterial infection was suspected to be from gut bacteria invading the haemocoel, following damage to the gut wall by extrusion of the microsporidial polar filament. These findings are supported by studies performed by Weiser & Lysenko (1956), Maddox (1966), Fowler & Reeves (1975), Fuxa (1981), Canning (1982) and Haque *et al.* (1999).

In this study the larvae were pooled before spores were extracted, therefore the cause of mortality of each larva could not be determined. Further research is required to investigate whether larval mortality was caused by the microsporidium, the bacterium, or an interaction between the two organisms. The most likely scenario is that an acute microsporidian infection was induced in hosts that were stressed due to sub-optimal rearing conditions, which has been reported by Walters & Kfir (1993). This would result in larval mortality and release of spores concentrated onto a part of the diet, which were then ingested by other larvae. Ingestion of a highly concentrated dose of microsporidian spores may lead to death by bacterial septicaemia. This would mean that the microsporidium is the primary cause of infection and bacteria are present due to a secondary infection.

In conclusion, a crude extraction method was developed in order to isolate possible causes of carob moth larval mortality. One of the organisms was determined by morphological analysis using transmission electron microscopy, to be a microsporidium. Initial identification of microsporidian infection usually requires morphological identification of spores, however morphological methods of classifying microsporidia tend to be unreliable for classification to species level and should be paired with molecular methods of classification. In Chapter 3 the identity of the microsporidium isolated from carob moth is confirmed using molecular methods.

Chapter 3: Genetic characterisation of the microsporidium isolated from carob moth, *Ectomyelois ceratoniae*

3.1 Introduction

Carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae), is an economically important pest of fruit and nut crops in many tropical and subtropical regions (Millar, 1990; Lange, 1991; Blumberg, 2008). In South Africa it is a pest of citrus, pomegranates and nuts. Little is known about the biology of the pest in South Africa, therefore a laboratory colony was initiated by Thackeray *et al.* (2017) at Citrus Research International (CRI), Port Elizabeth, South Africa, in order to study the basic biology and possible monitoring and control methods for the pest. The colony collapsed due to microbial contamination. In Chapter 2, the cause of the contamination was identified as a microsporidium based on morphology viewed by transmission electron microscopy. This chapter describes genetic confirmation of microsporidian infection of carob moth and further genetic characterisation of the microsporidium by PCR amplification of the full rRNA cistron consisting of the small subunit (SSU) rRNA gene, internal transcribed spacer (ITS), the large subunit (LSU) rRNA gene, intergenic spacer (IGS) and 5S rRNA gene.

Traditional taxonomic studies and species classification of microsporidia were mainly based on biological characters such as spore morphology and development, microsporidium-host relationships and life cycle viewed using a light microscope or transmission electron microscope (TEM) (da Silva *et al.*, 1999; Tsai *et al.*, 2003; Huang *et al.*, 2004; Chan & Koh, 2008; Ghosh *et al.*, 2014) (See Chapter 2). Classifying new microsporidia species using only these biological characters can be problematic and unreliable. Electron microscopy can be insensitive as only a small sample can be studied, and expensive equipment and expertise is required. In order to classify to species level based on morphology, all stages of the life cycle

need to be studied, which is problematic, as often only mature spores are recovered. Even if all life stages are present, it is still difficult to determine the lower classification of the microsporidium based on morphology as spore morphology can differ in alternative hosts, as well as within different tissues in the same host (da Silva *et al.*, 1999; Chan & Koh, 2008; Dong *et al.*, 2010). A more specific and sensitive method for phylogenetic studies is amplification and sequencing of selected genes.

Microsporidia have extremely small genomes ranging in size from 2.9 to 19.5 Mb (Franzen & Müller, 1999). Very few genes have been located and only a few full genomes are available. It is believed that the genome underwent extreme reduction in size as an adaptation to an obligate parasitic lifestyle (Nakjang *et al.*, 2013; Corradi, 2015). Genes used to resolve taxonomic relationships among microsporidia include the ribosomal RNA genes (small subunit (SSU), internal transcribed spacer (ITS), large subunit (LSU), the intergenic spacer (IGS) and 5S rRNA gene), the largest subunit of RNA polymerase II (RPB1), alpha- and beta-tubulin, polar tubule proteins, and elongation factors EF-1α and EF-2 (Kamaishi *et al.*, 1996a, 1996b; Hirt *et al.*, 1999; Delbac *et al.*, 2001; Keeling, 2003; Corradi & Keeling, 2009).

The genes most commonly used for classification of microsporidia are the rRNA gene sequences (Tsai *et al.*, 2009). Microsporidian rRNAs are prokaryote-like as they do not possess a distinct 5.8S rRNA gene, however they are shorter than prokaryotic rRNA (Gatehouse & Malone, 1998; Huang *et al.*, 2004). The rRNA genes usually occur in tandemly repeated units consisting of a transcription unit (SSU rRNA gene, ITS and LSU rRNA gene) and an intergenic spacer (IGS) (Gatehouse & Malone, 1998). The SSU and LSU rRNA genes are highly conserved in microsporidia, making them suitable for genus determination but not species determination. The spacer regions tend to be more variable, even between closely

related species, and may be more beneficial for species identification (Gatehouse & Malone, 1998; Tsai *et al.*, 2002; Ku *et al.*, 2007). Recently the arrangement of the subunits of the rRNA cistron has become important for identification of microsporidia (Huang *et al.*, 2004; Tsai *et al.*, 2009). The arrangement of the rRNA genes of most species of microsporidia is 5'-SSU-ITS-LSU-IGS-5S-3' (**Fig.3.2.A**), however Huang *et al.* (2004) discovered that the arrangement of the rRNA genes of *Nosema bombycis* was reversed (5'-LSU-ITS-SSU-IGS-5S-3') (**Fig.3.2.B**). The *Nosema* clade is divided into two groups: the "true" *Nosema* group infecting Lepidoptera, and the *Nosema* / *Vairimorpha* group infecting Lepidoptera and other insects (Baker *et al.*, 1994). *Nosema bombycis* belongs to the "true" *Nosema* group. Since the first discovery in *N. bombycis* other species within the "true" *Nosema* have been found to possess the reversed rRNA organisation (Tsai *et al.*, 2005; Wang *et al.*, 2006; Ku *et al.*, 2007; Dong *et al.*, 2010). It appears the reversed organisation of the rRNA genes is unique to members of the "true" *Nosema* group and not to members of the *Nosema* / *Vairimorpha* group, which means the organisation of the rRNA genes can be used to distinguish between the two groups.

The aim of this chapter was to genetically confirm the presence of a microsporidium within the carob moth colony and consequently to genetically characterise the microsporidium. The first objective was to amplify the SSU rRNA gene, ITS, LSU rRNA gene, IGS and 5S rRNA gene using PCR. The second objective was to clone the resulting PCR products into a plasmid vector and sequence the products to obtain full sequence data for each gene. The third objective was to compare the sequences with known sequences in the GenBank sequence database via the Basic Local Alignment Search Tool (BLAST) to determine the closest available relatives. The fourth objective was to produce a phylogenetic tree to determine the relationship of the isolated microsporidium with other species of microsporidia.

The fifth and final objective was to determine the organisation of the rRNA cistron to provide further information on the taxonomic position of the isolated microsporidium.

3.2 Materials and methods

3.2.1 Spore isolation and purification

Microsporidian spores were isolated from carob moth larvae from a culture kept at Citrus Research International (CRI) (Thackeray *et al.*, 2017). Spores were extracted using a method modified by Tsai *et al.* (2003) which was specific for microsporidia. Larvae were pooled into groups of five, homogenised in 2 ml 0.1 % SDS using a mortar and pestle, and then filtered through cheese cloth. The resulting filtrate was centrifuged at 1000 x *g* for 10 minutes to form a concentrated spore pellet. The pellet was resuspended in 500 µl double distilled water (ddH_20) and centrifuged for a further 10 minutes at 1000 x *g*. Each pellet was resuspended in 150 µl ddH_20.

3.2.2 DNA extraction

DNA was extracted from spores using two methods. The first method was a modified Cetyl trimethylammonium bromide (CTAB) extraction protocol described by Opoku-Debrah *et al.* (2013) for viral DNA extraction. This method was used initially because viral infection of carob moth larvae was suspected. The second method involved extracting DNA using a commercial kit, the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, USA). This kit was readily available, and has been used successfully to extract DNA from microsporidia (Yamashiro *et al.*, 2017).

For the CTAB DNA extraction, 100 µl of the extracted spores was added to 1 M sodium carbonate (Na_2CO_3) and incubated at 37 °C for 30 minutes. Following this step, 1M Tris-HCl (pH 6.8), SDS (10 % w/v) and proteinase K (25 mg/ml) were added and incubated at 37°C

for 30 minutes. After the incubation, RNase A (10 mg/ml) was added and the sample was incubated at 37 °C for 30 minutes. The spore mixture was centrifuged at 12 100 x *g* for 3 minutes and the supernatant was transferred to 400 µl preheated CTAB buffer (54 mM CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM Na_2EDTA, 1.4 M NaCl). The solution was incubated at 70 °C for 45 minutes. Following this step, a single chloroform extraction was performed. The DNA was precipitated in ice cold isopropanol overnight, washed with 70 % ethanol, air dried and resuspended in 20 µl ddH_20 and stored at -20 °C.

The second method involved extracting DNA using the ZR Fungal/Bacterial DNA MiniPrep™ kit. DNA was extracted from 200 µl of spores according to the ZR Fungal/Bacterial DNA MiniPrep™ kit instructions. The DNA was eluted in 25 µl ddH_20 and stored at -20 °C.

3.2.3 Amplification of selected genes

The primer sets used for amplification of regions of the SSU rRNA gene, ITS, LSU rRNA gene, IGS and 5S rRNA gene and the approximate size of the amplicons are shown in **Table 3.1**. The region of the rRNA cistron to which each primer binds is shown in **Fig.3.1**. The primer set 18f and 1537r are universal microsporidian primers that amplify the entire SSU rRNA gene (Baker *et al.*, 1995; Tsai *et al.*, 2002) (**Table 3.1, Fig.3.1**). The primers LS228f and 580r bind near the beginning of the LSU rRNA gene and the primer MPr-R2 binds near the end, allowing for amplification of the major sequence of the LSU rRNA gene (**Table 3.1, Fig.3.1**). The primer set S1129f and L1328r bind to regions within the SSU and LSU rRNA genes respectively, and was designed for amplification of the ITS region within the *Nosema ceranae* rRNA transcription unit which has the 5'-SSU-ITS-LSU-3' organisation (Huang *et al.*, 2008) (**Table 3.1, Fig.3.1**). The primer set ILSUF and 5SR bind to the end of the LSU

rRNA gene and at the putative end of the 5S rRNA gene respectively, amplifying the IGS and 5S region (Huang *et al.*, 2004; Liu *et al.*, 2012) (**Table 3.1, Fig.3.1**).

Table 3. 1 Primer sets used for amplification of rRNA genes of a microsporidium isolated from the carob moth.

Primer	Primer sequence	Amplicon size (bp)	Reference
Small subunit (SSU)			
18f		1200	
	5'-CAC CAG GTT GAT TCT GCC-3'		(Baker *et al.*, 1995)
1537r			
	5'-TTA TGA TCC TGC TAA TGG TTC-3'		(Tsai *et al.*, 2002)
Internal transcribed spacer (ITS)		200	
S1129f	5'-TGA ATG TGT CCC TGT TCT TTG-3'		(Huang *et al.*, 2008)
L1328r	5'-GGT ATC CTA TTG ATC CCA TGT G-3'		(Huang *et al.*, 2008)
SSU-ITS		1400	
18f	5'-CAC CAG GTT GAT TCT GCC-3'		(Baker *et al.*, 1995)
			(Huang *et al.*, 2008)
L1328r	5'-GGT ATC CTA TTG ATC CCA TGT G-3'		
Large subunit (LSU)		2400	
LS228f	5'-GGA GGA AAA GAA ACT AAC-3'		(Tsai *et al.*, 2002)
MPr-R2	5'-CCT CAA AAT GTC GGC ATA CA-3'		(Chen *et al.*, 2012)
Internal sequencing primer LSU-Internal-F:	5'-CTA AGA AAC GTG TTA CAA CG-3'		
SSU-ITS-partial LSU		1600	
18f	5'-CAC CAG GTT GAT TCT GCC-3'		(Baker *et al.*, 1995)
580r	5'-GGT CCG TGT TTC AAG ACG G-3'		(Zhu *et al.*, 1994)
Intergenic spacer (IGS)-5S		700	
ILSUF	5′-TGG GTT TAG ACC GTC GTG AG-3′		(Liu *et al.*, 2012)
5SR	5′-TAC AGC ACC CAA CGT TCC CA AG-3′		(Liu *et al.*, 2012)

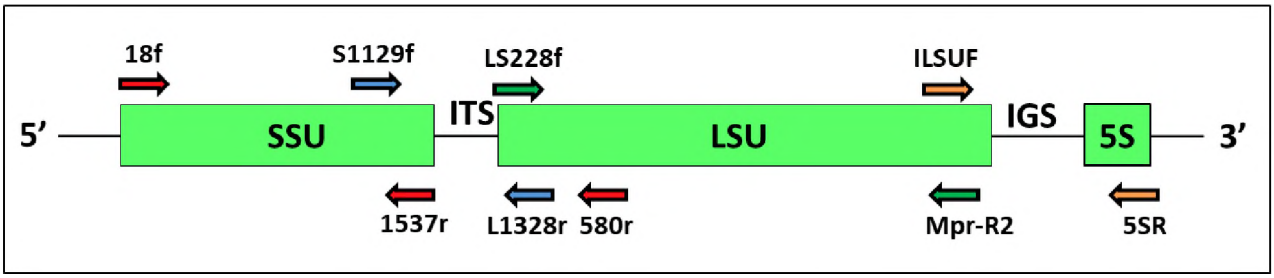


Fig.3. 1 A schematic diagram of the organisation of the rRNA cistron of most species of microsporidia (adapted from (Chen *et al.*, 2012)). The arrows indicate the region of the rRNA cistron to which each primer binds. Primers chosen for amplification were based on this rRNA organisation.

3.2.3.1 SSU, partial SSU-ITS-partial LSU and SSU-ITS amplification

The primer sets used to amplify regions of the SSU rRNA gene, partial SSU-ITS-partial LSU and combined SSU and ITS region were 18f and 1537r, S1129f and L1328r, and 18f and L1328r respectively (**Table 3.1**). Each 25 µl reaction consisted of 1 µl template DNA, 12.5 µl KAPA Taq ReadyMix (KAPA Biosystems, USA), 1 µl of each 10 mM primer and 9.5 µl ddH_2O. The amplification of the genes was performed in a SimpliAmp thermal cycler (Applied Biosystems, USA) with the following profile: initial denaturation at 94 °C for 2 minutes, 40 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 45 seconds, and a final elongation time of 5 minutes at 72 °C. A 5 µl aliquot from each reaction was analysed on a 1.0 % agarose gel.

3.2.3.2 SSU-ITS-partial LSU amplification

The primer set 18f and 580R was used to amplify the SSU rRNA gene, ITS and part of the LSU rRNA gene. The 25 µl reaction consisted of 1 µl template DNA, 12.5 µl KAPA Taq ReadyMix (KAPA Biosystems, USA), 1 µl of each 10 mM primer and 9.5 µl ddH_2O. The amplification was performed in a SimpliAmp thermal cycler (Applied Biosystems, USA) with the following profile: initial denaturation at 95 °C for 3 minutes, 35 cycles of 94 °C for 30 seconds, 57 °C for 30 seconds and 72 °C for 1.30 minutes, and a final elongation time of 5

minutes at 72 °C. The product was analysed on a 1 % agarose gel, and was extracted from the gel using the GeneJET Gel Extraction kit (Thermo Fisher Scientific, USA).

3.2.3.3 LSU amplification

The primer set used to amplify a region of the LSU rRNA gene was LS228f and MPr-R2 (**Table 3.1**). Each 25 µl reaction consisted of 1 µl template DNA, 12.5 µl Ampliqon Taq 2X DNA Polymerase Master Mix RED (Ampliqon, Denmark), 1 µl of each 10 mM primer and 9.5 ddH_2O. The amplification was performed in a SimpliAmp thermal cycler (Applied Biosystems, USA) with the following profile: initial denaturation at 94 °C for 3 minutes, 40 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 2 minutes, and a final elongation time of 5 minutes at 72 °C. A 5 µl aliquot of the reaction was analysed on a 1.0 % agarose gel. The PCR product was purified using the GeneJET PCR Purification kit (Thermo Fisher Scientific, USA).

3.2.3.4 IGS-5S amplification

The primer set used to amplify the IGS region and the 5S region was ILSUF and 5SR (**Table 3.1**). Each 25 µl reaction consisted of 1 µl template DNA, 12.5 µl Ampliqon Taq 2X DNA Polymerase Master Mix RED (Ampliqon, Denmark), 1 µl of each 10 mM primer and 9.5 µl ddH_2O. The amplification of the genes was performed in a SimpliAmp thermal cycler (Applied Biosystems, USA) with the following profile: initial denaturation at 95 °C for 2 minutes, 40 cycles of 95 °C for 20 seconds, 50 °C for 20 seconds and 72 °C for 45 seconds, and a final elongation time of 5 minutes at 72 °C. A 5 µl aliquot from each reaction was analysed on a 1.0 % agarose gel.

3.2.4 Determination of rRNA organisation

The primer sets 18f and L1328r, and 18f and 580r were used to determine the rRNA gene arrangement (**Fig.3.2**). The forward primer 18f is designed to bind at the beginning of the SSU rRNA gene. The reverse primers L1328r and 580r are designed to bind within the LSU rRNA gene. If the arrangement was SSU-ITS-LSU, then a product would be obtained using both primer sets (**Fig.3.2.A**). If the arrangement was reversed, LSU-ITS-SSU, then no product would be obtained (**Fig.3.2.B**). Each 25 µl reaction consisted of 1 µl template DNA, 12.5 µl KAPA Taq ReadyMix (KAPA Biosystems, USA), 1 µl of each 10 mM primer and 9.5 µl ddH_2O. The amplification of the genes was performed in a SimpliAmp thermal cycler (Applied Biosystems, USA) with the following profile: initial denaturation at 94 °C for 2 minutes, 40 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 2 minutes, and a final elongation time of 5 minutes at 72 °C. A 5 µl aliquot from each reaction was analysed on a 1.0 % agarose gel.

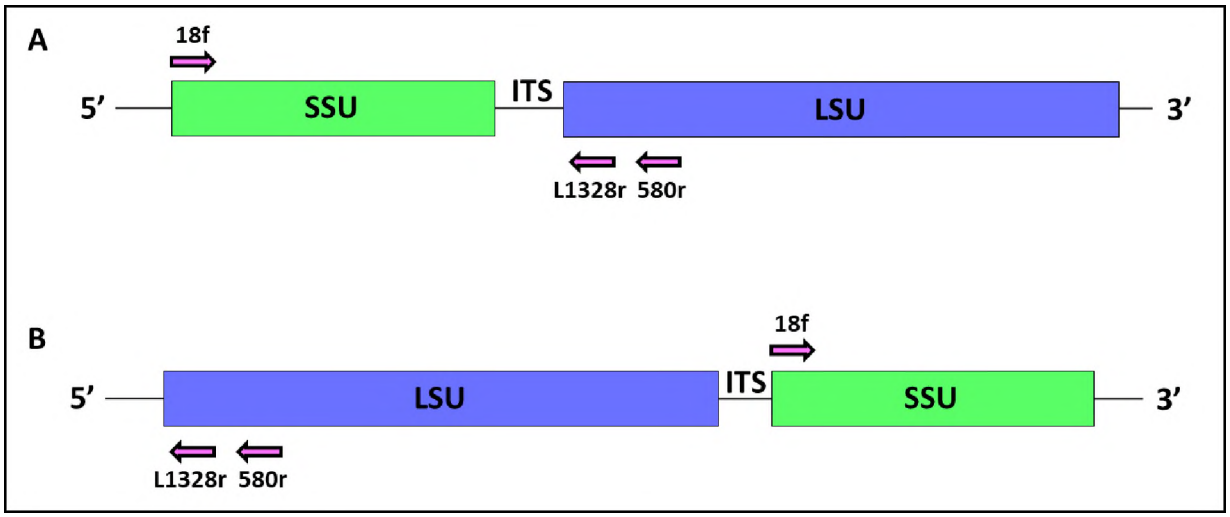


Fig.3. 2 Strategy for determining the organisation of the rRNA cistron in the microsporidium isolated from carob moth. **A)** If the organisation was SSU-ITS-LSU then the primer sets 18f and L1328r and 18f and 580r would return PCR products. **B)** If the organisation was LSU-ITS-SSU then no PCR products would be obtained.

3.2.5 Cloning of PCR products into a vector

The PCR products were ligated into either pGEM®-T Easy (Promega, USA) or into the pJET1.2/blunt cloning vector using the CloneJET PCR cloning kit (Thermo Scientific, USA) in order to obtain full sequence data. The ligations were transformed into DH5α competent cells and sequenced.

3.2.5.1 Cloning vectors

Two different vectors, pGEM®-T Easy and pJET1.2/blunt were used. pGEM®-T Easy is a 3015 bp TA cloning vector (**Fig.3.3.A**). The T-overhangs at the insertion site improve ligation efficiency by preventing vector recircularization, and provide a compatible overhang for PCR products generated by some thermostable polymerases. The multiple cloning region is situated within an α-peptide coding region of the enzyme β-galactosidase. The α-peptide coding region is disrupted if a product is ligated into the vector successfully, allowing identification of recombinants by blue/white screening on Luria agar plates containing X-Gal (5-Bromo-4-chloro-3-indolyl-D-galactopyranoside) and IPTG (Isopropyl β-D-1-thiogalactopyranoside). Inserts can be released from the vector by single-enzyme digestion using EcoR1, BstZ1 or Not1, or by a double-enzyme digest.

pJET1.2/blunt is a 2974 bp positive selection blunt-end cloning vector that contains a lethal gene, which is disrupted when a product is ligated into the cloning site (**Fig.3.3.B**). The lethal gene is expressed if a ligation is unsuccessful, killing the cell into which it was transformed. Only cells containing recombinant plasmids are able to form colonies on a Luria agar plate, eliminating the need for blue/white screening. PCR products are blunted with a DNA blunting enzyme before ligation. Ligations are rapid, taking only 5 to 30 minutes, depending on the size of the product. Inserts can be released from the vector by a double-enzyme digest using

restriction enzymes with sites included in the cloning site. The LSU, SSU-ITS-partial LSU and IGS-5S sequences were cloned into pJET1.2/blunt.

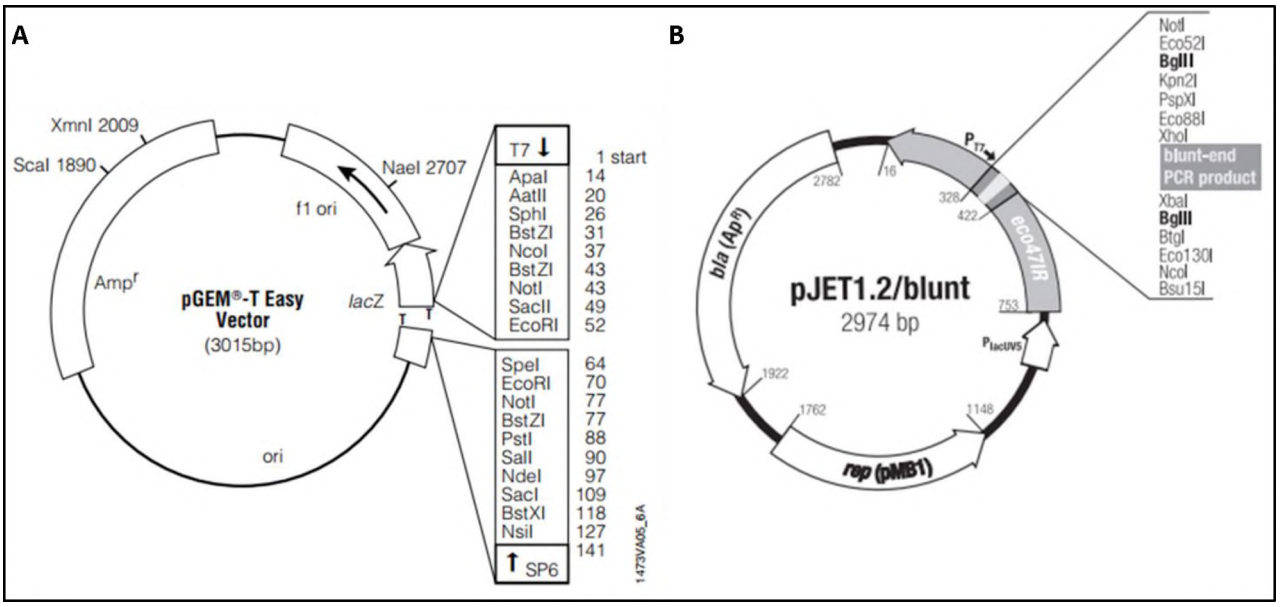


Fig.3. 3 Map of vectors into which PCR products were ligated for cloning. **A**) Vector map of pGEM®-T Easy (Promega, USA). **B**) Vector map of pJET1.2/blunt (Thermo Scientific, USA).

3.2.5.2 Ligation of PCR products into vector

The partial SSU-ITS-partial LSU and SSU-ITS sequences were ligated into the pGEM®-T Easy vector. The reaction mixture consisted of 2 µl of the PCR product, 1 µl pGEM®-T Easy vector, 1 µl T4 DNA ligase, 1 µl 10 x rapid ligation buffer for T4 DNA ligase, and ddH_20 to a final volume of 10 µl. The reactions were mixed by pipetting and were incubated overnight at 4 °C to obtain the maximum number of transformants.

The LSU, SSU-ITS-partial LSU and IGS-5S sequences were ligated into pJET1.2/blunt. The ligation is divided into 2 parts: the blunting reaction and the ligation reaction. The blunting reaction mixture consisted of 1 µl PCR product, 10 µl 2 x reaction buffer, 1 µl DNA blunting enzyme and ddH_20 to make up a final volume of 18 µl. The tubes were vortexed briefly,

centrifuged and incubated at 70 °C for 5 minutes, after which they were chilled on ice. For the ligation reaction, 1 µl pJET1.2/blunt cloning vector and 1 µl T4 DNA ligase were added to the blunting reaction mixture. The tubes were vortexed briefly, centrifuged and incubated at room temperature for 20 minutes.

3.2.5.3 Transformation of plasmids into competent cells

100 µl of competent DH5α competent cells was added to each ligation. The tubes were left on ice for 30 minutes, and then heat shocked for 45 seconds at 42 °C. The reactions were placed back on ice for 2 minutes. After this step, 500 µl of Luria broth without antibiotic was added to each reaction, and these were incubated at 37 °C for 45 minutes. The samples were centrifuged at 6000 rpm for 60 seconds. Most of the supernatant was discarded, except for approximately 100 µl. The cells were resuspended in the remaining 100 µl. Cells that had been transformed with pGEM®-T Easy were plated onto Luria agar plates containing a final concentration of 40 µg/ml X-Gal diluted in Dimethylformamide (DMF), 0.1 mM IPTG and 100 µg/ml ampicillin. Cells that had been transformed with pJET1.2/blunt were plated onto LA plates containing 100 µl/ml ampicillin. The plates were incubated at 37 °C for 16 hours. Colonies were selected using sterile forceps and toothpicks and added to 5 ml Luria broth containing ampicillin with a final concentration of 100 µl/ml in Sterilin™ bottles. The bottles were incubated overnight at 37 °C for 16 hours. A plasmid extraction was performed using the GeneJET Plasmid MiniPrep Kit (Thermo Fisher Scientific, USA), and 5 µl of each plasmid was analysed on a 1.0 % agarose gel to verify the presence of plasmids.

3.2.5.4 Restriction analysis of plasmids

The pGEM®-T Easy vector contains two EcoR1 sites in the multiple cloning site, therefore a single restriction digest could be performed to determine whether an insert was present in the plasmid. A restriction digest was performed using FastDigest EcoR1 (Thermo Fisher

Scientific, USA), as this excises the insert from pGEM®-T Easy. The reaction consisted of 3 µl plasmid, 1 µl FastDigest buffer, 1 µl EcoR1 FastDigest enzyme, and ddH_2O to a final volume of 10 µl. The reaction was incubated at 37 °C in a water bath for 30 minutes. To determine whether an insert was present, 3 µl of the restriction reaction was analysed on a 1.0 % agarose gel.

A single digest cannot be performed to extract an insert from the pJET1.2/blunt vector, therefore a double digest was performed using FastDigest Xho1 and FastDigest Xba1 (Thermo Fisher Scientific, USA). These two enzymes were selected because the vector contains sites for these enzymes on either side of the insert, excising the insert from the vector. The reaction consisted of 0.5 µl plasmid, 2 µl FastDigest buffer, 1 µl Xho1 FastDigest enzyme, 1 µl Xba1 FastDigest enzyme and ddH_20 to a final volume of 20 µl. The reaction was incubated at 37 °C in a water bath for 20 minutes. To determine whether an insert was present, 5 µl of the restriction reaction was analysed on a 1.0 % agarose gel.

3.2.6 Sequence analysis and alignments

Plasmids were sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing. Once the sequences had been obtained, they were submitted to be compared to other sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). The resulting sequence data also was used to create plasmid maps using SnapGene® v.4.0.2 software (from GSL Biotech; available at snapgene.com).

A subset of the small subunit rRNA sequences used for phylogenetic analysis by Vossbrinck & Debrunner-Vossbrinck (2005) was obtained from the GenBank database (**Table 3.2**); these were edited and aligned with the SSU sequence obtained from the microsporidium infecting carob moth in MEGA v.5.2 (Tamura *et al.*, 2011). The available LSU sequences for the species included in Vossbrinck & Debrunner-Vossbrinck (2005) as well as other

supplementary LSU sequences were obtained from the GenBank database (**Table 3.3**) and were subjected to the same methods of analysis as the SSU sequences. The sequences from the species in **Table 3.2** and **Table 3.3** were uploaded and aligned in Geneious v.7.2 (Kearse *et al.*, 2012) in order to obtain images of the alignments (**S2** and **S3**).

Table 3. 2 Microsporidium and GenBank accession number used for SSU rRNA phylogenetic analysis

Microsporidium species	**GenBank accession number**
Cystosporogenes legeri	AY233131
Cystosporogenes operophterae	AJ302320
Endoreticulatus bombycis	AY009115
Endoreticulatus schubergi	L39109
Nosema apis	X73894
Nosema bombi	AY008373
Nosema bombycis	L39111
Nosema carpocapsae	AF426104
Nosema ceranae	U26533
Nosema furnacalis	U26532
Nosema granulosis	AJ011833
Nosema oulemae	U27359
Nosema portugal	AF033316
Nosema pyrausta	AY958071
Nosema spodopterae	AY211392
Nosema trichoplusiae	U09282
Nosema tyriae	AJ012606
Nosema vespula	U11047
Vairimorpha lymantriae	AF033315
Vairimorpha necatrix	Y00266

Table 3. 3 Microsporidium and GenBank accession number used for LSU rRNA phylogenetic analysis

Microsporidium species	**GenBank accession number**
Encephalitozoon cuniculi	AJ005581
Glugoides intestinalis	DQ680158
Nosema apis	U97150
Nosema bombi	AY741110
Nosema bombycis	AY259631
Nosema ceranae	DQ486027
Nosema granulosis	DQ996239
Nosema plutellae	AY960987
Nosema sp. HR	KP100640
Nosema sp. MPr	HQ399665
Nosema sp. PA	FJ969508
Nosema sp. PX1	AY960986
Nosema sp. SC	FJ767862
Nosema spodopterae	AY747307
Nosema trichoplusiae	DQ996243
Ordospora colligata	XR001377307
Vairimorpha necatrix	EU544672
Vairimorpha sp. SD	HQ891818

The full rRNA cistron consisting of 5'-SSU-ITS-LSU-IGS-5S-3' rRNA sequences was obtained using Geneious. The sequence data returned for each PCR product was uploaded into Geneious and aligned using the sequence data from the closest relatives as a reference. The rRNA cistron sequence from the microsporidium isolated from carob moth was aligned with annotated complete rRNA cistrons from Microsporidium 57864 (Acc. No. U90885), *Nosema* sp. CO1 (Acc. No. AY383655) and *Vairimorpha* sp. SD (Acc. No. HQ891818), as well as the partial rRNA cistron from its closest relative, *Nosema* sp. MPr (Acc. No. HQ399665). The carob moth microsporidium's rRNA cistron was annotated and the size of each unit was determined based on the alignment of these sequences, therefore the accuracy of the annotations is dependent on the accuracy of the sequences that have been submitted to GenBank.

3.2.7 Phylogenetic analysis

Two phylogenetic trees were constructed in Mega v.5.2.; one from the SSU rRNA sequence data and one from the LSU rRNA sequence data. A neighbour-joining tree was constructed for each gene using the Kimura 2-parameter method and 1000 bootstraps. Bootstrap values lower than 50 were excluded. The SSU tree was rooted using the clade containing the *Cystosporogenes* spp. and the *Endoreticulatus* spp. The LSU tree was rooted with the clade containing *Glugoides intestinalis*, *Ordospora colligata*, and *Encephalitozoon cuniculi*. The resulting phylogenetic trees were compared to determine whether both supported the phylogenetic placement of the microsporidium isolated from carob moth within the microsporidia.

3.3 Results

3.3.1 DNA extraction

The methods used for DNA extraction were the CTAB method and the ZR Fungal/Bacterial DNA MiniPrep™ kit. No DNA was visible on the gel for either method (data not shown). In order to confirm that template DNA was present, PCR of the SSU rRNA gene was performed using the primer set 18f and 1537r and analysed on a 1.0 % agarose gel. Bright bands of similar intensity were visible for the PCR of both methods, therefore both DNA extraction methods were shown to be effective (data not shown).

3.3.2 Amplification of rRNA genes

3.3.2.1 SSU, partial SSU-ITS-partial LSU and SSU-ITS

A region of approximately 1200 bp of the SSU rRNA gene was amplified successfully using the primer set 18f and 1537r (**Fig.3.4, lane 2**). A region of approximately 200 bp consisting of the end part of the SSU rRNA gene, the ITS and the beginning part of the LSU rRNA gene

was amplified successfully using the primer set S1129f and L1328r (**Fig.3.4, lane 3**). The combined SSU-ITS region was amplified successfully using the primer set 18f and L1328r (**Fig.3.4, lane 4**).

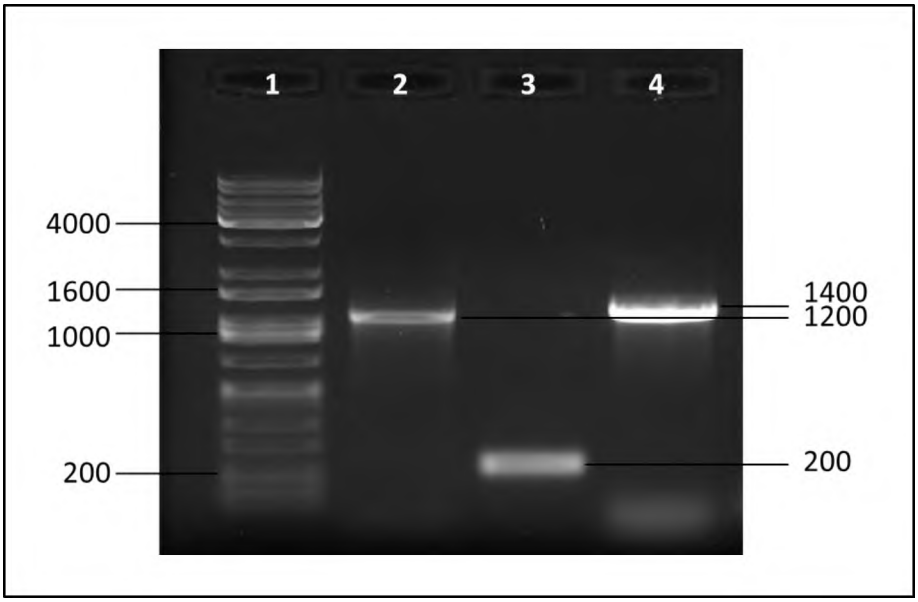


Fig.3. 4 A 1.0 % agarose gel of the results of the PCR amplification of the SSU rRNA gene, partial SSU-ITS-partial LSU and SSU-ITS region. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: SSU rRNA amplicon, Lane 3: partial SSU-ITS-partial LSU amplicon, Lane 4: SSU-ITS amplicon.

3.3.2.2 SSU-ITS-partial LSU

The SSU-ITS-partial LSU region was amplified using the primer set 18f and 580r, generating a product of approximately 1600 bp (**Fig.3.5.A, lane 2**). Multiple bands were present (**Fig.3.5.A, lane 2**), therefore the band of approximately 1600 bp was extracted from the gel and purified. The extraction and purification was successful, resulting in a single band of approximately 1600 bp (**Fig.3.5.B, lane 2**).

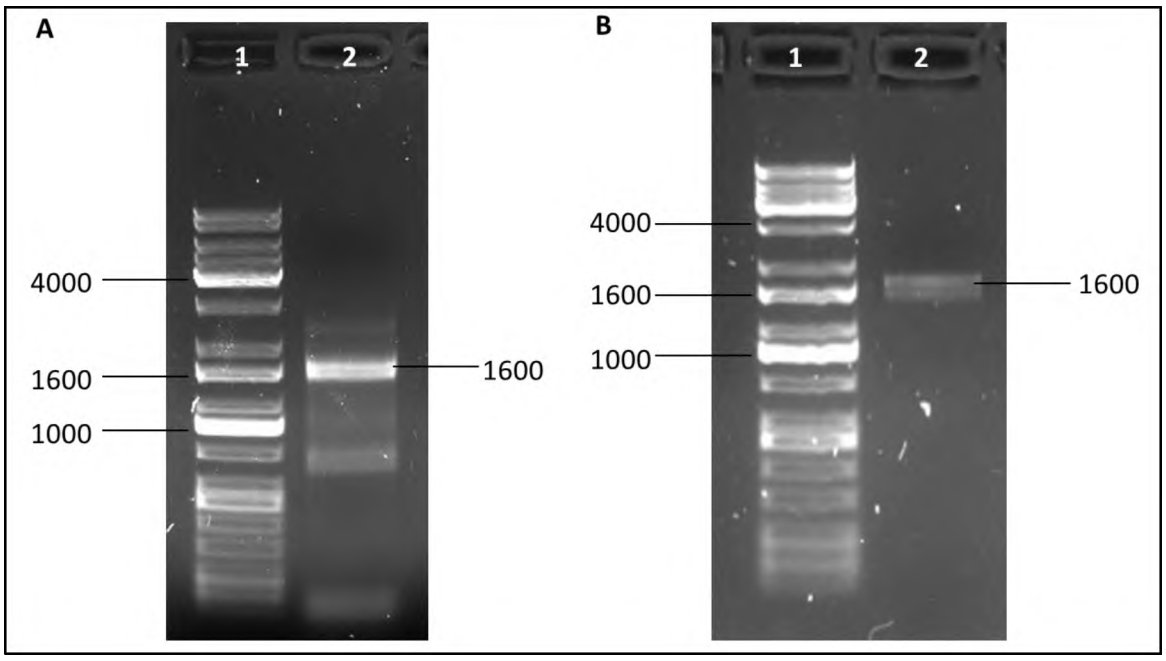


Fig.3. 5 A) A 1.0 % agarose gel of the results of the PCR amplification of the SSU-ITS-partial LSU ribosomal RNA region. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: SSU-ITS-partial LSU rRNA region. **B)** A 1.0 % agarose gel of the 1600 bp gel extracted PCR product containing the SSU rRNA gene, ITS and part of the LSU rRNA gene. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: gel extracted 1600 bp PCR product.

3.3.2.3 LSU

A major part of the LSU rRNA gene, approximately 2400 bp, was amplified using the primer set LS228f and Mpr-R2. The resulting PCR product was purified and analysed on a 1.0 % agarose gel (**Fig.3.6, lane 2**).

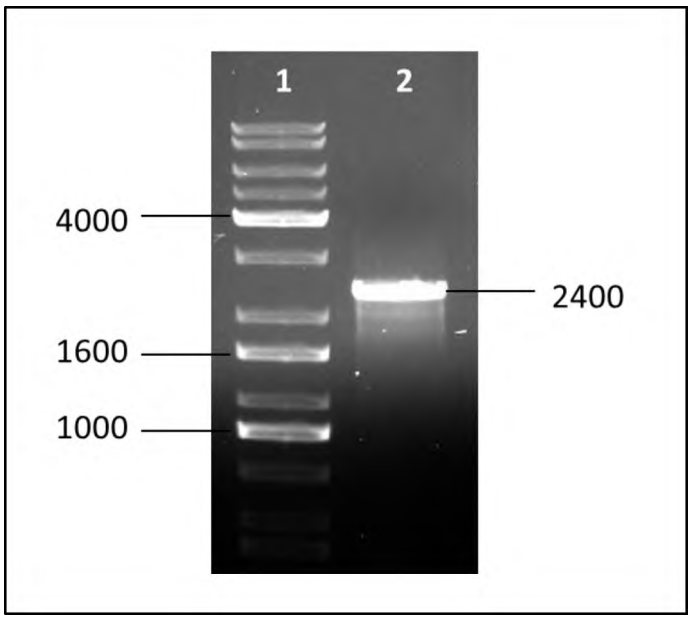


Fig.3. 6 A 1.0 % agarose gel of the gel extracted LSU rRNA PCR product. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: region of the LSU rRNA gene.

3.3.2.4 IGS-5S

The IGS region and the 5S rRNA region were amplified using the primer set ILSUF and 5SR.

The resulting PCR product was analysed on a 1.0 % agarose gel (**Fig.3.7, lane 2**).

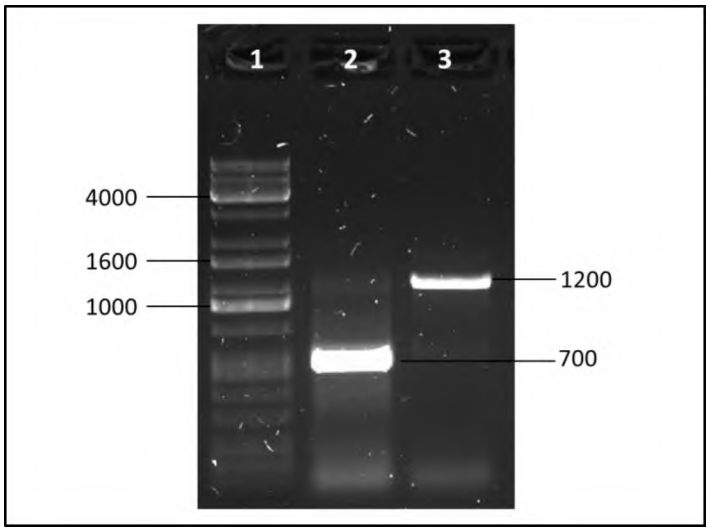


Fig.3. 7 A 1.0 % agarose gel of the IGS-5S PCR product. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: IGS-5S rRNA region, Lane 3: SSU rRNA control.

3.3.3 Organisation of the rRNA cistron

In order to determine the rRNA organisation, specific primer sets were used and the resulting products run on a 1.0 % agarose gel. The primer set 18f and L1328r returned a product of approximately 1400 bp (**Fig.3.8, lane 2**), which is larger than the SSU rRNA gene alone, therefore it most likely contained regions from both the SSU rRNA gene and ITS. The primer set 18f and 580r returned a product of approximately 1600 bp (**Fig.3.8, lane 3**). This was larger than both the SSU and SSU-ITS products, therefore it most likely contained the SSU, ITS and part of the LSU rRNA. The predicted organisation of the rRNA genes is 5'-SSU-ITS-LSU-IGS-5S-3' because both the primer set 18f and L1328r and 18f and 580r returned products.

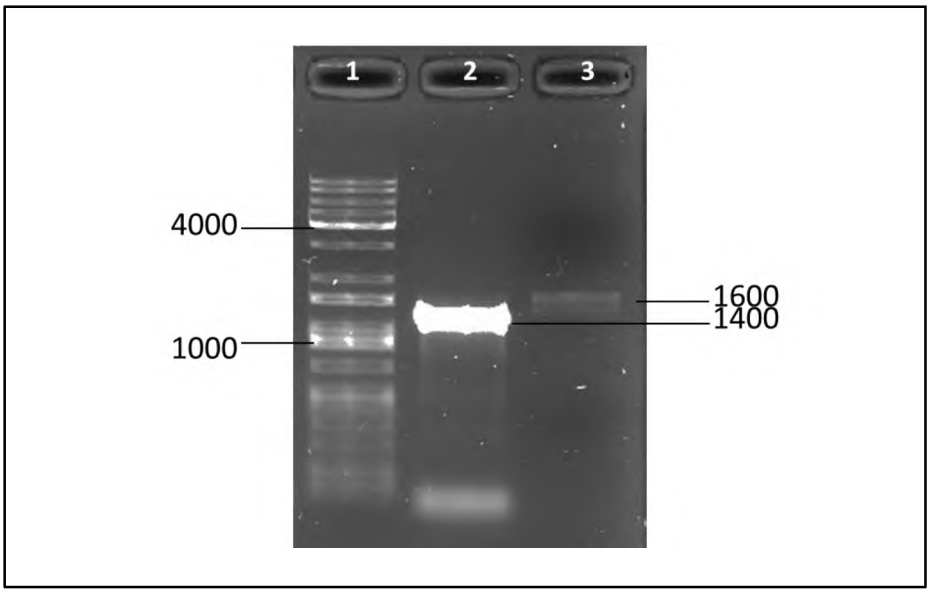


Fig.3. 8 A 1.0 % agarose gel of the products generated from PCR using primers to determine rRNA organisation. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: PCR product generated using primer set 18f and 1328r; Lane 2: PCR product generated using primer set 18f and 580r.

3.3.4 Cloning and restriction analysis

3.3.4.1 ITS and SSU-ITS

The plasmids were subjected to restriction analysis using the restriction enzyme EcoR1, which excises an insert from pGEM®-T Easy in a single digest, and analysed on a 1.0 % agarose gel to determine whether the ligation and cloning procedures were successful.

The unrestricted partial SSU-ITS-partial LSU plasmids were very bright on the gel (**Fig.3.9, lanes 3, 5, 7, 9, 11**). After restriction of the partial SSU-ITS-partial LSU plasmids with EcoR1, a band of approximately 3000 bp and one of 200 bp was observed (**Fig.3.9, lanes 2, 4, 6, 8, 10**). The 200 bp band is approximately the same size as the PCR product of the partial SSU-ITS-partial LSU region (**Fig.3.9, lane 12**). It was determined that the partial SSU-ITS-partial LSU region was cloned successfully into pGEM®-T Easy, and plasmid 4 (**Fig.3.9, lane 9**) was sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing. The resulting sequence data were used to create a plasmid map (**Fig.3.10**).

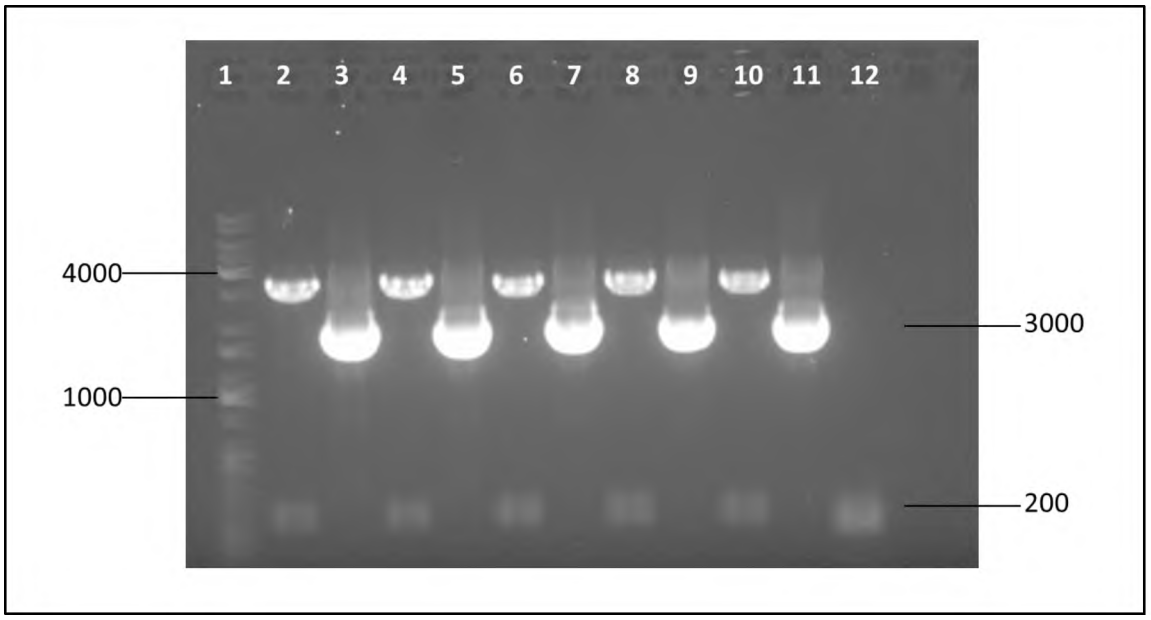


Fig.3. 9 Restriction analysis of the partial SSU-ITS-partial LSU amplicon cloned into the vector pGEM®-T Easy. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: cut plasmid 1, Lane 3: uncut plasmid 1, Lane 4: cut plasmid 2, Lane 5: uncut plasmid 2, Lane 6: cut plasmid 3, Lane 7: uncut plasmid 3, Lane 8: cut plasmid 4, Lane 9: uncut plasmid 4, Lane 10: cut plasmid 5, Lane 11: uncut plasmid 5, Lane 12: PCR product.

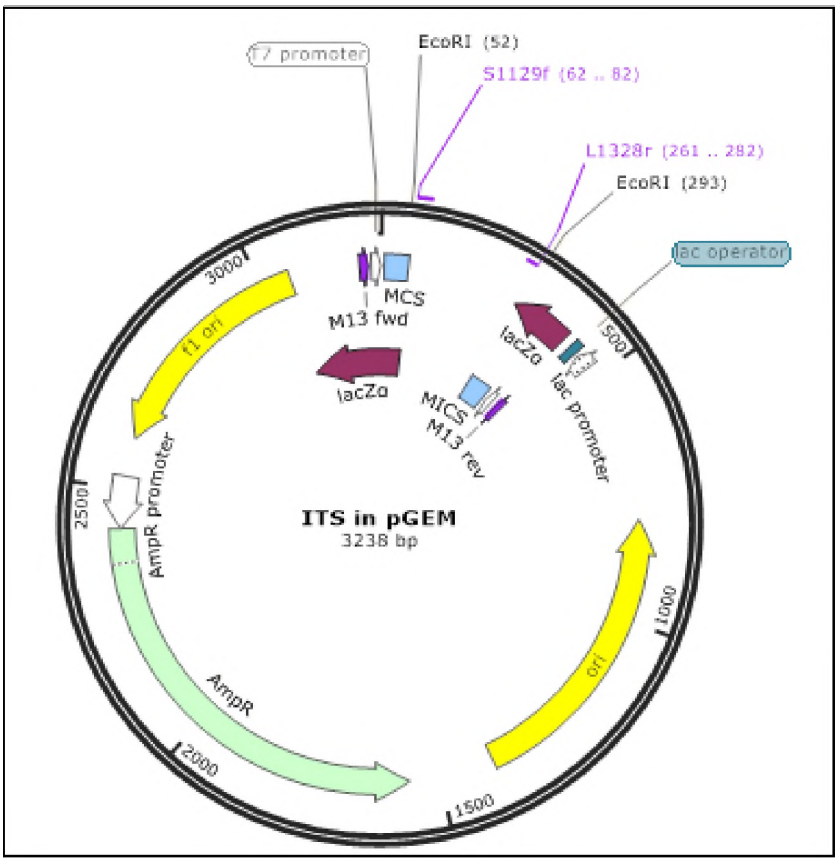


Fig.3. 10 Plasmid map of the partial SSU-ITS-partial LSU PCR product cloned into the pGEM®-T Easy vector. The primer set S1129f and L1328r show the beginning and the end of the ligated product. The plasmid was sequenced in the forward and reverse directions using the pGEM®-T Easy sequencing primers M13 fwd and M13 rev. The EcoR1 restriction sites flank the insert, allowing for excision in a single digest.

After restriction of the SSU-ITS plasmids with EcoR1, a band of approximately 3000 bp and one of 1400 bp were observed (**Fig.3.11, lanes 3, 5,** 7). The 1400 bp band is approximately the same size as the PCR product of the SSU-ITS region (**Fig.3.11, lane 8**). It was determined that the SSU-ITS region was cloned successfully into pGEM®-T Easy, and plasmid 1 (**Fig.3.11, lane 2**) was sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing. The resulting sequence data was used to create a plasmid map (**Fig.3.12**).

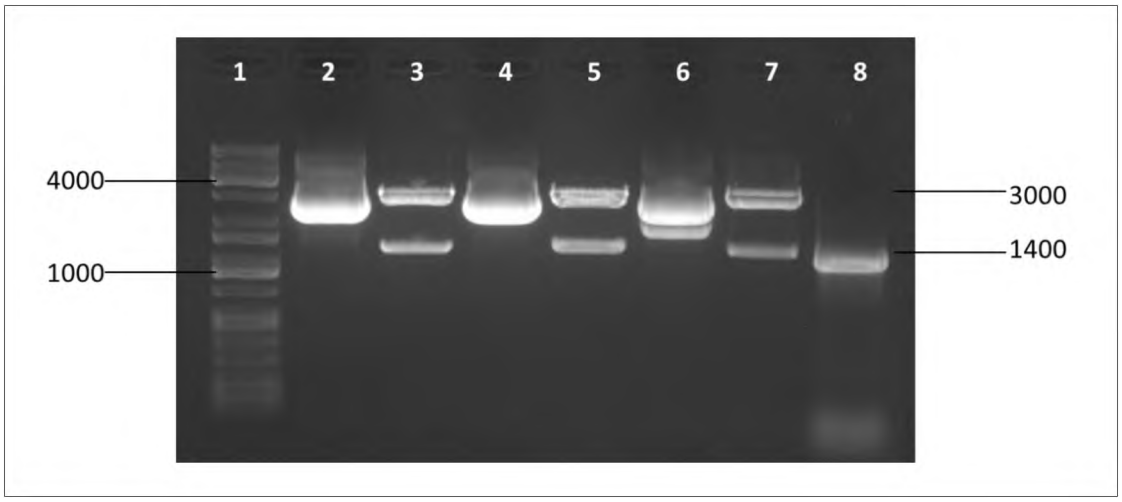


Fig.3. 11 Restriction analysis of the combined SSU-ITS region cloned into the vector pGEM®-T Easy. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: uncut plasmid 1, Lane 3: cut plasmid 1, Lane 4: uncut plasmid 2, Lane 5: cut plasmid 2, Lane 6: uncut plasmid 3, Lane 7: cut plasmid 3, Lane 8: SSU-ITS PCR product.

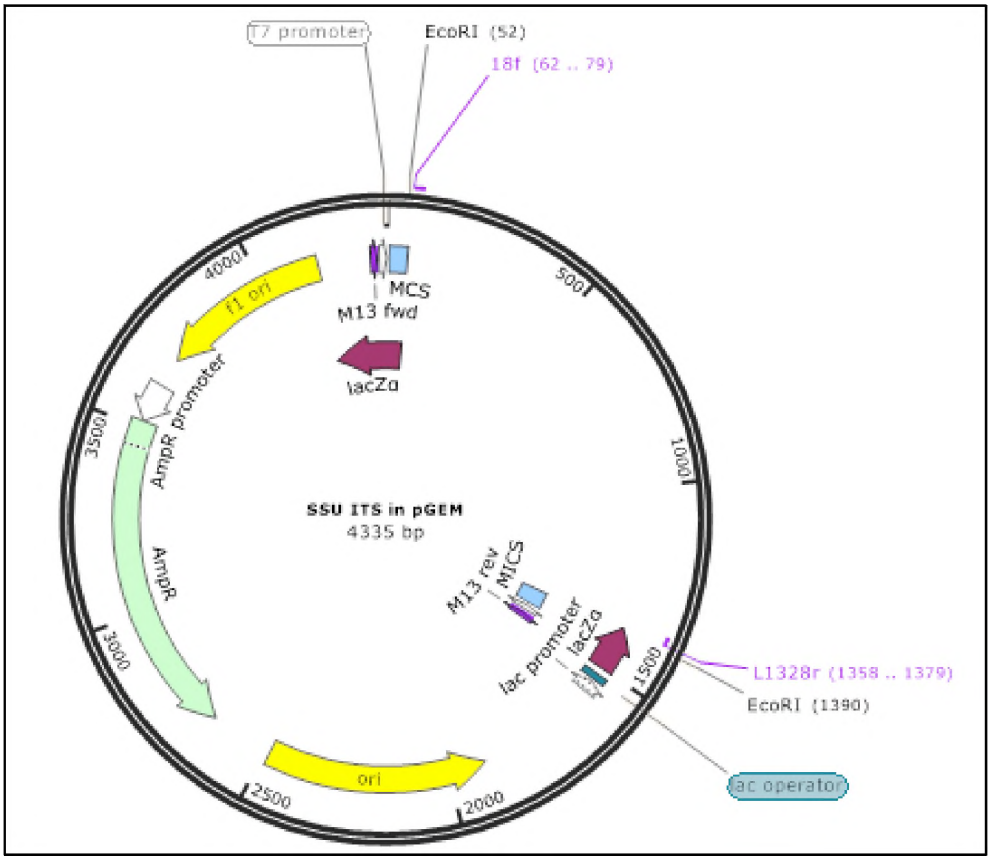


Fig.3. 12 Plasmid map of the SSU-ITS PCR product cloned into the pGEM®-T Easy vector. The primer set 18f and L1328r show the beginning and the end of the insert. The plasmid was sequenced in the forward and reverse directions using the pGEM-T Easy sequencing primers M13 fwd and M13 rev. The EcoR1 restriction sites flank the insert, allowing for excision in a single digest.

3.3.4.2 SSU-ITS-partial LSU, LSU and IGS-5S

The plasmids were subjected to restriction analysis using the restriction enzymes Xho1 and Xba1, which excise the insert from pJET1.2/blunt, and were analysed on a 1.0 % agarose gel to determine whether the ligation and cloning procedures were successful. The combined SSU-ITS-partial LSU (**Fig.3.13**), LSU (**Fig.3.15**) and IGS-5S (**Fig.3.17**) were cloned into pJET1.2/blunt successfully.

After restriction of the SSU-ITS-partial LSU plasmids with Xho1 and Xba1, a band of approximately 3000 bp and one of 1600 bp were observed (**Fig.3.13, lanes 3, 5**). The 1600 bp band is approximately the same size as the PCR product of the SSU-ITS-partial LSU region. It was determined that the SSU-ITS-partial LSU PCR product was cloned successfully into pJET1.2/blunt, and plasmid 2 (**Fig.3.13, lane 4**) was sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing. The resulting sequence data were used to create a plasmid map (**Fig.3.14**).

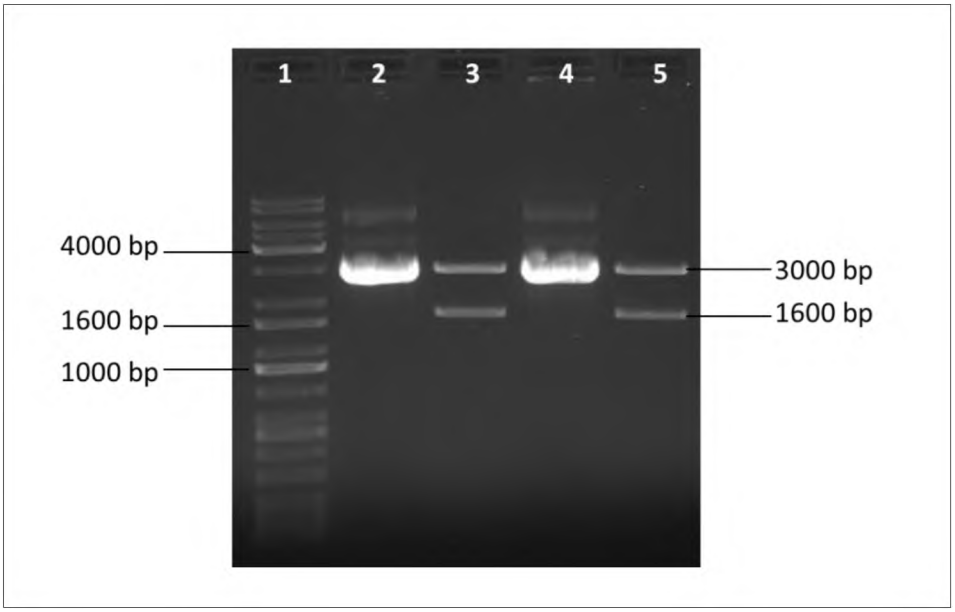


Fig.3. 13 Restriction analysis of the combined SSU-ITS-LSU region cloned into the vector pJET1.2/blunt. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: uncut plasmid 1, Lane 3: cut plasmid 1, Lane 4: uncut plasmid 2, Lane 5: cut plasmid 2.

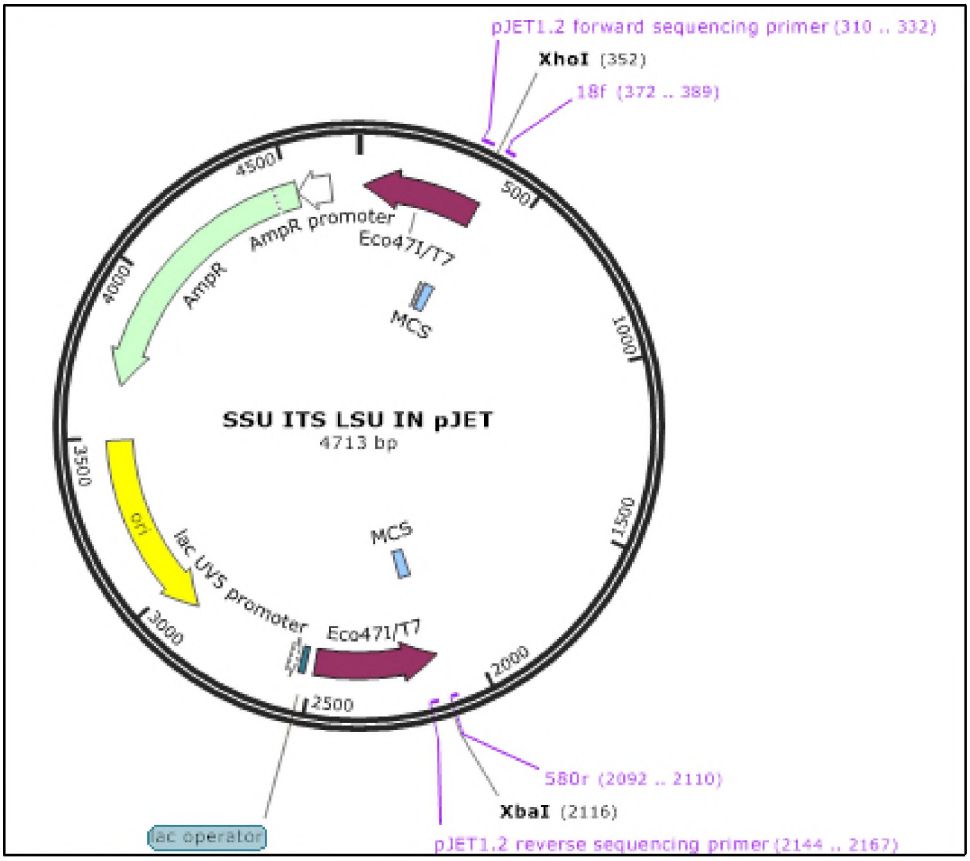


Fig.3. 14 Plasmid map of the SSU-ITS-partial LSU PCR product cloned into the pJET1.2/blunt vector. The primer set 18f and 580r show the beginning and the end of the insert. The plasmid was sequenced in the forward and reverse directions using the pJET1.2/blunt sequencing primers pJET1.2 forward and pJET1.2 reverse. The Xho1 and Xba1 restriction sites flank the insert, allowing for excision in a double digest.

One of the unrestricted LSU plasmids appeared to be smaller than the other two (**Fig.3.15, lane 4**). This may be due to vector religation. After restriction of the LSU plasmids with Xho1 and Xba1, a band of approximately 3000 bp was observed in lanes 3, 5 and 7 and a band of 2400 bp was observed in lanes 3 and 7 (**Fig.3.15**). The 2400 bp band is approximately the same size as the PCR product of the LSU region (**Fig.3.15, lane 8**). It was determined that the LSU PCR product was cloned successfully into pJET1.2/blunt plasmids 1 and 3, and plasmid 3 (**Fig.3.15, lane 6**) was sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing. The resulting sequence data were used to create a plasmid map (**Fig.3.16**).

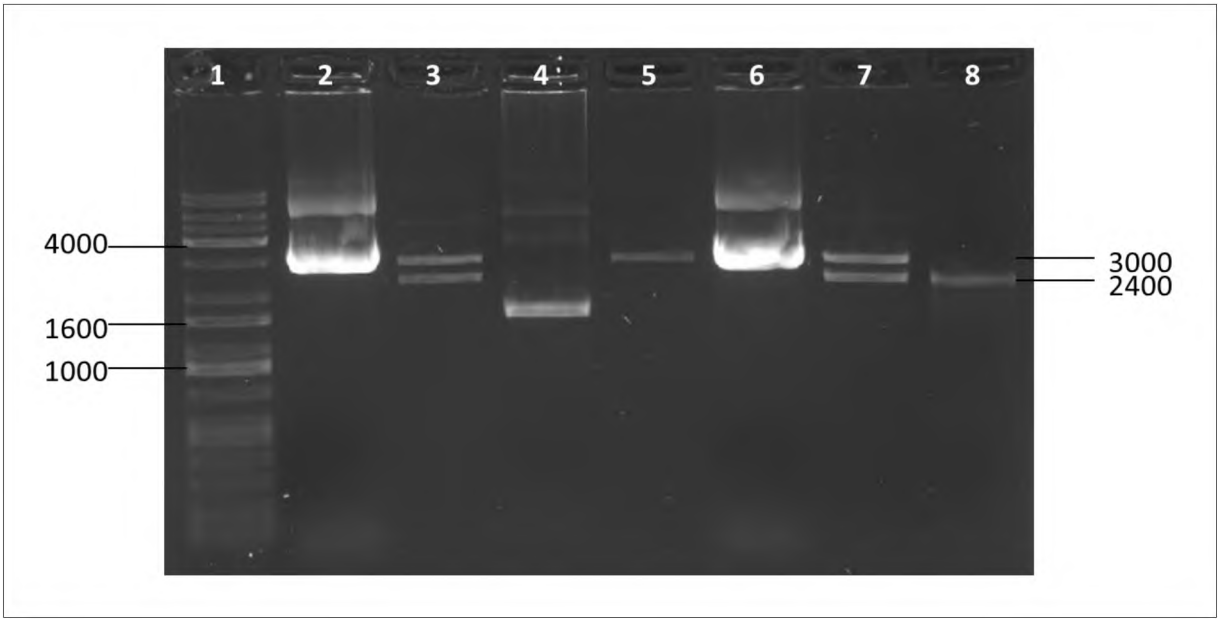


Fig.3. 15 Restriction analysis of the LSU rRNA gene cloned into the vector pJET1.2/blunt. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: uncut plasmid 1, Lane 3: cut plasmid 1, Lane 4: uncut plasmid 2, Lane 5: cut plasmid 2, Lane 6: uncut plasmid 3, Lane 7: cut plasmid 3, Lane 8: LSU PCR product.

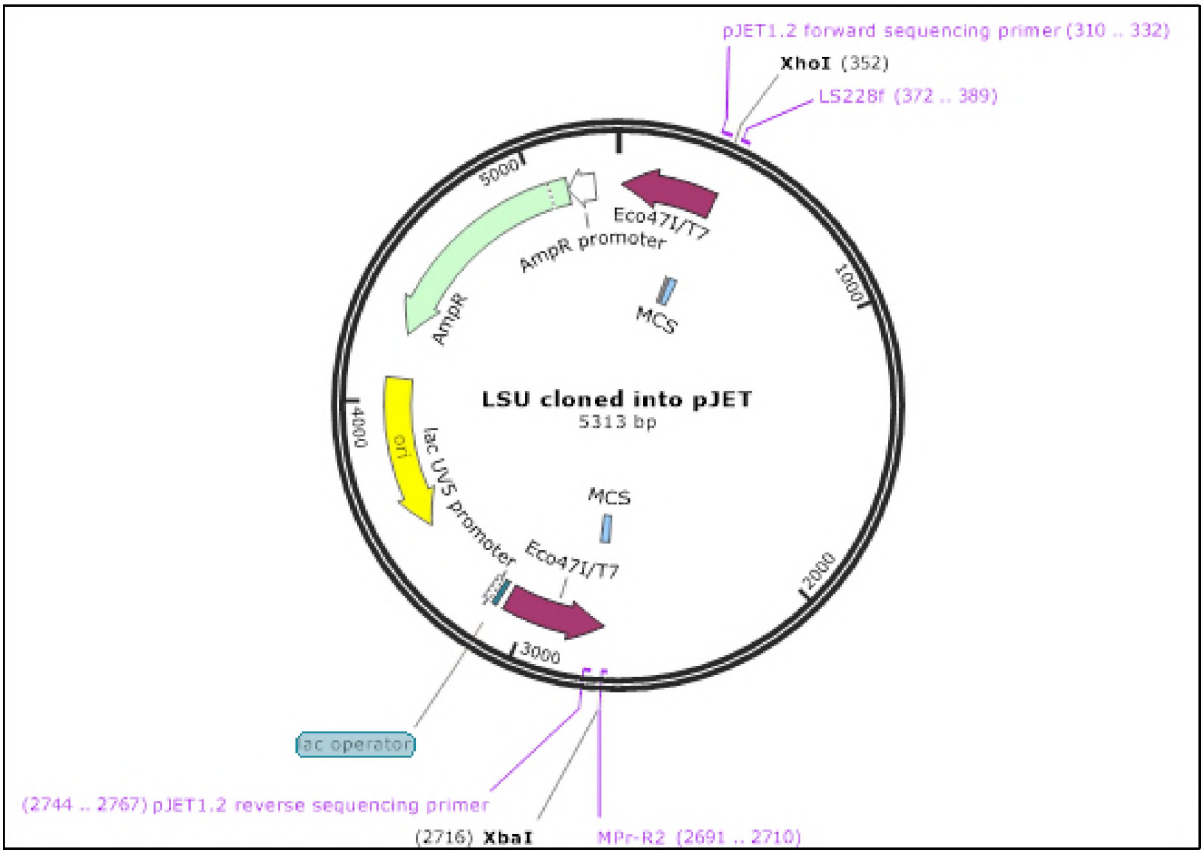


Fig.3. 16 Plasmid map of the LSU PCR product cloned into the pJET1.2/blunt vector. The primer set LS228f and MPr-R2 show the beginning and the end of the insert. The plasmid was sequenced in the forward and reverse directions using the pJET1.2/blunt sequencing primers pJET1.2 forward and pJET1.2 reverse. The Xho1 and Xba1 restriction sites flank the insert, allowing for excision in a double digest.

After restriction of the IGS-5S plasmids with Xho1 and Xba1, a band of approximately 3000 bp was observed in lanes 3 and 5 and a band of approximately 700 bp was observed in lanes 3 and 5 (**Fig.3.17**). The 700 bp band is approximately the same size as the PCR product of the IGS region (**Fig.3.17, lane 6**). It was determined that the IGS-5S PCR product was cloned successfully into pJET1.2/blunt plasmids 1 and 2, and plasmid 1 (**Fig.3.17, lane 2**) was sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing. The resulting sequence data was used to create a plasmid map (**Fig.3.18**).

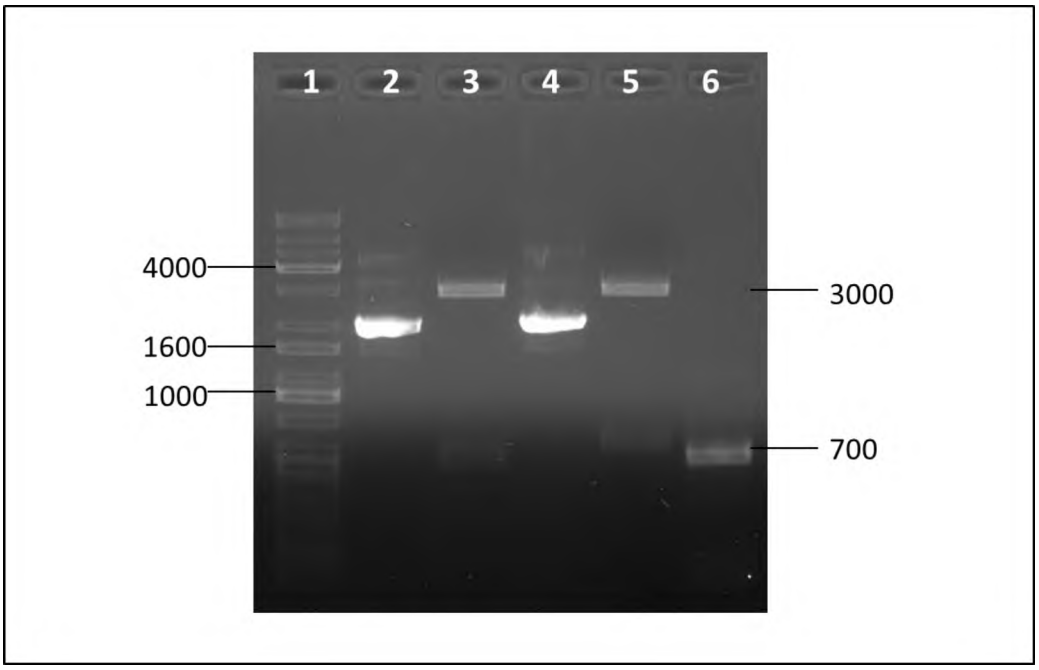


Fig.3. 17 Restriction analysis of the IGS-5S rRNA region cloned into the vector pJET1.2/blunt. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: uncut plasmid 1, Lane 3: cut plasmid 1, Lane 4: uncut plasmid 2, Lane 5: cut plasmid 2, Lane 6: IGS-5S PCR product.

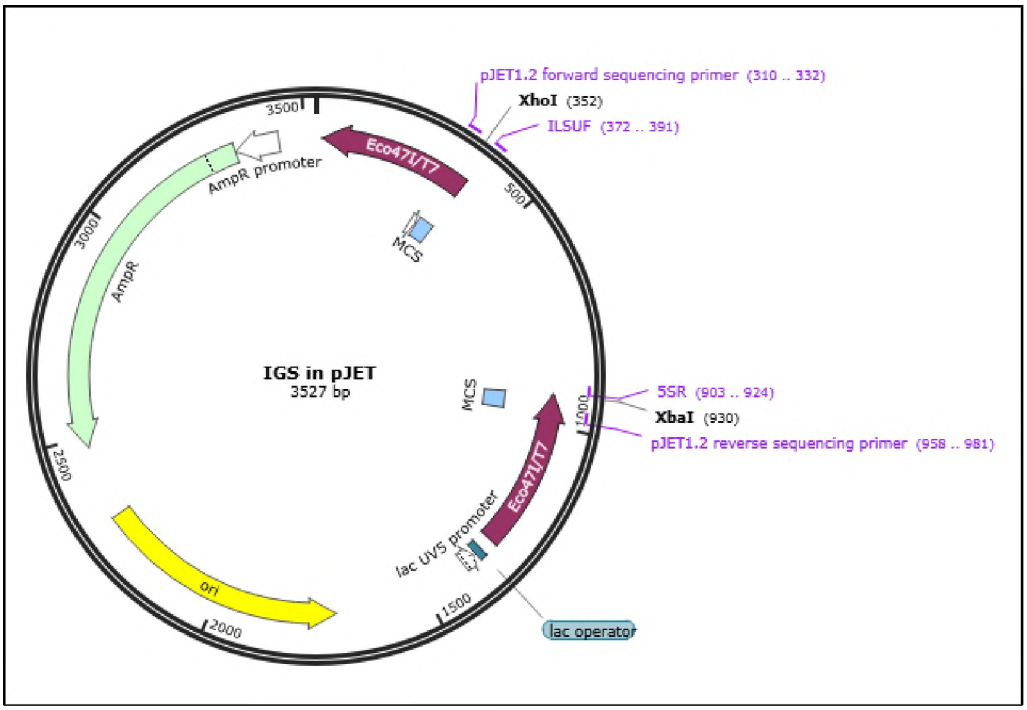


Fig.3. 18 Plasmid map of the IGS-5S PCR product cloned into the pJET1.2/blunt vector. The primer set ILSUF and 5SR show the beginning and the end of the insert. The plasmid was sequenced in the forward and reverse directions using the pJET1.2/blunt sequencing primers pJET1.2 forward and pJET1.2 reverse. The Xho1 and Xba1 restriction sites flank the insert, allowing for excision in a double digest.

3.3.5 Sequence analysis and alignment

The total size of the SSU rRNA gene amplified was 1248 bp and the GC content was 37.1 %. The BLAST search of the SSU sequence returned a 99 % similarity with uncultured *Nosema* clone M-Pr (HQ399665), *N. carpocapsae* (AF426104) and *N. oulemae* (U27359). The size of the ITS was 39 bp and the GC content was 2.6 %. This was too small to perform a BLAST search, therefore the 200 bp region amplified was submitted. The BLAST search returned a 96 % similarity with uncultured *Nosema* clone M-Pr (HQ399665), *N. carpocapsae* (AF426104) and *Nosema* sp. CO1 (AY383655.1). The total size of the LSU rRNA gene was 2547 bp and the GC content was 33.4 %. The BLAST search returned a 99 % similarity with uncultured *Nosema* clone M-Pr (HQ399665) and *Nosema* sp. CO1 (AY383655). The total size of the IGS region was 104 bp and the GC content was 45.5 %. The total size of the 5S

rRNA gene was 116 bp and the GC content was 43.1 %. The BLAST search of the IGS-5S region returned a 99 % similarity with Microsporidium 57864 (U90885), *Nosema* sp. CO1 (AY383655) and *Nosema bombi* (AY741111). The total length of the rRNA gene amplified was 4054 bp (**Fig.3.19**).

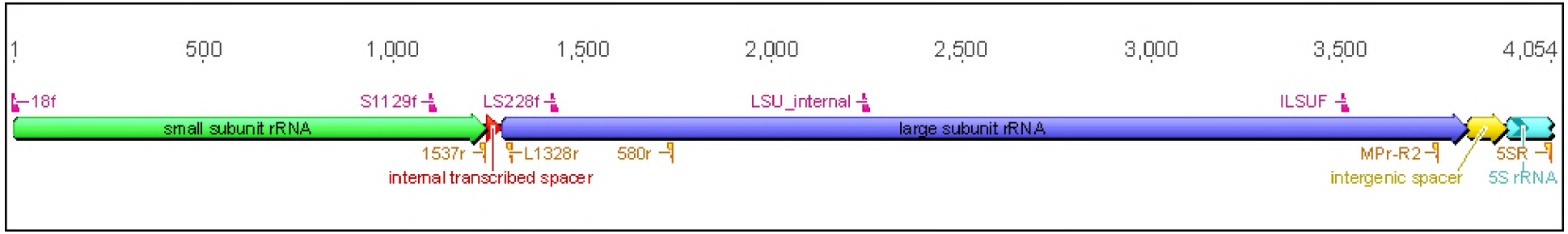


Fig.3. 19 Diagram of the rRNA cistron. Forward primer names and binding sites are indicated in pink and reverse primer names and binding sites are indicated in orange.

3.3.6 Phylogenetic analysis

Phylogenetic analysis of the SSU rRNA gene indicated that the microsporidium isolated from carob moth does belong to the Terresporidia group, also known as microsporidia of terrestrial origin, proposed by Vossbrinck & Debrunner-Vossbrinck (2005) (**Fig.3.20**). Phylogenetic analysis of the SSU rRNA gene also indicated that the microsporidium isolated from carob moth clustered with the *Nosema* / *Vairimorpha* group (**Fig.3.20**). This result is supported by phylogenetic analysis of the LSU rRNA gene (**Fig.3.21**). The divergence at the node separating the "true" *Nosema* from the *Nosema* / *Vairimorpha* group is strongly supported by a bootstrap value of 100 on both trees (**Fig.3.20, Fig.3.21**).

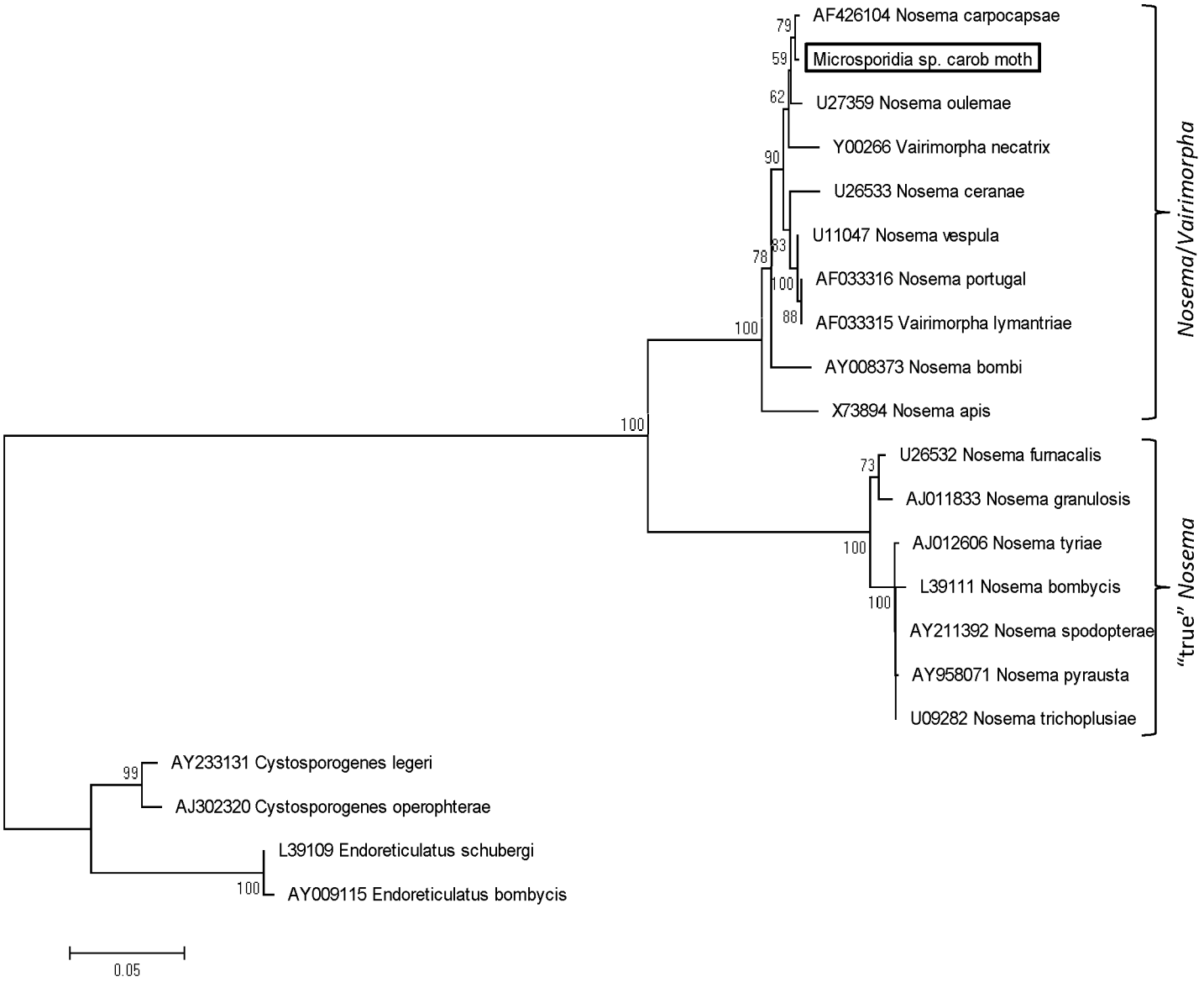


Fig.3. 20 Neighbour-joining phylogenetic tree with 1000 bootstraps and a Kimura 2-parameter substitution model based on the SSU rRNA gene. The black box indicates the position of the microsporidian isolated from carob moth. GenBank accession numbers are included in the species name. Bootstrap values lower than 50 were excluded. The tree was rooted using the clade containing the *Cystosporogenes* spp. and the *Endoreticulatus* spp. (Lloyd *et al.*, 2017).

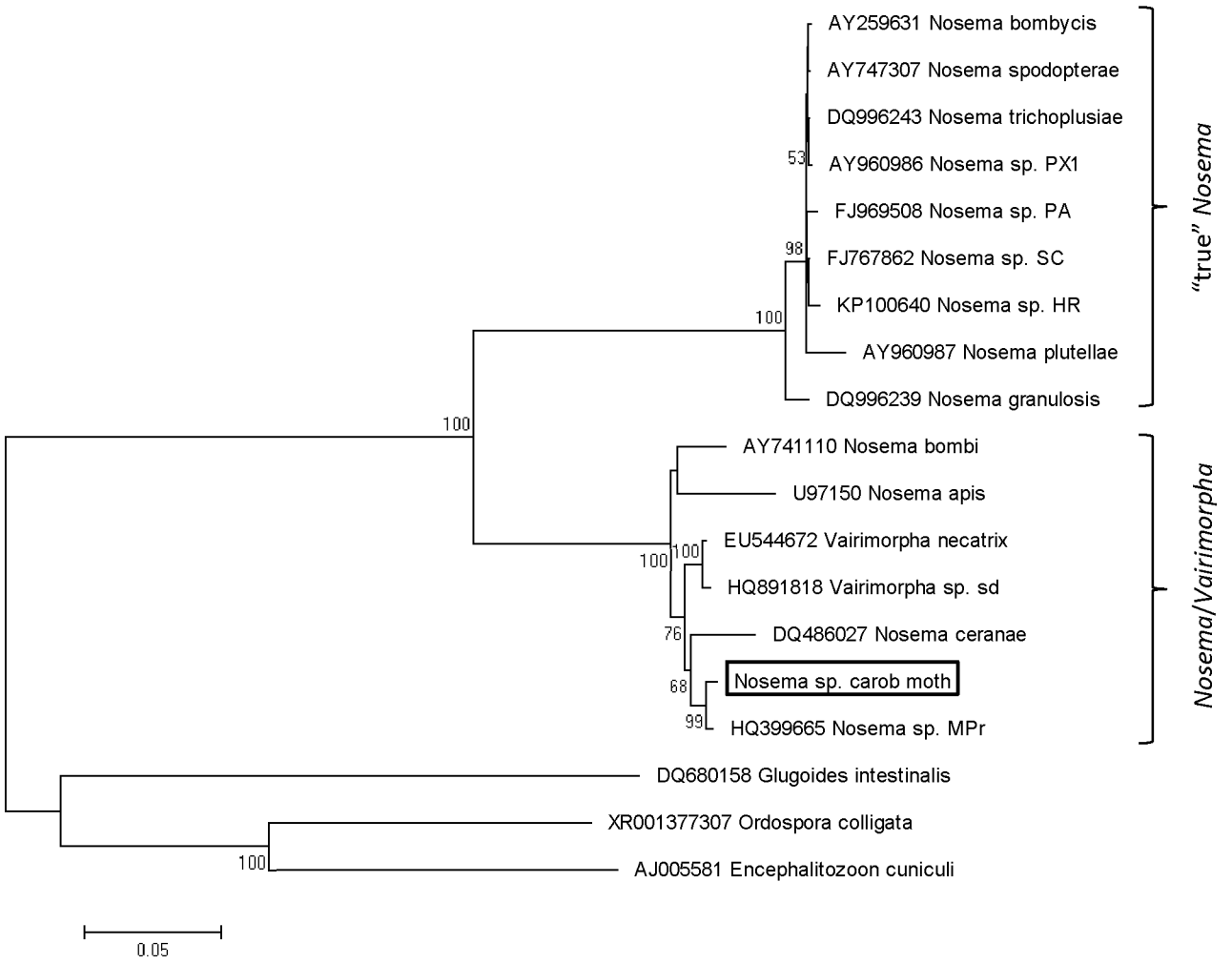


Fig.3. 21 Neighbour-joining phylogenetic tree with 1000 bootstraps and a Kimura 2-parameter substitution model based on the LSU rRNA gene. The black box indicates the position of the microsporidian isolated from carob moth. GenBank accession numbers are included in the species name. Bootstrap values lower than 50 were excluded. The tree was rooted with the clade containing *Glugoides intestinalis*, *Ordospora colligata*, and *Encephalitozoon cuniculi*.

3.4 Discussion

Pathogens within insect colonies need to be identified in order to develop mitigation strategies. Initial identification of pathogens usually involves visualisation using transmission electron microscopy (see Chapter 2). Once the initial identification is complete, methods to confirm the identification need to be implemented. Molecular techniques such as PCR and sequencing of specific regions of the genome provide a sensitive, specific and unbiased method of classifying organisms. In this chapter the identity of a potential mortality-causing agent isolated from carob

moth larvae was confirmed to belong to the microsporidia by sequencing of the rRNA cistron consisting of the small subunit rRNA gene, the internal transcribed spacer, the large subunit rRNA gene, the IGS and the 5S rRNA gene, and the organisation of the rRNA cistron was determined. Furthermore, phylogenetic analysis of the SSU and LSU rRNA genes showed that that the microsporidium isolated from carob moth shared a high identity with species belonging to the *Nosema* / *Vairimorpha* clade.

Before PCR and sequencing of genes can be performed, DNA needs to be extracted from the target organism. The lack of DNA on the gel was most likely due to low concentrations of DNA. This may have been due to sub-optimal DNA extraction methods. Microsporidian spores have a thick wall made up of three layers: the proteinaceous exospore, the chitinous endospore and a plasma membrane (Franzen & Müller, 1999). This coat needs to be ruptured in order to liberate the DNA, therefore adaptations may need to be made to typical DNA extraction methods (Franzen & Müller, 1999; Solter *et al.*, 2012).

Initially a modified CTAB DNA extraction method for viral DNA extraction was used because viral infection of carob moth larvae was suspected. The CTAB method has been used successfully to extract DNA from microsporidia, with some modifications (Chen *et al.*, 2013; Holt *et al.*, 2013). Holt *et al.* (2013) incubated spores in CTAB buffer containing 0.2 % 2-mercaptoethanol (dissolves polar tubules) and proteinase K overnight. DNA was then extracted using a commercial kit (Allprep DNA/RNA/Protein Mini Kit, Qiagen). Chen *et al.* (2013) included 0.2 % 2-mercaptoethanol in the CTAB buffer, and ruptured spores using glass beads. The mixture was incubated with proteinase K for 5 hours, and then chitinase for 16 hours. In order to optimise the DNA extraction method for this study, longer incubation times could be used, 2-mercaptoethanol could be included into the CTAB buffer, chitinase could be included to dissolve chitin in the spore wall, and mechanical disruption using glass beads could be

attempted. Mechanical disruption can cause shearing of the DNA if too vigorous, therefore care needs to be taken (Undeen & Cockburn, 1989).

The ZR Fungal/Bacterial DNA MiniPrep Kit™ was used by Yamashiro *et al.* (2017) to extract DNA from *Enterocytozoon bieneusi* spores, however to the best of the author's knowledge, this kit has not been used to extract DNA from *Nosema* spores. The kit claims to be able to extract DNA from tough-to-lyse fungi, bacteria, algae and protozoa; samples are lysed by mechanical disruption using ceramic BashingBeads™. Vesty *et al.* (2017) compared four different methods of extracting DNA from fungal spores in saliva. They found that the ZR Fungal/Bacterial DNA MiniPrep Kit™ was not as efficient as other commercially available kits. The most efficient kit was the QIAamp DNA Mini Kit (Qiagen, Germany), which relies on enzymatic cell lysis. This kit has been used by a number of researchers to extract DNA from microsporidia (Huang *et al.*, 2004; Franzen *et al.*, 2005; Ghani *et al.*, 2013). Franzen *et al.* (2005) extracted the DNA according to the kit instructions, whereas Huang *et al.* (2004) and Ghani *et al.* (2013) pre-treated the spores before using the kit. Huang *et al.* (2004) mechanically disrupted the mixture using zirconia / silica beads before applying it to the column. Ghani *et al.* (2013) mechanically disrupted the spores using glass beads, and then incubated the mixture in proteinase K, Tris-HCl (pH 9.5), 10 % SDS and 0.1 % 2-mercaptoethanol for 1 hour before extracting the DNA using the kit. In order to optimise the DNA extraction protocol using a commercial kit in this study, some pre-treatment may be required.

Despite no DNA being present on the gel, sufficient DNA was extracted for PCR analysis of the selected genes. The SSU rRNA gene, ITS, LSU rRNA gene and IGS-5S region of the microsporidium isolated from carob moth were successfully amplified and cloned into vectors. Two different vectors, pGEM®-T Easy and pJET1.2/blunt were used. pGEM®-T Easy is a TA cloning vector that allows for blue/white screening of recombinants. This is useful as it is easy to distinguish between vectors containing the desired insert (white colonies) and religated

vector (blue colonies), however it requires special agar plates containing X-Gal and IPTG, and the ligation time is relatively long. The pJET1.2/blunt cloning vector is a blunt-end cloning vector that contains a lethal restriction enzyme gene that is only expressed when not disrupted by a DNA insert. This feature should exclude growth of any cells that do not contain an insert, eliminating the need for blue/white screening. However, many colonies still needed to be screened as some grew without an insert. The ligation time required was very rapid, which is useful when time is a constraint. PCR products were cloned successfully into both vectors, therefore the use of different vectors was not detrimental to the study.

The SSU rRNA gene, ITS, LSU rRNA gene, IGS and 5S rRNA gene share a high identity with other members of the *Nosema* group, which has been proposed to be paraphyletic (Sprague, 1977; Baker *et al.*, 1994). The group is divided into two clades: the clade with *Nosema bombycis* as the type species, also known as the "true" *Nosema* infecting Lepidoptera, and the clade with *Vairimorpha necatrix* as the type species, also known as the *Nosema / Vairimorpha* clade (Zhu *et al.*, 2011). Phylogenetic analysis of the SSU and LSU rRNA genes revealed that this isolate clustered with the *Nosema / Vairimorpha* clade rather than the "true" *Nosema* clade. Furthermore, analysis of the SSU rRNA gene and LSU rRNA gene showed that the microsporidia from carob moth belongs to the Terresporidia, or microsporidia of terrestrial origin, proposed by Vossbrinck & Debrunner-Vossbrinck (2005). The "true" *Nosema* consists mostly of microsporidia that infect lepidopteran hosts. The "true" *Nosema* have a potentially novel rRNA organisation of 5'-LSU-ITS-SSU-IGS-5S-3', and usually have a longer ITS region than members belonging to the *Nosema / Vairimorpha* clade (Choi *et al.*, 2011). The microsporidian isolated from carob moth in this study clusters with the *Nosema / Vairimorpha* clade consisting of species that have a smaller ITS region and the 5'-SSU-ITS-LSU-IGS-5S-3' rRNA arrangement, which was confirmed. The microsporidium shared a high level of identity with species within the genus *Nosema* and therefore is most likely a *Nosema* species, however

the *Nosema* group is under taxonomic revision as some authors believe species within the "true" *Nosema* clade should be separated from the *Nosema* / *Vairimorpha* clade (Baker *et al.*, 1994; Tsai *et al.*, 2003, 2005; Kyei-Poku *et al.*, 2008).

Amplification and analysis of a region of the largest subunit of RNA polymerase II (RPB1) was attempted, however this region failed to amplify using primers designed by Hirt *et al.* (1999). RPB1 is a single-copy house-keeping gene that is divided into conserved domains labelled A-H (Cheney *et al.*, 2001; Kyei-Poku & Sokolova, 2017). The RPB1 gene has a greater level of polymorphism than the more conserved ribosomal genes. Therefore Cheney *et al.* (2001) proposed that it be used as an alternative molecular marker for determining phylogenetic relationships among the microsporidia. The degenerate RPB1 primers RPB-F1 and RPB-R1 designed by Hirt *et al.* (1999) amplified a 1100 bp fragment of the D-F region of the *Vairimorpha necatrix* RPB1 gene, but failed to amplify in this study. Failure of the amplification of the RPB1 gene may be caused by polymorphisms in the region where the primers should bind, indicating that the primers are not appropriate for amplification of the RPB1 region of the microsporidium isolated from carob moth. It may be necessary to design new RPB1 primers based on conserved regions of alignments of the RPB1 region from species within the *Nosema* / *Vairimorpha* group in order to use this gene for further classification of the microsporidium isolated from carob moth.

Molecular identification based on rRNA genes can be used to identify a microsporidium to genus level, however these genes are not sufficiently polymorphic to determine whether a new species has been discovered (Zhu *et al.*, 2011). Lange (1991) is the only other study in which a microsporidium was isolated from the carob moth. The rRNA sequences from the carob moth microsporidium analysed in this study could not be compared with those of the microsporidium isolated by Lange (1991) to determine whether the species were the same, because no molecular identification of that microsporidium was performed.

In conclusion, the SSU rRNA gene, ITS, LSU rRNA gene, IGS and 5S rRNA gene from the microsporidium isolated from carob moth were amplified successfully, cloned into a vector, sequenced and analysed. In addition, these sequences were assembled into the full rRNA cistron. The identity of the organism isolated from a laboratory carob moth colony was confirmed to be a microsporidium, specifically one belonging to the *Nosema* / *Vairimorpha* clade. The microsporidium shared a high level of identity with species within the genus *Nosema* and therefore is most likely a *Nosema* species. However, the *Nosema* group is under taxonomic revision as some authors believe species within the "true" *Nosema* clade should be separated from the *Nosema* / *Vairimorpha* clade. The next chapter describes the experimental infection of codling moth, *Cydia pomonella*, larvae with the microsporidium isolated from the carob moth colony. Codling moth was chosen because the microsporidium isolated from carob moth shares a high identity with *N. carpocapsae* isolated from codling moth, therefore it is considered likely that codling moth may be susceptible to infection.

Chapter 4: Experimental infection of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), with the microsporidium isolated from carob moth

4.1 Introduction

In the previous chapter, the identity of the organism isolated from carob moth was determined by amplification and sequence analysis of ribosomal RNA genes. Sequence and phylogenetic analysis showed that the organism in question is a microsporidium that is closely related to members of the *Nosema* / *Vairimorpha* clade and shares a high identity with members of the genus *Nosema*. One microsporidium which is closely related to the microsporidium isolated from carob moth is *Nosema carpocapsae*, a pathogen isolated from codling moth, *Cydia pomonella* (Malone & Wigley, 1981a; b). This chapter investigated the ability of the microsporidium isolated from carob moth to infect codling moth, which is the host of a closely related microsporidium.

Positive identification of a suspected pathogen from a diseased insect does not always implicate the organism as the cause of the disease. In order to confirm whether the organism isolated from a diseased host is the cause of the infection, the organism needs to be studied further. One way of making a conclusive diagnosis is establishing whether Koch's postulates are satisfied. The process for establishing whether Koch's postulates are satisfied is discussed by Lacey & Solter (2012) and is summarised below. The steps taken to establish whether Koch's postulates are satisfied include (1) confirming that the pathogen is isolated from all of the diseased organisms, (2) culturing the pathogen on a nutrient medium if the pathogen is facultative or in a susceptible host if the pathogen is an obligate pathogen, and it must be identified and characterised, (3) inoculation of the pathogen into healthy insects of the same species as the original or another susceptible host, resulting in similar symptoms of disease, and (4) the pathogen must be re-isolated from the inoculated insects and must have the same characteristics

as those established in step 2. Microsporidia are obligate parasites and cannot be cultured on a nutrient medium (Lacey & Solter, 2012). Therefore to confirm pathogenicity the microsporidium isolated from carob moth needed to be able to re-infect the carob moth or another susceptible host. Due to the collapse of the carob moth colony, no larvae were available for experimentation therefore an alternative host was required. In Chapter 3 it was discovered that the microsporidium isolated from carob moth was closely related to *Nosema carpocapsae*, which infects codling moth. Codling moth is also readily available, relatively easy to culture and larvae can be reared on an artificial diet. For the above reasons, codling moth was selected as a potential alternative host to determine the pathogenicity of the microsporidium isolated from carob moth.

In order to conduct infection assays, a pure inoculum is required to ensure all effects observed in the treatment are due to the target organism alone (Pertoft, 2000). Density gradient centrifugation using Percoll® (Sigma-Aldrich, USA) as a density gradient medium is commonly performed for fractionation of cells and subcellular particles. The use of Percoll® as a density gradient centrifugation medium is reviewed by Pertoft (2000). Percoll® is a silica colloid that does not interfere with viability and biological function of cells and microorganisms, making it ideal for purification of cells and microorganisms. Particles are separated by density and form distinct bands, allowing for isolation of particles of a particular density. Gradient centrifugation using Percoll® is used regularly for purification of microsporidian spores (Solter *et al.*, 2012). Although Percoll® purification usually yields very pure suspensions of microsporidia, contamination by bacteria can occur (Ghosh *et al.*, 2014). Growth of bacterial contaminants can be inhibited by antibiotics (Pilley, 1978; Kurtti *et al.*, 1990; Ghosh *et al.*, 2014). Pilley (1978) found that the addition of neomycin sulphate (final concentration of 500 µg/ml) or tetracycline hydrochloride (100 µg/ml) to suspensions of *Vairimorpha necatrix* spores inhibited bacterial growth without affecting the viability of the

microsporidian spores. In order to obtain purified spore suspensions, spores should be purified by gradient centrifugation using Percoll® and should contain an antibiotic to inhibit bacterial growth.

Infection assays can be conducted using various inoculation methods including (1) droplet feeding where a droplet of inoculum is fed to the target organism, (2) surface contamination (used in this study) where a known volume of inoculum is spread over the surface of the diet, (3) the diet plug method where one small piece of artificial diet containing inoculum is ingested by each larva and (4) the diet incorporation method which involves mixing a known concentration of the pathogen with artificial diet (Lacey, 2012). Factors influencing the decision of which inoculation method is most suitable for the host population tested include the instar of the larvae, the pathogen-host interaction, feeding habits and diet of the larvae and the availability of the pathogen stock (Lacey, 2012).

Assays to determine infectivity of a microsporidium isolate involve inoculation of potential hosts with a known spore dose or concentration. The concentration of spores to which the larvae are exposed needs to be determined by experimentation. If the concentration of the inoculum is too high, larval mortality may be caused by bacterial septicaemia. This is caused by invasion of the haemocoel by opportunistic gut bacteria liberated when microsporidian polar filaments damage the insect gut wall (Pilley, 1976; Vávra & Maddox, 1976; Chakrabarty *et al.*, 2012). A concentration that is too low may result in little or no infection (Solter *et al.*, 2012).

The aim of this chapter was to determine whether codling moth was susceptible to infection by the microsporidium isolated from carob moth. The objectives were to purify the microsporidium isolated from carob moth to obtain an inoculum that is free of contaminants, to enumerate the spore suspension for inoculation using a haemocytometer, and to attempt to infect codling moth with the microsporidium isolated from carob moth in order to determine its pathogenicity.

4.2 Materials and methods

4.2.1 Source of *Cydia pomonella*

Cydia pomonella egg sheets were obtained from ENTOMON Technologies (Pty) Ltd, Stellenbosch, South Africa, which rears *C. pomonella* for a sterile insect release programme. Eggs were incubated in petri dishes lined with damp filter paper and sealed with Parafilm, and were stored in a controlled environment room at 25 °C until eggs hatched into larvae.

4.2.2 Diet preparation

Pre-mixed artificial diet containing whole wheat flour, wheat germ, Brewer's yeast, methyl paraben, ascorbic acid and benzoic acid, was supplied by River Bioscience (Pty) Ltd, Port Elizabeth, South Africa. The diet was sterilised in an oven at 180 °C for 10 minutes. Further steps were conducted under a laminar flow to avoid contamination. 0.1 M propionic acid and 0.1 M phosphoric acid was added to 400 ml of a cooled but still molten agar solution consisting of 13 g of agar. Water was added to the mixture for consistency before the mixture was poured into 24-well plates and allowed to cool.

4.2.3 Purification and enumeration of microsporidian spores

Spores were extracted from diseased larvae according to the method used in section 3.2.1 of Chapter 3. The spore suspension was purified further according to a Percoll® cushion method modified from Kurtti *et al.* (1990), Gisder *et al.* (2010) and Remadevi *et al.* (2010). The Percoll® was prepared according to the manufacturer's instructions. Briefly, Stock Isotonic Percoll® (SIP) was prepared using 9 parts Percoll® to 1 part 1.5 M NaCl. The SIP was diluted to a final concentration of 70 % using 0.15 M NaCl. The solution was layered gently on top of the 70% (v/v) Percoll® cushion and centrifuged at 13400 rpm for 30 minutes. After centrifugation, the supernatant was discarded. The spores were washed by adding ddH_2O and

centrifuging at 6600 rpm. This step was repeated and the spores were resuspended in a final volume of 100 µl ddH_2O.

Spores were enumerated according to the method of Hunter-Fujita (1998) with some modifications. The pooled spore stock was prepared and counted using a phase contrast microscope (Olympus BX40 phase contrast microscope) set to dark phase. The spore stock was vortexed briefly to ensure the spores were evenly distributed throughout the medium, and then a 1 in 10 dilution of the stock was prepared using distilled water. 5 µl of the diluted stock was pipetted onto a 0.02 mm depth Helber bacterial counting chamber with Thoma ruling (Hawksley, UK) and using a coverslip. It was viewed using a 40x objective and 10x eyepiece lenses. Spores were counted on 5 large squares, each of which was divided into 16 small squares. The squares chosen were the top left, top right, bottom left, bottom right and one random large square in the middle. Spores touching the top and left boundary of each large square were counted. The spore stock was counted in triplicate and the mean spore count was used to determine the concentration of spores per ml. The concentration of spores per ml was determined using the following formula:

$$Spores/ml = (Dilution \times Mean\ number\ of\ spores) \div (Number\ of\ small\ squares \times 5 \times 10^{-8})$$

The number of small squares is 80, and 5×10^{-8} is the volume of each small square.

4.2.4 Microsporidia spore dose preparation

Codling moth larvae were exposed to two spore concentrations: A high spore concentration of 1.0×10^7 spores/ml and a low concentration of 1.1×10^4 spores/ml. The stock spore concentration was adjusted using ddH_2O to the working concentrations using the following equation:

$$C_1V_1 = C_2V_2$$

C_1 is the initial spore concentration in spores/ml, C_2 is the required spore concentration in spores/ml, V_1 is the volume of the initial spore suspension to be added to make up the desired concentration, and V_2 is the final volume of the spore suspension required.

Tetracycline, an antibiotic, was added to selected treatments at a final concentration of 100 µg/ml.

4.2.5 Assay to determine the susceptibility of *Cydia pomonella* to the microsporidium

4.2.5.1 Exposure of neonate larvae to a high spore concentration

Surface-treated bioassays were conducted in 24-well plates using modified methods described by Malone & Wigley (1981a) and Siegel *et al.* (2001). The trial consisted of two treatments: a control consisting of ddH_2O and a treatment consisting of ddH_2O containing a final concentration of 1.0×10^7 spores/ml, which is one of the higher concentrations reported by Malone & Wigley (1981a). 25 µl of each treatment was added to cover the entire surface of the diet in each well and allowed to dry for 30 minutes. Twenty neonate larvae were subjected to each treatment. One neonate larva was added into each well using a 000 paint brush. Paper towel was folded and placed under the lid of each plate to prevent the larvae from escaping, after which the plates were sealed, wrapped in layers of paper towel secured with tape, and incubated in a controlled environment room set at 25 °C for 2 weeks. After 2 weeks, the plates were inspected and the number of live larvae was recorded. Larvae from each treatment were surface sterilised in 70 % ethanol, pooled and subjected to the spore extraction method described in Chapter 3. DNA was extracted using the CTAB method outlined in section 3.2.2 of Chapter 3 and PCR amplification of the small subunit rRNA gene was performed as described in section 3.2.3 of Chapter 3. PCR amplification of the bacterial 16S rDNA was also performed using the primer set fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Weisburg *et al.*, 1991). Each 25 µl reaction consisted of 1 µl

template DNA, 12.5 µl KAPA Taq ReadyMix, 1 µl of each 10 mM primer and 9.5 µl ddH_2O. The amplification of the genes was performed in a SimpliAmp thermal cycler with the following profile: initial denaturation at 95 °C for 10 minutes, 30 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 45 seconds, and a final elongation time of 10 minutes at 72 °C. The resulting products were analysed on a 1.0 % agarose gel to determine whether spores or bacteria were present in any of the larvae. If a bacterial band was present, it was sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing, and the identity was determined using BLAST. The experiment was only replicated twice since few larvae were recovered and the cause of mortality was bacterial infection.

4.2.5.1 Exposure of second instar larvae to a low spore concentration

Surface-treated bioassays were conducted in 24-well plates using modified methods described by Malone & Wigley (1981a) and Siegel *et al.* (2001). Once the *C. pomonella* eggs had hatched, the neonate larvae were transferred to un-inoculated diet-containing 24-well plates until they reached second instar. The trial consisted of four treatments: a control consisting of ddH_2O, a treatment consisting of ddH_2O containing a final concentration of 100 µg/ml tetracycline, a treatment of 1.1 x 10^4 spores/ml, and a treatment of 1.1 x 10^4 spores/ml containing a final concentration of 100 µg/ml tetracycline. This concentration was selected as it was the IC_{50} value (median concentration required to infect 50 % of the larvae) for second instar codling moth larvae inoculated with *N. carpocapsae*, as determined by Malone & Wigley (1981a). Tetracycline at a concentration of 100 µg/ml has been found to inhibit bacterial growth without affecting microsporidian spore viability (Pilley, 1978). 25 µl of each treatment was added to cover the entire surface of the diet in each well and allowed to dry for 30 minutes. Twenty second instar larvae were subjected to each treatment. One second instar larva was added into each well using a 000 paint brush. Paper towel was folded and placed under the lid of each plate to prevent the larvae from escaping, after which the plates were sealed, wrapped in

layers of paper towel secured with tape, and incubated in a controlled environment room set at 25 °C for 2 weeks. After 2 weeks, the plates were inspected and the number of live larvae was recorded. Larvae were weighed and the head capsule width was measured using an Olympus SZX 16 stereo microscope with Olympus Stream Motion software. The head capsule width was used to determine the instar of each larva according to Blomefield & Giliomee (2009). Larvae from each treatment were surface sterilised in 70 % ethanol, pooled and subjected to the spore extraction method described in Chapter 3. DNA was extracted using the CTAB method outlined in section 3.2.2 of Chapter 3 and PCR amplification of the small subunit rRNA gene was performed as described in section 3.2.3 of Chapter 3. The resulting products were analysed on a 1.0 % agarose gel to determine whether spores were present in any of the larvae. The experiment was replicated 3 times.

4.2.6 Statistical analysis

All statistical analyses were performed in R v. 3.4.1 (R Core Team, 2017). The percentage mortality data for the low and high spore concentrations and mass data were subjected to a Shapiro-Wilk test of normality to determine whether the data met the assumption of normality required for parametric statistical analyses. The null hypothesis for the Shapiro-Wilk test is that the data are normally distributed. The alternative hypothesis is that the data follow some other distribution. The null hypothesis is rejected in favour of the alternative hypothesis if the p-value is smaller than 0.05. The null hypothesis failed to be rejected for the high concentration mortality data, therefore Bartlett's test of homoscedasticity was conducted to determine whether the high concentration mortality data met the assumption of equal variances required for parametric statistical analyses. The null hypothesis for Bartlett's test is that all variances are equal. The alternative hypothesis is that variances differ to some degree. The null hypothesis is rejected in favour of the alternative hypothesis if the p-value is smaller than 0.05. The high concentration percentage mortality data met the assumptions of normality and homoscedasticity

required for parametric statistical analyses, therefore a parametric test was selected. The high concentration percentage mortality data consisted of two treatments, were unpaired and met the assumption of normality and equal variance, therefore Welch's t-test was performed in order to determine whether any differences in mortality between treatments were significant.

The percentage mortality data for the low concentration mortality failed to meet the assumption of normality required for parametric statistical analyses, therefore a non-parametric test was selected. The low concentration percentage mortality data consisted of more than three treatments, were unpaired and did not meet the assumption of normality. Therefore, a Kruskal-Wallis test was performed in order to determine whether any differences in percentage mortality between treatments were significant.

The mass data failed to meet the assumption of normality required for parametric statistical analyses, therefore a non-parametric test was selected. The independent variable was the treatment and the dependent variable was the mass of the larvae. The mass data consisted of more than three treatments, were unpaired and did not meet the assumption of normality. Therefore a Kruskal-Wallis test was performed in order to determine whether any differences in mass between treatments were significant.

The instar data were categorical, nominal, unpaired and the expected number in each cell was less than five. Therefore the data were analysed using Fisher's exact test to determine whether the instar reached was different between treatments. The null hypothesis for Fisher's exact test is that the variables are independent. The alternative hypothesis is that there is a significant association between variables. The null hypothesis is rejected in favour of the alternative hypothesis if the p-value is smaller than 0.05. Failure to reject the null hypothesis means that there is no significant interaction between the variables.

4.3 Results

4.3.1 Purification and enumeration of microsporidian spores

Microsporidian spores were subjected to Percoll® purification in order to obtain a contaminant-free inoculum to be used in experiments to determine whether the microsporidium isolated from carob moth was able to infect codling moth. After Percoll® purification some bacteria were still present in the inoculum. Bacteria were smaller (2.05 ± 0.04 μm in length) than microsporidia therefore only spores that were longer than 2.5 μm in length were enumerated.

4.3.2 Determination of microsporidian infection

4.3.2.1 High concentration experiment

Neonate codling moth larvae were inoculated with water or a high concentration of spores and water. The larvae were analysed for microsporidian infection by PCR amplification of the SSU rRNA gene region and for bacterial infection by PCR amplification of the 16S rDNA region. No SSU bands were observed for all replicates of every treatment (**Fig.4.1, lane 2**) and bacterial 16S bands were observed in the larvae inoculated with spores (**Fig.4.1, lane 3**).

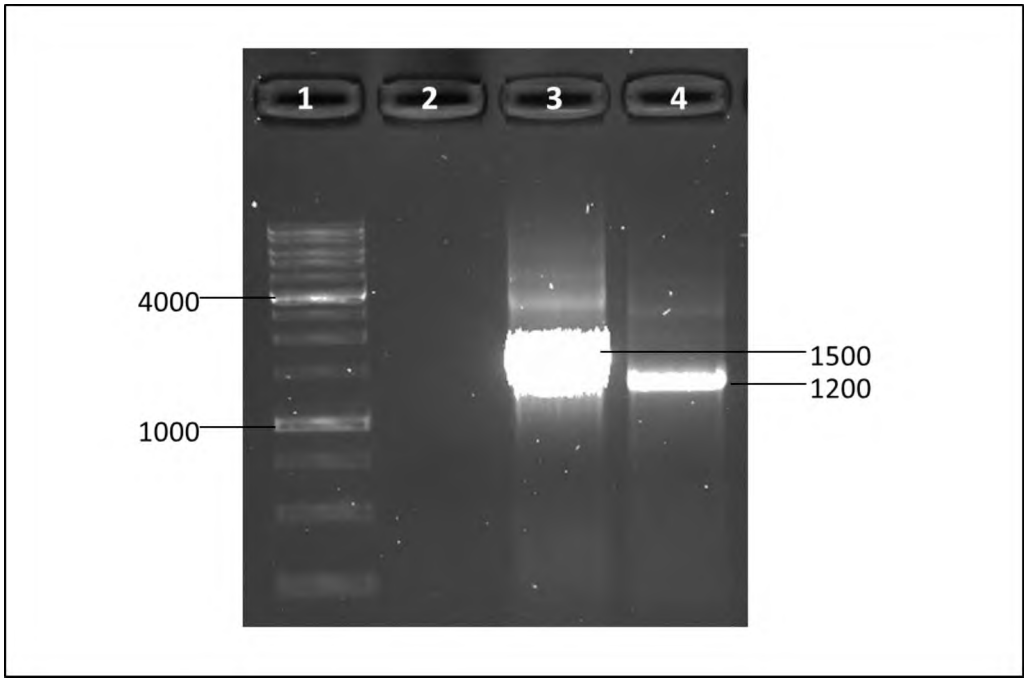


Fig.4. 1 A representative 1.0 % agarose gel of the results of the PCR of the microsporidian SSU rRNA gene and bacterial 16S rDNA region to determine whether microsporidian and bacterial infections were present after inoculation of codling moth larvae with a high microsporidian spore concentration. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: SSU rRNA amplification from larvae inoculated with spores, Lane 3: 16S rDNA amplification from larvae inoculated with spores, Lane 4: positive SSU rRNA control.

The 16S rDNA product was sequenced and the generated sequence was compared with GenBank archived sequences using BLAST. The bacterium in the infected codling moth larvae was identified as *Bacillus cereus* (99 % identity, e-value = 0.0).

4.3.2.2 Low concentration experiment

Second instar codling moth larvae were inoculated with water, water and antibiotic, spores and water, or spores and antibiotic. The larvae were analysed for microsporidian infection by PCR amplification of the SSU rRNA gene region. No SSU bands were observed for all replicates of every treatment (**Fig.4.2, lanes 2-5**).

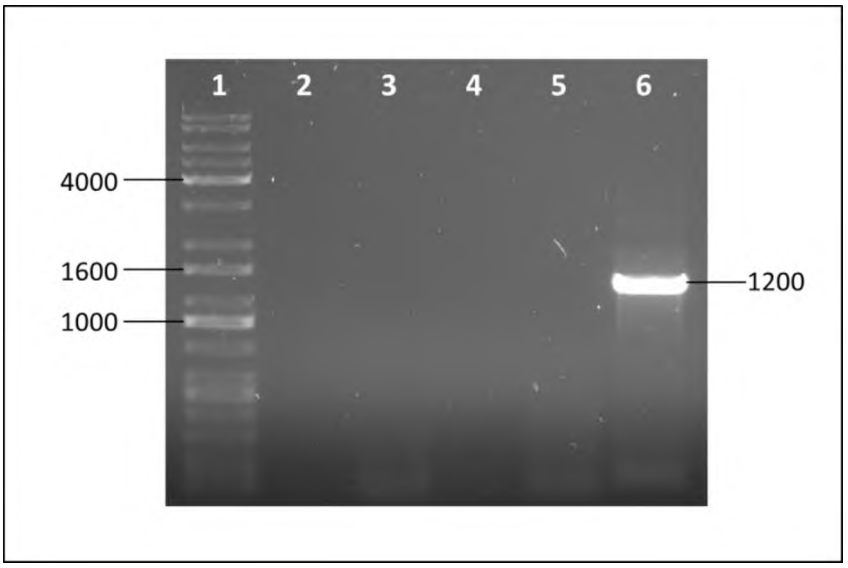


Fig.4. 2 A representative 1.0 % agarose gel of the results of the PCR of the microsporidian SSU to determine whether microsporidian infection was present after inoculation of codling moth larvae with a low microsporidian spore concentration. Lane 1: KAPA^{TM} Universal ladder in base pairs, Lane 2: water, Lane 3: water and antibiotic, Lane 4: spore and water, Lane 5: spore and antibiotic, Lane 6: positive SSU control.

4.3.3 Susceptibility of *Cydia pomonella* to the microsporidium

Larval mortality for each treatment in the high concentration experiment (**Table 4.1**) and low concentration experiment was calculated (**Table 4.2**). The average percentage mortality for each treatment was not calculated and adjusted according to the control mortality using Abbott's formula (Abbott, 1925) because in some cases the treatment mortality was lower than the control mortality. Since it was sufficiently clear that the microsporidium was not infecting codling moth larvae at the lower dose, the bioassays were not repeated. Second instar larvae in the low concentration experiment were weighed and their head capsule width was measured to determine the instar that each larva reached (**Table 4.3**).

Table 4. 1 Percentage mortality of neonate *Cydia pomonella* larvae in the high concentration experiment exposed to different treatments

	Treatment	Number exposed	Number dead	Mortality %
Replicate 1	Water (control)	20	2	10
Replicate 1	Spore and water	20	15	75
Replicate 2	Water (control)	20	6	30
Replicate 2	Spore and water	20	20	100

Table 4. 2 Percentage mortality of second instar *Cydia pomonella* larvae in the low spore concentration experiment exposed to different treatments

Replicate	Treatment	Number exposed	Number dead	Mortality %
Replicate 1	Water (control)	20	3	15
Replicate 1	Water and antibiotic	20	0	0
Replicate 1	Spores and water	20	4	20
Replicate 1	Spores and antibiotic	20	1	5
Replicate 2	Water (control)	20	1	5
Replicate 2	Water and antibiotic	20	0	0
Replicate 2	Spores and water	20	1	5
Replicate 2	Spores and antibiotic	20	1	5
Replicate 3	Water (control)	20	0	0
Replicate 3	Water and antibiotic	20	0	0
Replicate 3	Spores and water	20	3	15
Replicate 3	Spores and antibiotic	20	5	25

Table 4. 3 Average mass, head capsule width and instar of *Cydia pomonella* larvae in the low spore concentration experiment

	Treatment	Average mass	Standard error	Average head capsule width	Standard error	Instar
Replicate 1	Water	0.089	0.007	1.620	0.032	5
Replicate 1	Water and antibiotic	0.066	0.004	1.558	0.049	5
Replicate 1	Spore and water	0.084	0.005	1.570	0.044	5
Replicate 1	Spore and antibiotic	0.079	0.006	1.531	0.044	5
Replicate 2	Water	0.090	0.005	1.647	0.025	5
Replicate 2	Water and antibiotic	0.082	0.004	1.631	0.017	5
Replicate 2	Spore and water	0.091	0.006	1.702	0.015	5
Replicate 2	Spore and antibiotic	0.133	0.040	1.682	0.017	5
Replicate 3	Water	0.080	0.005	1.613	0.020	5
Replicate 3	Water and antibiotic	0.080	0.005	1.664	0.018	5
Replicate 3	Spore and water	0.087	0.005	1.705	0.031	5
Replicate 3	Spore and antibiotic	0.083	0.007	1.671	0.020	5

A Shapiro-Wilk test was conducted to determine whether the mass data and the high and low spore concentration mortality data were normally distributed. The null hypothesis that the mass data ($W = 0.33525$, $p < 2.2 \times 10^{-16}$) and low concentration mortality data ($W = 0.8366$, $p = 0.02518$) were normally distributed was rejected in favour of the alternative hypothesis at the 5 % level of significance, therefore the data did not follow a normal distribution. The null hypothesis that the high concentration mortality data was normally distributed failed to be rejected at the 5 % level of significance ($W = 0.94853$, $p = 0.707$), therefore the data followed a normal distribution.

Since the assumption of normality was not met for the low concentration mortality data, a non-parametric test was conducted to determine whether there were statistically significant differences between treatments. The null hypothesis that there were no statistically significant

differences between the treatments failed to be rejected at the 5 % level of significance (Kruskal Wallis X^2 = 6.4063, p = 0.0934), therefore treatment had no statistically significant effect on low concentration mortality.

Since the assumption of normality was met for the high concentration percentage mortality data, Bartlett's test of homogeneity of variances was conducted to determine whether variances did not differ significantly. The null hypothesis failed to be rejected at the 5 % level of significance (Bartlett's K^2 = 0.0329, p = 0.856), therefore the high concentration mortality data was homoscedastic. The assumptions of normality and equal variances were met, therefore a parametric statistical test could be performed to analyse the data. A Welch's t-test was conducted to determine whether there were statistically significant differences in mortality between treatments. The null hypothesis that there were no statistically significant differences between the treatments just failed to be rejected at the 5 % level of significance (t = 4.2167, p = 0.057). The difference in mortality between treatments was taken to be significant because the p-value was close to 0.05.

Fisher's exact test was used to determine whether treatment had a significant effect on the instar reached, which is an indication of larval development. The null hypothesis that the variables were independent failed to be rejected at the 5 % level of significance (p = 0.24), meaning treatment had no significant effect on larval development.

4.4 Discussion

In order to determine whether an organism is the cause of disease and mortality, it needs to be able to induce a similar infection when inoculated into the same host or an alternative host. In this chapter an alternative host, codling moth, was inoculated with either a high or low concentration of the microsporidium isolated from carob moth. Mortality in the high concentration experiment was significant, however the cause of mortality was a bacterial

infection. No signs of infection were observed and no microsporidia were isolated from codling moth larvae inoculated with a low spore concentration. No statistically significant mortality was observed in any of the treatments, and inoculation with spores, antibiotic, or spores and antibiotic did not affect larval mass or development significantly.

There are no standard methods for infection of alternative hosts with microsporidia. Infective concentrations need to be determined by experimentation: a concentration that is too high may result in death from bacterial septicaemia instead of microsporidian infection (Pilley, 1976; Vávra & Maddox, 1976; Chakrabarty *et al.*, 2012), while a concentration that is too low may result in little or no infection (Solter *et al.*, 2012). An initial experiment was conducted in which neonate codling moth larvae were inoculated with a high spore concentration of 1×10^7 spores/ml. Mortality was significant, but it was determined that the larvae died from infection by *Bacillus cereus*. *Bacillus cereus* is commonly found in the gut of invertebrates where it forms a symbiotic relationship with the host (Jensen *et al.*, 2003).

The cause of bacterial infection may be due to the concentration of the microsporidia inoculum being too high. If a susceptible host ingests a large number of spores, septicaemia may arise from gut bacteria invading the haemocoel due to damage caused to the wall of the insect gut by microsporidian polar filaments (Pilley, 1976; Vávra & Maddox, 1976). Other possible causes of infection may be contaminating bacteria in the inoculum or an interaction between the microsporidium isolated from the carob moth and the bacterium found in the inoculum. Pierce *et al.* (2001) found that larvae of the European corn borer, *Ostrinia nubilalis*, infected with *Nosema pyrausta* were more susceptible to infection by the bacterium *Bacillus thuringiensis*.

In order to ensure bacterial contaminants were not affecting results, a treatment containing spores with tetracycline was included in the experiment. Tetracycline has been found to inhibit bacteria without affecting microsporidian spore viability (Pilley, 1978). A treatment consisting of water and tetracycline was also included to ensure the antibiotic did not have a negative

effect on codling moth larval mass and development, which was confirmed by the results. Second instar larvae were inoculated with a lower concentration of spores to determine whether the spore concentration was causing bacterial septicaemia. No significant mortality occurred in any of the treatments, and none of the treatments had a significant effect on larval mass or development. The presence of bacteria in the inoculum did not have an effect on larval mortality or development as the differences in mortality and development between the spore and water treatment and spore and antibiotic treatment were not significant.

No microsporidian infection was detected in any larvae, therefore the concentration of spores to which the larvae were exposed may be too low, or codling moth may not be susceptible to the species of microsporidium isolated from the carob moth. The spore concentration was chosen as it was the IC_{50} value determined for second instar codling moth larvae infected with *Nosema carpocapsae* (Malone & Wigley, 1981a). *Nosema carpocapsae* is closely related to the microsporidium isolated from carob moth (see Chapter 3) and infects codling moth, therefore it was hypothesised that codling moth may be susceptible to the carob moth microsporidium. Second instar larvae were selected as Malone & Wigley (1981a) reported that they required the lowest concentration of *N. carpocapsae* to infect 50 % of the codling moth larvae. The surface inoculation method has been used to infect lepidopteran hosts with microsporidia in other studies (Fuxa, 1979; Solter *et al.*, 1997; Solter & Maddox, 1998; Pierce *et al.*, 2001), however this method may not be as effective as other methods such as droplet feeding and the diet plug method, and requires a large number of spores. Due to the collapse of the carob moth colony, the number of microsporidian spores available for use in experiments was limited. Ideally a dose-response bioassay in which larvae are exposed to a series of dilutions should be conducted to determine whether a host is susceptible to microsporidian infection and to determine the point at which bacterial septicaemia occurs (Solter *et al.*, 2012). A dose-response bioassay could not be conducted in this study because few spores were available for experimentation.

Inoculation of codling moth larvae with a higher spore concentration may result in infection, however more microsporidian spores would need to be obtained or an alternative feeding method would have to be utilised. The droplet feeding method involves offering a starved host a small droplet of water containing a known number of microsporidian spores on a bacterial inoculating loop (Solter *et al.*, 2012). Larvae are required to consume the entire droplet otherwise they are excluded from the experiment. This method is advantageous as an Infective Dose (ID) can be calculated and only a small amount of inoculum is required.

Codling moth may not be susceptible to infection by the microsporidium isolated from carob moth. Research on the host range of different species of microsporidia isolated from Lepidoptera has been conducted by Solter *et al.* (1997), Solter *et al.* (2000) and Chakrabarty *et al.* (2012). Solter *et al.* (1997) tested the susceptibility of 49 non-target lepidopteran species to a high dose (10^5 spores/µl) or a low dose (10^3 spores/µl) of five closely related biotypes of microsporidia: *Microsporidium* sp. MP, *Microsporidium* sp. MR, *Microsporidium* sp. MS, *Nosema lymantriae* and *Endoreticulatus* sp. EP. The responses were placed in the following categories: 1) no infection, 2) atypical infection, and 3) heavy infection. They found that certain non-target hosts were not susceptible to infection by some of the microsporidium isolates, even when exposed to a high spore dose. The isolates varied in their ability to infect the non-target hosts: more of the non-target species were resistant to the MP biotype and *N. lymantriae* was the most virulent isolate, indicating that *N. lymantriae* may have a broader host range. This research indicates that even closely related microsporidia vary in their ability to infect alternative hosts. Solter *et al.* (2000) found that several *Nosema* and *Vairimorpha* species were unable to infect *Lymantria dispar* and *Spodoptera exigua*, and postulated that the host range of lepidopteran microsporidia may be narrower than previously thought. Chakrabarty *et al.* (2012) found that *Nosema ricini* isolated from the eri silkworm, *Philosamia ricini*, was unable to infect the mulberry silkworm, *Bombyx mori*, and the tasar silkworm, *Antheraea mylitta*, despite being

administered a dose of 1.52 x 10^8 spores/ml. They also found that *Nosema bombycis*, *N. mylitta* and *N. assamensis* were unable to infect *P. ricini*. The above studies give evidence that non-target hosts may not be susceptible to infection by certain species of microsporidia but are susceptible to a closely related species, and that the host ranges of microsporidia vary. This may explain why the codling moth is susceptible to infection by *Nosema carpocapsae*, but no infection by the closely related microsporidium species isolated from carob moth was detected.

In conclusion, codling moth was unable to be infected with the microsporidium isolated from carob moth. This may be due to exposure to a dose that is too low, or codling moth may not be susceptible to the microsporidium. The pathogenicity of the microsporidium isolated from the carob moth could thus not be confirmed.

Chapter 5: General discussion

Carob moth is an important pest of a variety of crops, yet the pest status of carob moth on citrus in South Africa was poorly understood. In order to mitigate damage caused by a pest in the field, its biology needs to be investigated under laboratory conditions. A carob moth colony was initiated for this purpose, but failed to establish due to microbial infection. The aim of this study was to isolate, identify and genetically characterise the causal agent of colony collapse as this information is invaluable for understanding the organism in question, and can aid the development and application of protocols to suppress microsporidian infection from cultures and prevent further contamination.

An unknown organism was isolated from diseased carob moth larvae reared under laboratory conditions. The organism was viewed using transmission electron microscopy and was similar in morphology to microsporidian spores observed in studies by Malone & Wigley (1981b), Lange (1991), Vavra *et al.* (2006), Kwak *et al.* (2013) and Ptaszyńska *et al.* (2014). The morphological identification allowed for selection of a more robust method of classification using molecular methods. The region most commonly used for species identification of microsporidia is the region encoding the ribosomal rRNA consisting of the small subunit (SSU) rRNA gene, the internal transcribed spacer (ITS) and the large subunit (LSU) rRNA gene. These form part of the rRNA cistron consisting of the SSU, ITS, LSU, IGS and 5S regions. Microsporidia-specific primer sets were selected based on published literature (Zhu *et al.*, 1994; Baker *et al.*, 1995; Tsai *et al.*, 2002; Huang *et al.*, 2008; Chen *et al.*, 2012; Liu *et al.*, 2012) and were used to amplify the region encoding the rRNA . The PCR was successful and the full rRNA cistron was assembled. BLAST analysis showed that the microsporidium isolated from carob moth is a novel and genetically uncharacterised species that has a high identity with members of the genus *Nosema*. The *Nosema* group consists of the "true" *Nosema* clade and the *Nosema* / *Vairimorpha* clade (Baker *et al.*, 1994). Phylogenetic analysis based on SSU and LSU

sequence data revealed that the microsporidium isolated from carob moth clustered with the *Nosema* / *Vairimorpha* clade, which has the typical rRNA arrangement of 5'-SSU-ITS-LSU-IGS-5S-3' (Choi *et al.*, 2011). This arrangement was confirmed using specific primer sets. Few full LSU and IGS sequences are available for the *Nosema* / *Vairimorpha* clade on GenBank, therefore sequence information about the entire rRNA cistron of a novel *Nosema* species can contribute important genetic information for classification of species within the group.

The cause of carob moth larval mortality is unknown since both microsporidian spores and bacteria were isolated from cadavers. The bacterium in the infected codling moth larvae was identified as *Bacillus cereus*, using PCR amplification and sequencing of the 16S rDNA region. *Bacillus cereus* is commonly found in the gut of invertebrates where it forms a symbiotic relationship with the host (Jensen *et al.*, 2003). Microsporidia usually cause sub-lethal effects, however the host can become more susceptible to other opportunistic microbial infections, which ultimately result in mortality. Pierce *et al.* (2001) found that larvae of the European corn borer, *Ostrinia nubilalis*, were more susceptible to infection by the bacterium *Bacillus thuringiensis* when already infected by the microsporidium *Nosema pyrausta*. It has been suggested that lethal bacterial infection could be caused by the release of opportunistic bacteria from the insect gut due to damage caused by exposure to a high dose of microsporidian spores (Pilley, 1976; Vávra & Maddox, 1976; Chakrabarty *et al.*, 2012). The extrusion of a large number of microsporidian polar filaments damages the insect gut, releasing gut bacteria which kill the host before microsporidian infection can establish. Fuxa (1981) reported that the cause of mortality was different when six lepidopteran pest species were inoculated with a high and low dose of *Vairimorpha necatrix* spores. Mortality of larvae exposed to a low dose of spores was caused by microsporidiosis, while mortality of larvae exposed to a large spore dose was caused by bacterial septicaemia. Two scenarios may explain the presence of two potentially pathogenic organisms in the deceased carob moth larvae. Firstly, the laboratory rearing

procedures may have been sub-optimal, causing larvae to become stressed and allowing the microsporidium to multiply and cause an acute infection. Walters & Kfir (1993) found that stressed *Chilo partellus* (Lepidoptera: Pyralidae) larvae exhibited higher mortality rates from an acute rather than chronic infection by the microsporidium *Nosema partelli*. Mortality from acute microsporidium infection could have resulted in a high dose of microsporidian spores being released onto a concentrated part of the artificial diet, either through frass or when a larva died, and a high dose of spores could be ingested by other larvae. Ingestion of numerous spores may have resulted in damage to the gut, releasing an opportunistic bacterium, which infected the host and resulted in mortality. Secondly, the microsporidium infection may have caused larvae to become more susceptible to infection by *B. cereus* present in the environment, which ultimately caused mortality. Spore extractions in this study were performed on pooled sets of cadavers, therefore some larvae may have died from microsporidium infection and others from a secondary bacterial infection. Further research is required to establish the ultimate cause of mortality. However, whether the ultimate cause of death or simply a catalyst, the microsporidian is still contributing towards the collapse of the host culture and must be brought under control.

In order to determine whether an organism is the cause of mortality, it must be able to induce the same symptoms in the host upon reinfection (Lacey, 2012). The carob moth colony collapsed, therefore no hosts were available to test the pathogenicity of the microsporidium and an alternative host had to be selected. The microsporidium isolated from carob moth shares a high identity with *Nosema carpocapsae*, a microsporidium known to infect codling moth, *Cydia pomonella* (Malone & Wigley, 1981a; b). Codling moth is an important pest species that is easy to rear and eggs could be sourced, therefore it was selected as a potential alternative host for infectivity studies. A spore concentration of 1×10^7 spores/ml was administered to neonate codling moth larvae using the surface dose method (Malone & Wigley, 1981a). The percentage

mortality was high, but microsporidian infection was not the ultimate cause of death. Upon examination of the deceased larvae, mortality was observed to be as a result of a bacterial infection and not infection by the microsporidian spores fed to the larvae. By sequencing the 16S rDNA region, the bacterium was identified as *B. cereus*, which is the same bacterium discovered in diseased carob moth larvae from this study. *Bacillus cereus* spores were identified in the purified inoculum used to infect the neonate larvae, but the microsporidian spores were observed to far outnumber the bacterial spores. As mentioned above, ingestion of relatively high doses of microsporidian spores can lead to bacterial septicaemia. Neonate mortality may have been caused by ingestion of a large number of microsporidian spores, resulting in mortality caused by bacterial septicaemia, or mortality may have been caused by *B. cereus* spores in the inoculum.

A second experiment to investigate pathogenicity towards the alternative host was conducted where a spore concentration of 1.1×10^4 spores/ml was administered to second instar codling moth larvae following the method used by Malone & Wigley (1981a). Since the inoculum contained bacteria after purification, tetracycline was included in two of the treatments to ensure that bacterial infection was not caused by contaminated inoculum. Tetracycline has been found to inhibit bacteria without affecting microsporidian spore viability (Pilley, 1978). No significant mortality occurred in any of the treatments and no microsporidian infection was detected. No bacterial infection was detected in any of the treatments, including the treatments that did not contain tetracycline. Malone & Wigley (1981a) did not observe significant mortality in codling moth larvae inoculated with *Nosema carpocapsae*, but did manage to establish an infection. It is possible that the spore concentration administered to the codling moth larvae in this study was too low to cause microsporidian infection or that second instar larvae are more resistant to microsporidian infection, which has been observed in *Spodoptera littoralis* and *Spodoptera exigua* (Lepidoptera: Noctuidae) (Al Fazairy, 1986). The fact that no

bacterial infection was observed in larvae inoculated with spores that were not treated with tetracycline may indicate that the bacterium is not the primary cause of mortality or that older larvae are more resistant to bacterial infections. To establish whether codling moth is in fact susceptible to infection by the microsporidium isolated from carob moth, further investigation is required.

One of the main challenges faced in this study was annotation of the rRNA cistron. No general rules and regulations exist for annotation of microsporidian rRNA cistrons, resulting in discrepancies between annotated cistrons available on GenBank. This means that the annotations for the rRNA cistron of the carob moth microsporidum based on alignments with the rRNA cistron of a closely related species may differ from the annotations produced when aligned with annotated rRNA cistrons from an alternative closely related species. The sequence information can only be submitted to the GenBank database once the annotation problem is resolved. The use of annotation software is also not feasible as most genome annotation software relies on trends in the genome to determine coding and intergenic regions (Peyretaillade *et al.*, 2012). Since only the rRNA cistron was sequenced, no trends could be identified by the software to "learn" how to annotate such a small region. The microsporidian intergenic spacer region differs greatly within a group, therefore annotating by alignment with related species is difficult and largely unsuccessful. The annotations produced in this study are based on alignments of the SSU, ITS, LSU and 5S rRNA regions of annotated sequences from closely related species, from which the position of the IGS was inferred by determining the end of the LSU and the beginning of the 5S rRNA regions. Consequently, the accuracy of the rRNA cistron annotations produced in this study is dependent on the accuracy of the related rRNA cistrons' annotations.

Future work requires the establishment of a new carob moth colony in order to obtain specimens for experimentation and to isolate more spores. Sequencing of additional genes

should be performed in order to contribute to the limited genetic data available for members of the *Nosema* / *Vairimorpha* group. Full genome sequencing will be the next step to obtain more information about the microsporidium. The first microsporidian genome sequenced was from *Encephalitozoon cuniculi* and was approximately 2.9 Mbp (Katinka *et al.*, 2001). There are now 27 constructed full genomes available on GenBank, with only three of these belonging to *Nosema* species. *Nosema bombycis*, *N. apis* and *N. ceranae* have a genome size of 15.7 Mbp, 8.6 Mbp and 6.7 Mbp respectively. The scarcity of microsporidian full genome data sets may be due to the extremely divergent nature of their genome and that conventional approaches to annotating these genomes are not suitable (Peyretaillade *et al.*, 2012). Additional sequence data will provide valuable information for further comparison with other microsporidia and for understanding the complex nature of the microsporidian genome, and can provide an understanding of biological processes that govern microsporidian development (Peyretaillade *et al.*, 2012). Currently full genome sequencing is expensive, time-consuming and requires a significant amount of genomic DNA therefore it could not be attempted in this study. However the development of next-generation DNA sequencing technology is advancing rapidly and soon will enable inexpensive and routine methods for comprehensive genome analysis (Shendure & Ji, 2008).

The biological activity of the microsporidium against carob moth should be determined using a dose response assay. If the larvae can be reinfected, more spores would be available for further experimentation. The effects of microsporidian infection on various life cycle parameters could be investigated to determine whether the microsporidium is the cause of colony collapse. Bioassays in which carob moth larvae are inoculated with different ratios of the microsporidium and the bacterium could be conducted to see how the two organisms interact and the effects they have on percentage mortality and median lethal time (LT_{50}).

The utility of microsporidia as biological control agents has been reported by Bjørnson & Oi (2014). Microsporidia generally cause chronic infections in insects, affecting fecundity, longevity and fitness. Since most species of microsporidia that are hosted by insects do not cause acute infections that result in rapid mortality, they generally are not considered to be useful as biopesticides. Three species of microsporidia have established and spread after being released as biological control agents: *Paranosema locustae*, *Nosema pyrausta*, and *Knealhazia solenopsae* (Bjørnson & Oi, 2014). The use of *Paranosema locustae* as a biological control agent has been reviewed by Lockwood *et al.* (1999) and is summarised below. *Paranosema locustae* is released for control of various species of rangeland grasshoppers, and causes a slow debilitating disease (Lockwood *et al.*, 1999; Bjørnson & Oi, 2014). Initial reports stated that the microsporidium caused chronic effects and was efficiently transmitted, therefore should be considered as a biological control agent. Recent field studies to examine the efficacy of *P. locustae* reported that the microsporidium caused inconsistent reductions in fecundity between generations, transmission between seasons was low, and mortality was low. *Nosema pyrausta* is naturally present in the European corn borer, *Ostrinia nubilalis*, and has a regulatory effect on natural populations (Lewis *et al.*, 2009; Bjørnson & Oi, 2014). *Nosema pyrausta* was considered as a biological control agent as it causes chronic, debilitating effects on the host, and mortality occurs when the host is stressed or when other control methods are implemented in conjunction with inoculative spore release (Lublinkhof & Lewis, 1980; Lewis *et al.*, 2009). The problem with using *Nosema pyrausta* as a biological control agent is that it is difficult to mass-produce, and has been found to negatively impact parasitoids of *O. nubilalis* (Sajap & Lewis, 1988; Lewis *et al.*, 2006). *Knealhazia solenopsae* is naturally present in populations of the red fire ant, *Solenopsis invicta*, so was considered as a biological control agent. *Knealhazia solenopsae* causes reduction in oviposition rates and longevity, but transmission is dependent on the number of queens and whether colonies are territorial, or whether they share resources and move between different nests (Williams *et al.*, 1999; Oi & Williams, 2003; Bjørnson & Oi,

2014). Transmission of *Knealhazia solenopsae* depends on interactions occurring between different nests, otherwise the microsporidium is not spread (Oi, 2006).

Thus, if they present little promise as biological control agents against pest species, is it possible to reduce their impact on beneficial species? It is possible to eliminate microsporidia from laboratory colonies, but there is no standard method. One of the earliest methods for removing microsporidia from insect cultures was the Pasteur and Cantoni technique devised to eliminate *Nosema bombycis* in silkworm cultures (Boohene *et al.*, 2003). The method involved isolating individual females for oviposition and subsequently determining the infection status of the females (Boohene *et al.*, 2003). The progeny of healthy females were pooled to found an uninfected colony (Boohene *et al.*, 2003). The problem with this technique is that it is labour-intensive and requires a large number of healthy insects to ensure a genetically diverse colony. Heat therapy has also been used successfully in some cases to reduce or eliminate infection from insects. Heat therapy works on the principle that the thermal tolerance of the host is greater than that of the parasite, however this is not true in all cases (Becnel & Andreadis, 2014). Kfir & Walters (1997) reported that *Nosema partelli* infection levels in the stem borer, *Chilo partellus*, larvae were significantly reduced when larvae hatched from eggs that were exposed to 47 °C for 25 minutes or 49 °C for 20 minutes. The larvae that survived the heat treatment were used as founders of a new clean colony. A number of drugs have been used including Fumagillin, an antibiotic derived from the fungus *Aspergillus fumigatus*, the antibiotic Rifampicin, Benomyl and Albendazole (Boohene *et al.*, 2003; Van Frankenhuyzen *et al.*, 2004; Becnel & Andreadis, 2014). Fumagillin was found to suppress microsporidian infections caused by *Nosema* species in honey bees, *Apis mellifera* and the boll weevil, *Anthonomus grandis*; however it did not have a significant effect on *Nosema muscidifuracis* in *Muscidifurax raptor* (Becnel & Andreadis, 2014). Prolonged exposure to Fumagillin has been shown to delay larval development and caused significant reductions in larval survival to

pupation, fecundity and female pupal weight (Van Frankenhuyzen *et al.*, 2004). Rifampicin has been shown to reduce microsporidian infection in some species infected with a *Nosema* species, but not in others (Boohene *et al.*, 2003). Benomyl has also been effective to a degree, however prolonged exposure negatively affects larvae (Becnel & Andreadis, 2014). Sanitation is extremely important, especially if the microsporidium is not transmitted transovarially. All equipment and work stations must be sterilised, usually by wiping them down with a 2.5 to 5 % bleach solution (Rebelo, 2002). All dead specimens must be separated from the eggs in order to reduce horizontal transmission. In some cases eggs can be treated with bleach in order to destroy spores attached to the egg surface, however the optimum bleach concentration and exposure time needs to be established to ensure microsporidia are destroyed while not damaging the eggs.

In order to start a new carob moth colony, moths need to be collected from the field. These moths should then be used as the founders of a new colony. Research needs to be performed to determine the temperature and duration of exposure that will inhibit microsporidia without causing damage to the moths. Once this has been determined, eggs should be heat treated, and the larvae that hatch should be checked for microsporidian infection. Egg batches in which microsporidian infection is discovered should be discarded, and microsporidian-free batches should be used to establish a new clean colony. Strict sanitation protocols must be put in place to ensure that transmission of microsporidia is limited. The colony should be checked often, and dead larvae should be removed and discarded. Surfaces and equipment should be bleached to prevent accidental contamination. Moths obtained from the field to supplement the laboratory colony should be quarantined and checked for microsporidia before being introduced into the colony. The conditions in which the carob moth is reared may need to be optimised to ensure moths are healthy and able to fight off infection.

The pathogenicity of the microsporidium against codling moth or other related species requires further study and the experimental design needs to be optimised. Once more spores are obtained it will be possible to inoculate later instars with higher spore concentrations. The insects may need to be stressed in order to become infected, therefore the effect of stressors such as starvation, over-crowding and extreme temperatures on susceptibility of codling moth larvae should be investigated. An alternative feeding method such as droplet feeding may be more appropriate as smaller volumes are required and lethal doses rather than concentrations can be determined. Since bacterial contaminants were still present after Percoll® purification, the addition of antibiotics to inhibit bacteria should be included in the purification protocol. Once the experimental procedures have been optimised, the infectivity of the microsporidium against other closely related hosts, such as hosts within the family Pyralidae, could be tested.

In conclusion, a microsporidium was isolated successfully from diseased carob moth larvae and was identified as a microsporidium belonging to the *Nosema* / *Vairimorpha* group. This group has not been studied extensively and few full cistron sequences have been deposited in GenBank. This study is therefore significant as it can provide additional valuable information to other researchers working on microsporidia infecting insects. The results of this study are a first step towards initiating a healthy carob moth colony for further study of the pest in South Africa.

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Supplementary material

S 1 Nucleotide sequence of amplified regions

CACCAGGTTGATTCTGCCTGACGTAGACGCTATTCCCTAAGATTAACCCATGCATGT
TTTTGACATTTATTTGAAAAATGGACTGCTCAGTAATACTCACTTTATTTAATGTAC
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GCAGGATCATAATAGTTAATTTAATATTATATTTTTTTATTATTATTTATTTTTACC
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GTA

Small subunit rRNA gene

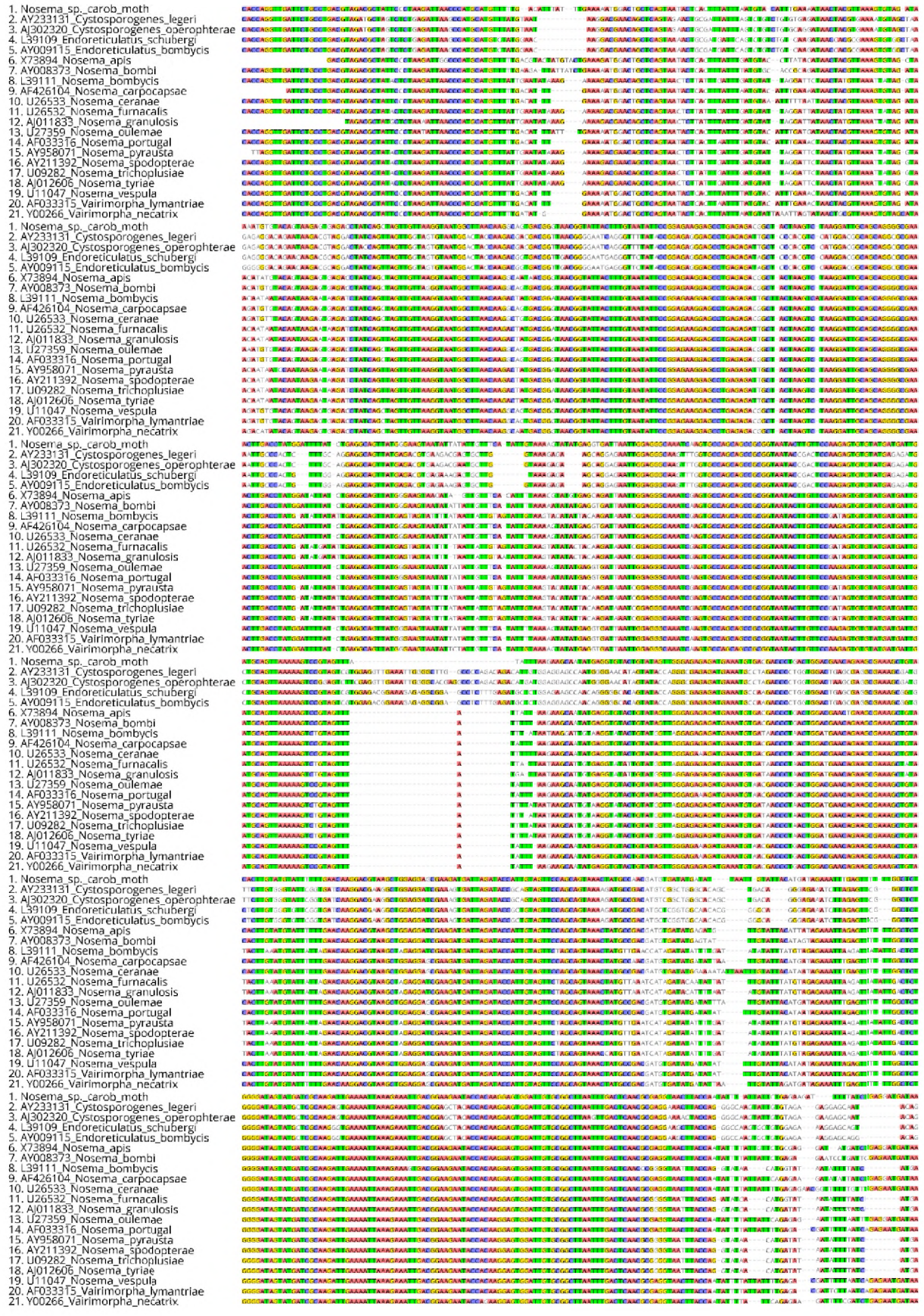
Internal transcribed spacer

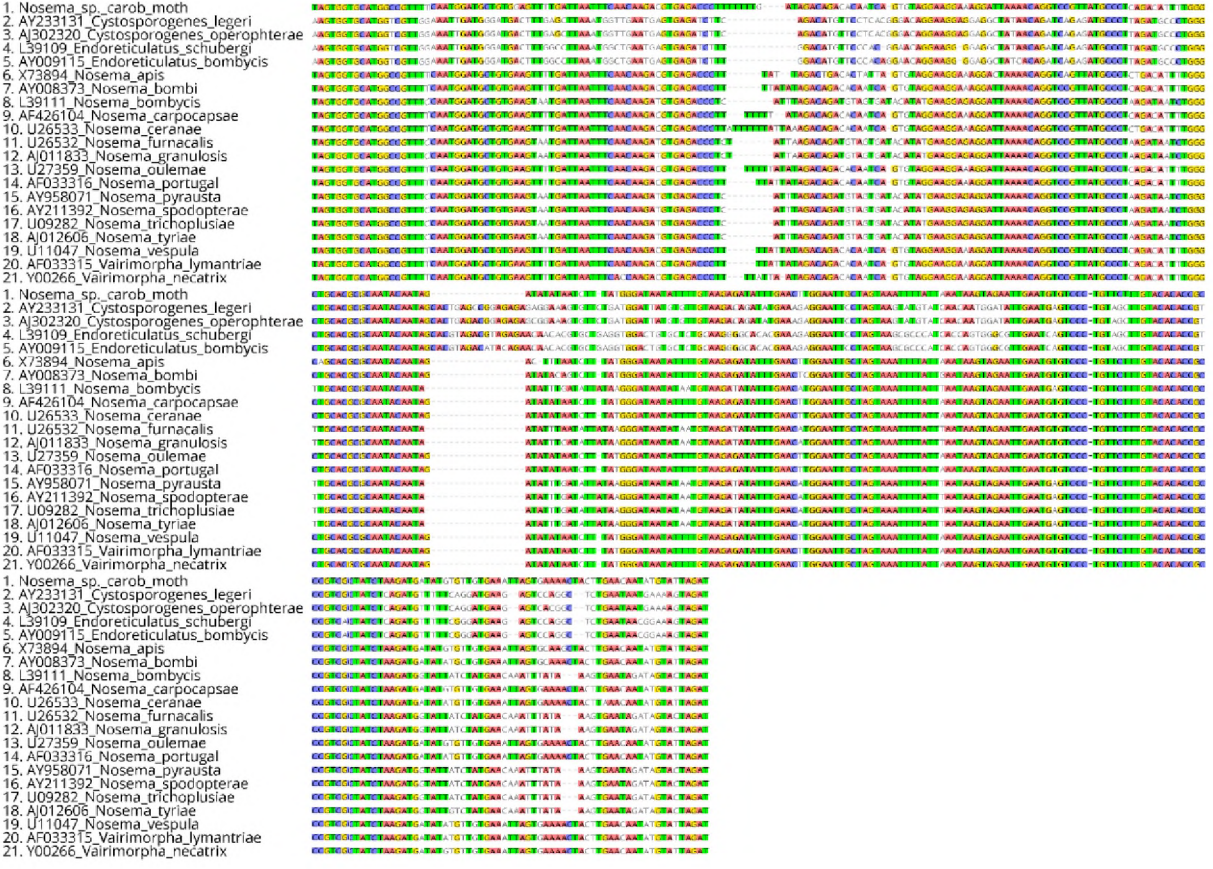
Large subunit rRNA gene

Intergenic spacer

5S rRNA gene

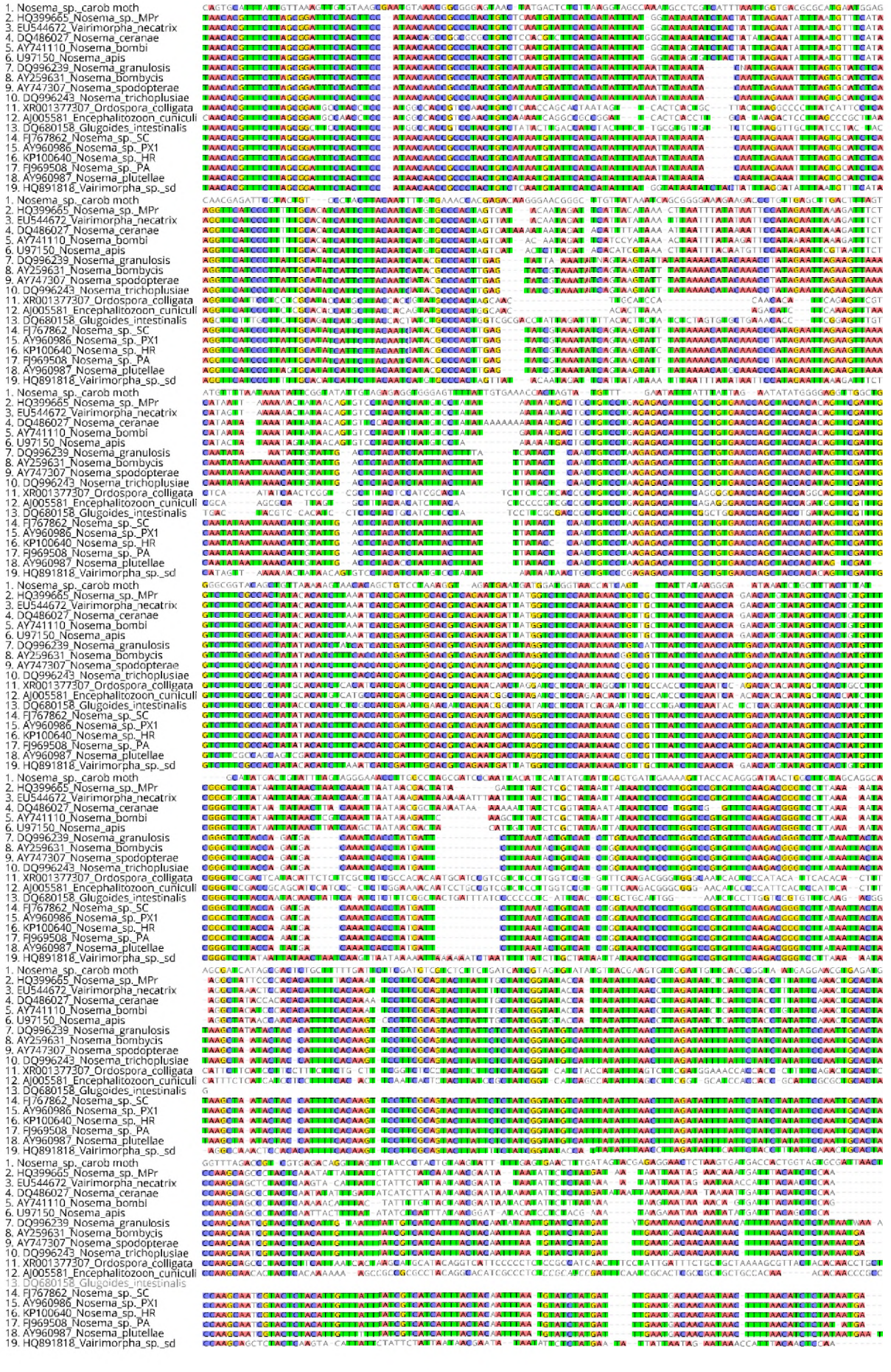
S 2 Alignment of SSU rRNA genes




1. Nosema_sp._carob_moth
2. AY233131_Cystosporogenes_legeri
3. AJ302320_Cystosporogenes_operophterae
4. L39109_Endoreticulatus_schubergi
5. AY009115_Endoreticulatus_bombycis
6. X73894_Nosema_apis
7. AY008373_Nosema_bombi
8. L39111_Nosema_bombycis
9. AF426104_Nosema_carpocapsae
10. U26533_Nosema_ceranae
11. U26532_Nosema_furnacalis
12. AJ011833_Nosema_granulosis
13. U27359_Nosema_oulemae
14. AF033316_Nosema_portugal
15. AY958071_Nosema_pyrausta
16. AY211392_Nosema_spodopterae
17. U09282_Nosema_trichoplusiae
18. AJ012606_Nosema_tyriae
19. U11047_Nosema_vespula
20. AF033315_Vairimorpha_lymantriae
21. Y00266_Vairimorpha_necatrix

S 3 Alignments of LSU rRNA genes


1. Nosema_sp._carob moth
2. HQ399665_Nosema_sp._MPr
3. EU544672_Vairimorpha_necatrix
4. DQ486027_Nosema_ceranae
5. AY741110_Nosema_bombi
6. U97150_Nosema_apis
7. DQ996239_Nosema_granulosis
8. AY259631_Nosema_bombycis
9. AY747307_Nosema_spodopterae
10. DQ996243_Nosema_trichoplusiae
11. XR001377307_Ordospora_colligata
12. AJ005581_Encephalitozoon_cuniculi
13. DQ680158_Glugoides_intestinalis
14. FJ767862_Nosema_sp._SC
15. AY960986_Nosema_sp._PX1
16. KP100640_Nosema_sp._HR
17. FJ969508_Nosema_sp._PA
18. AY960987_Nosema_plutellae
19. HQ891818_Vairimorpha_sp._sd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7. DQ996239_Nosema_granulosis
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9. AY747307_Nosema_spodopterae
10. DQ996243_Nosema_trichoplusiae
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17. FJ969508_Nosema_sp._PA
18. AY960987_Nosema_plutellae
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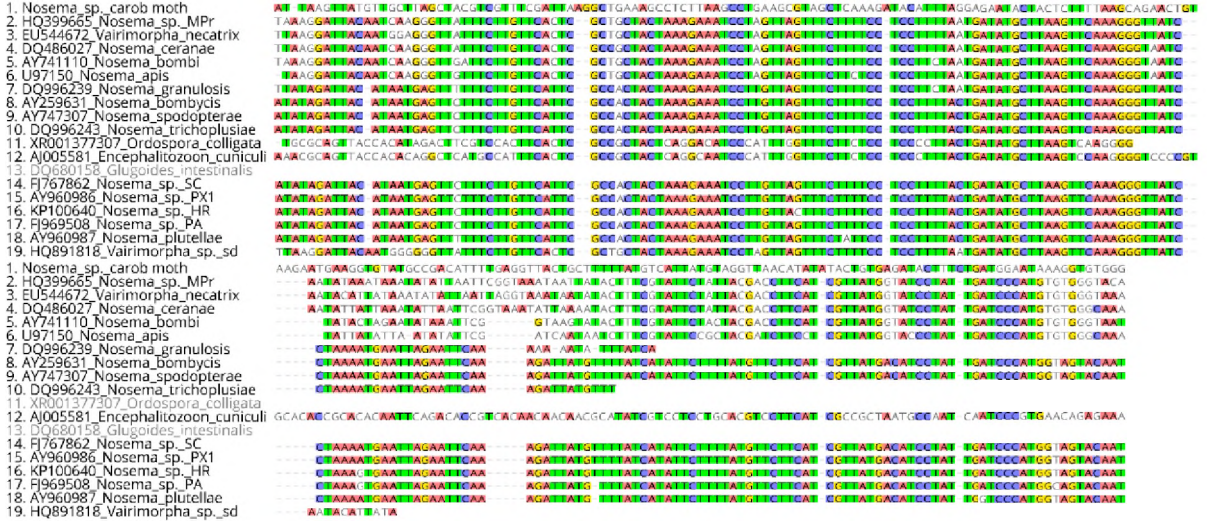
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16. KP100640_Nosema_sp._HR
17. FJ969508_Nosema_sp._PA
18. AY960987_Nosema_plutellae
19. HQ891818_Vairimorpha_sp._sd