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Nosema Disease in Honeybees

Genetic Variation and Control

A report for the Rural Industries Research and Development Corporation

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Foreword

The first objective of this report was to locate and then determine the base composition of structural DNA sequences specific to the adult honeybee parasite *Nosema apis. N. apis*, the causative agent of nosema disease in honeybees. This disease is considered the most economically important disease of adult honeybees in Australia and the world (Bailey and Ball, 1991; Matheson, 1993a).

The second objective was to use the DNA sequence data to (a) determine the taxonomic relatedness of *N. apis* to other microsporidia, (b) assess genetic variation between Australian isolates of *N. apis*, and (c) determine whether detection of virulent and avirulent isolates of *N. apis* is possible.

An additional objective was included to examine the potential of a novel control method for nosema disease and a model system was designed to test the effectiveness of thymol as a novel control agent for nosema disease.

This project was funded from industry revenue which is matched by funds provided by the federal government and is an addition to RIRDC's diverse range of over 600 research publications. It forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian beekeeping industry.

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Peter Core
Managing Director
Rural Industries Research and Development Corporation

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I would also like to thank the staff, both technical and administrative, of the CSIRO Division of Entomology, Black Mountain, Canberra, for allowing me access to the facilities of the Division to undertake the research.

I would also like to thank the Australian Beekeeping Industry, who, through its Honeybee Research and Development Council, provided a stipend and research funding (HBRDC Project Number CSE-3H) during the first two years of this study.

Contents

Executive Summary

The microsporidian *Nosema apis* is the causative agent of the European honeybee (*Apis mellifera*) nosema disease. This disease is considered the most economically important disease of adult honeybees in Australia and the world (Bailey and Ball, 1991; Matheson, 1993a). Nosema disease can significantly reduce yields of honey (Fries, 1993) and pollen (Anderson and Giacon, 1992). The most common method of control for nosema disease is the application of the antibiotic fumagillin (Fumidil B). This method of control is not practiced widely in Australia because of the associated risk of honey contamination.

The life cycle of *N. apis* has been extensively studied; so too have the affects of *N. apis* on colony health and productivity. However, little is known about the underlying genetic variability that must exist within this species. For example, are some strains more pathogenic? To explore genetic differences one must have a means of positive identification for each strain. Morphologically, the species *N. apis* appears to be invariant. Therefore, differences at the cellular level may be the only means of strain identification. The best candidates for strain identification are the differences found within the DNA sequence of all species. These so called 'molecular markers' are extensively used as strain identifiers within species. A collection of molecular markers that positively identify a strain is referred to as a genotype or genetic fingerprint.

The initial application for funding contained two objectives. The first was to locate and then determine the sequence of genes whose composition is specific to the adult honeybee parasite *N. apis.* The second objective was to use the DNA sequence data to (a) determine the taxonomic relatedness of *N. apis* to other microsporidia, (b) assess genetic variation between Australian isolates of *N. apis*, and (c) determine whether detection of virulent and avirulent isolates of *N. apis* is possible.

A further objective was included in the project at the end of the first year. This objective investigated an anecdotal report (Mr Ron Brown, personal communication) on the effects of thymol as a novel control method for nosema disease. Mr Brown had fed wintering honeybee colonies sugar syrup containing thymol as a preservative. During this time, his honeybee colonies had apparently remained free of nosema disease.

First Objective

The first objective was to locate and then determine the sequence of genes whose composition is specific to the adult honeybee parasite *N. apis.* Initially, it was intended that genomic libraries be constructed from the genomes of several geographically distinct isolates of *N. apis.* The genomic library would then be searched for DNA fragments containing the genes of the ribosomal RNA (rRNA) subunits. These genes are ubiquitous to all cellular organisms. The rRNA subunits, in conjunction with proteins and other factors, combine to form the ribosome. The ribosome is the cellular machinery responsible for the translation of messenger RNA into proteins. The ribosomal genes contain regions of DNA sequence that are highly conserved in all cellular organisms, while elsewhere in the genes are regions that are hypervariable. It was hoped that within the hypervariable regions, there would be sufficient genetic variation to discriminate different *N. apis* strains.

Rapid advances in molecular techniques, namely polymerase chain reaction (PCR), made it possible to obtain complete and partial rRNA gene sequences from several *N. apis* isolates plus the complete rRNA sequence from the microsporidian *Nosema vespula* without the construction of genomic libraries. PCR allows for the enzymic amplification of specific regions of DNA sequence directly from the genomic DNA of the target organism. This proved to be a cost-effective and rapid technique that increased the number of populations sampled then would have otherwise been possible.

Second Objective

The second objective used the DNA sequence data obtained from the first objective to (a) determine the taxonomic relatedness of *N. apis* to other microsporidia, (b) assess genetic variation between Australian isolates of *N. apis*, and (c) determine whether detection of virulent and avirulent isolates of *N. apis* might be possible.

Taxonomic relatedness

Phylogenetic analysis of the rRNA gene sequence data placed *N. apis* as a close relative of the microsporidian *Viarimorpha lymenantra* and *N. vespula*. The species of the genus *Viarimorpha* often parasitise butterfly and moth species (Malone and McIvor, 1996), while *N. vespula* was isolated from the European wasp *Vespula germanica* (D. Anderson, personal communication). A detailed, phylogenetic analyses (Rice, 1999) of the rRNA gene sequence data from *N. apis* and *N. vespula* suggests that the rRNA genes of the microsporidia are very bacteria-like in size and structure, yet are highly divergent. Excluding the highly conserved regions of the genes, the rRNA genes of *N. apis* and *N. vespula* are as significantly different from higher multicellular organisms, as are the bacterial rRNA genes. These data suggests that the microsporidia are a very ancient group of organisms diverging early in eukaryotic evolution.

Genetic variation

A region of the rRNA genes from nine geographically distinct isolates of *N. apis* was sequenced. Geographically distinct isolates were chosen because of the increased likelihood that genetic variation would exits in isolates from different localities. However, this does not preclude the possibility that genetic diversity could also exist in one population of *N. apis* obtained from one colony of honeybees. This possibility was not investigated in this work.

A region spanning approximately 670 base pairs (bp) of the rRNA genes was PCR-amplified and sequenced for nine *N. apis* isolates and one *N. vespula* isolate. This region extended from the end of the small subunit (SSU) rRNA gene to about 450 nucleotides inside the large subunit (LSU) rRNA gene and included the internal transcribed spacer (ITS). Within this region are several areas noted for their hypervariability and as such represented good candidates as possible strain discriminatory sequences.

Sequence length variation was observed amongst the PCR fragments obtained from the nine *N. apis* isolates. This length variation ranges from 663 bp for the Kangaroo Island (KI) isolate to 666 bp for the Canberra (CN) isolate. The PCR fragment amplified from *N. vespula* was 675 bp in length. Among the nine isolates of *N. apis*, most of the length variation was restricted to a region within the ITS and one other hypervariable region.

The genetic variation observed between the *N.* apis isolates, *N. vespula* and *V. lymentra* was used as the basis for phylogenetic analysis. The phylogenetic analysis was restricted to 501 sequence positions because of the inclusion of the *V. lymentra* sequence. There were only a few informative positions in the aligned sequence data. In the context of the analysis of these 11 sequences, a sequence position is informative in the alignment if at least two but not more than nine sequence positions share the same DNA sequence character-state (A, C, T, or G).

Results of phylogenetic analysis suggest that the taxa can be divided into four distinct phylogenetic groups: ‘Australia’, consisting of the Kangaroo Island (KI), Bateman Bay (BB), Canberra (CN), and Western Australia (WA) isolates; ‘Java’, consisting of the isolate obtained fro *Apis cerana* honeybees (AC) and the isolate from honeybees collected in Java (JV); ‘Vairimorpha’, consisting of the *V. lymentra* (VL), *N. vespula* (NV), and Canadian (CA) isolates; ‘International’, consisting of the

Swedish (SW) and New Zealand (NZ) isolates. Statistical analysis shows weak support for these groups. These grouping of taxa do reflect some natural association.

The Australian group for example, consists of geographically distinct isolates from the mainland Australia and Kangaroo Island. Honeybees are an exotic species to Australia. The introduction of honeybees (and presumably *N. apis*) occurred about 1810 (Warhurst and Goebel, 1995). The number of introductions of honeybees since that time has been limited because of geographical isolation and quarantine regulations designed to keep Australia free of the many exotic pests and disease of honeybees. It is plausible (and most parsimonious) that all these *Nosema* isolates have descended from one original founder population of *N. apis,* although statistical support for this is weak.

The Java group (AC and JV) indicates a true phylogenetic relationship between these two isolates despite only moderate statistical support. It is likely that the ancestor of these two isolates was a parasite of *A. mellifera* that subsequently switched species, and is now a parasite of *A. cerana*. This is not surprising as *A. mellifera* and *A. cerana* are very closely related and are believed to be in an immature stage of speciation (Ruttner and Maul, 1983). There are reports of *N. apis* being found in *A. cerana* (Singh, 1975; Yakobson *et al.*, 1992). The accuracy of these reports has been questioned because they were based on light microscopy (Fries *et al.*, 1996). Fries *et al.* recently isolated a new species of *Nosema* from *Apis cerana* and named it *N. ceranae*. The report presented here is the first to confirm the presence of *N. apis* in *Apis cerana* using DNA techniques.

The Vairimorpha group demonstrates the close phylogenetic relationship between *V. lymentra* and *N. vespula* and suggests that *N. vespula* may in fact belong to the genus *Vairimorpha* and not *Nosema.* The sequence alignments revealed that these two species shared 50 nucleotide positions.

The grouping of the Canadian isolate with the Vairimorpha group is considered to be an artefact, a consequence of the limited amount of sequence data available. Limited sequence data may have also been responsible for the grouping of the Swedish and the New Zealand isolates. By virtue of their sequence differences to the Australia isolates, they do however form a separate group. Statistical support for these groupings was low and considered dubious.

Strain identification markers

The data indicates that the region sequenced is not evolving at a rate that provides sufficient discriminatory information to allow for the positive identification of strains or isolates within *N. apis*. The data obtained is not suitable for differentiating virulent and non-virulent strains if such strains exist. Therefore, other molecular markers are required. Perhaps, the sequence of the non-transcribed gene spacer situated between the LSU and SSU rRNA genes would be more suitable. Alternatively, mini- or micro-satellites (small repetitive sequences $\leq$ 20 base pairs) may prove to be more informative and reliable as molecular markers.

Third Objective

Reports by beekeepers indicate that honeybees that forage on certain floral species are relatively immune to nosema disease. Paradoxically, honeybees that forage on other floral species are believed to be more susceptible to nosema disease. It is likely that these phenomena are linked to the nutritional value of the nectar and pollen that the honeybees collect from different plant species. Variation in nutritional qualities of pollen and nectar are likely to influence a honeybee's ability to resist infection.

Recently, it has been demonstrated that the nectar of many floral species contains organic substances known to be biologically active against organisms such as bacteria (Molan, 1992). These substances may also be biologically active against *N. apis.* One such candidate is thymol. Thymol, the oil that gives the herb thyme its distinctive taste and aroma, is used commercially in products such as

mouthwash because of its bactericidal properties. This oil is also known to occur in a number of Australian eucalypt plant species (Borland *et al.*, 1991). Anecdotal evidence suggests that thymol is biologically active against *N. apis* (Ron Brown, personal communication).

The third objective investigates the use of thymol to control nosema disease. Initially, *N. vespula* was chosen as a model organism to test the biological activity of thymol. This organism was chosen because it is easily maintained in the laboratory. The pest species *Helicoverpa armigera* was used as the host. The caterpillar of *H. armigera* can causes extensive damage to cotton crops, and its biology and rearing methods are well established for laboratory research.

The data obtained form this study can be extrapolated for nosema disease based on Brown's observations. Furthermore, the data could form the basis of further research. There are two areas of interest. First, the use of thymol in food supplements to control nosema disease. Second, the detection of thymol in nectar and pollen of economically important floral species used for live honeybee and honey production—those plant species that are believed to suppress nosema disease.

A feeding regime of food containing different concentrations of thymol was fed to third instar *H. armigera* caterpillars. These caterpillars were infected orally with approximately 10,000 *N. vespula* spores. The disease was allowed to develop for ten days. On the tenth day post-inoculation, the spore load was determined microscopically for each surviving caterpillar in the trial.

Statistical analysis of the data demonstrated that thymol was biologically active against *N. vespula.* A diet containing 0.15mM thymol was found to prevent *N. vespula* infections in more than 50% of caterpillars inoculated. Feeding of diet containing thymol prior to infection was not found to be of significant value in preventing infection. It was concluded that thymol works directly on spores rendering them non-viable.

1. *Nosema apis* – Intraspecific Comparison

1.0 Introduction

Nosema apis is a host specific microsporidian parasite of the adult European honeybee *Apis mellifera* (Bulla and Cheng, 1977) and is an endemic pest of honeybees throughout the world (Matheson, 1993a). The ultrastructure and life cycle of *N. apis* have been extensively studied using electron microscopy (Fries, 1993), but these studies have revealed nothing about the genetic diversity of this organism. More specifically, it is generally assumed that all microsporidians obtained from honeybee and observed using the light microscope are *N. apis*. This, however, may not be so. General disagreement about the classification of Microspora based on ultrastructure and life cycles pervade the literature (Baker *et al.*, 1994; Baker *et al.*, 1995; and Fries *et al.*, 1996). A case in point is a microsporidian found to parasitise honeybee larvae (Buys, 1972, 1977). Based on ultrastructure inferred from electron micrographs, this microsporidian was identified as a new species. However, Clark (1980) reported that the spore size and developmental stages of this microsporidian indicated that it was a unique strain of *N. apis* rather than a new species. Therefore, the use of the light or the electron microscope for identifying closely related species or inferring phylogenetic relationships is of limited use.

Other examples of incorrect diagnosis of microsporidial species have been documented. Vossbrinck *et al.* (1993) discussed the case of the incorrect identification of three isolates of a microsporidial species obtained from several AIDS patients. From morphological studies these isolates were initially identified as *Encephalitozoon cuniculi*. Later, it was demonstrated by SDS-PAGE and Western blot analysis that these isolates, while identical to each other, and were not *E. cuniculi.* They were subsequently assigned the name *Encephalitozoon hellem.* Vossbrinck *et al.* conclude that SDS-PAGE and Western blot analysis may not even be sensitive enough to distinguish between closely related species and that molecular techniques offer the most reliable method of identification.

In the absence of definitive morphological characters for species identification other techniques using molecular markers may greatly assist in the classification of the Microspora. Riboprinting is one such technique. This technique uses restriction fragment length polymorphism (RFLP's) of PCR-amplified ribosomal RNA (rRNA) genes as molecular markers to reveal inter- and intraspecific variation in the target species. The successful application of this technique to determine the phylogenetic relationships among 13 microsporidian species clearly demonstrated its usefulness to determine relationships at the species level (Pomport-Castillon *et al.*, 1997). However, no intraspecific genetic variation was detected in the region amplified. Therefore, to detect intraspecific genetic diversity in the Microsporidia, it may be necessary to extend the riboprinting technique to other regions of the genome. Alternately, it may be necessary to use more discriminatory techniques such as DNA sequencing.

In this section I report on the results obtained based on the first two objectives of the research proposal. More specifically, I compare the rRNA gene sequences for nine geographically distinct isolates of *N. apis* and also compare these data to sequences of the Microsporidia *Nosema vespula* and *Vairimorpha lymantriae* to infer phylogenetic relationships, using evolutionary tree building methods.

1.1 Methodology

1.1.1 Source of *Nosema spp.* spores

1.1.1.1 Nosema apis isolates

N. apis infects and replicates in the epithelial cells of the honeybee midgut (Fries, 1993). Nine isolates of *N. apis* were used in this study (Table 1.1). Distinct, geographical isolates were chosen to increase the probability that genetic variation would be detected. Except for the Java isolate, all were obtained from the European honeybee, *Apis mellifera* (Apoidea: Apidae). The isolate from Java was obtained from the Asian honeybee *Apis cerana* (Apoidea: Apidae). The isolates from Canada, New Zealand, and Sweden were provided as purified spores, while the remainder of the isolates were obtained as infections in whole bees from which the spores were recovered.

Samples of 35 honeybees, potentially infected with *N. apis,* were collected from the entrances of beehives. Five of the 35 honeybees were chosen at random. Crushing the thorax between the fingers, grasping the sting and terminal sclerites with tweezers, and gently pulling the alimentary tract away from the abdomen removed their alimentary tracts. A small piece of the midgut was removed from each honeybee, crushed between a microscope slide and a cover slip and microscopically examined at 400x magnification for the presence of *N. apis* spores. On confirmation of a *N. apis* infection within the beehive, the alimentary tracts of the remaining 30 honeybees were removed and stored at 4°C awaiting spore recovery and purification.

1.1.1.2 Nosema vespula isolate

The *N. vespula* isolate was a gift from Dr Denis Anderson, CSIRO Division of Entomology, Canberra, Australia. This as yet undescribed species of microsporidian was originally isolated by Dr Anderson from the infected larvae of the European wasp *Vespula germanica* (Vespoidea: Vespidae). From preliminary morphological and ultrastructure studies as well as studies on the reproductive cycle, this species was tentatively name "*Nosema vespula*".

Experimental research has shown that this isolate infects an extensive host range including hymenopterans, dipterans, and lepidopterans. Since discovery, this species has been maintained *in vivo* using *Helicoverpa armigera* caterpillars as hosts. The organism initially infects the host's epithelial gut cells and then moves to the host's fat bodies where parasite replication takes place (Dr Denis Anderson, personal communication). A stock of the organism had been reared in caterpillars that had in turn been stored at -20°C. Spore numbers, sufficient for use in molecular biological techniques, were obtained from a mass infection of 250 caterpillars. The caterpillars were a gift from Dr Peter Christian, CSIRO Division of Entomology, Canberra. The inoculum, used to infect these caterpillars, was recovered (Section 1.1.2) from frozen stocks. Host caterpillars were maintained on a soybean flour and wheat germ diet (Teakle and Jensen, 1985). Third instar caterpillars were starved for 3 hours and then fed 0.25 gm blocks of diet inoculated with 10µl of a 10^5 spores ml^{-1} dilution in distilled water. The caterpillars were maintained at 28°C and fed on demand for nine days. The infected caterpillars were then stored at -20°C awaiting spore recovery and purification.

1.1.2 Purification of *Nosema spp.* spores from host tissue

Spores grown in honeybees or caterpillars were liberated from tissue by macerating either five entire caterpillars or 30 alimentary tracts in 30ml of distilled water using a mortar and pestle. To remove large particulate matter, the spore suspension was filtered through four layers of Kimwipe (Kimberly-Clark, Australia). The filtered suspension was centrifuged at 1000 x g for 20 minutes and the supernatant removed. The pellet of spores was suspended in 1ml of distilled water and centrifuged into a discontinuous gradient of neutralised Percoll (Sigma) according to the method of Sato and Watanabe (1980). The Percoll gradient was constructed by the sequential layering of 7ml 100%, 8ml 75%, 8ml 50% and 8ml 25% Percoll in a 50ml ultracentrifuge tube (25 x 89 mm). A 1ml aliquot of spore suspension was immediately overlayed onto the gradient and centrifuged at 3,000 x g for 90 minutes using a Beckman 28S rotor in a Beckman L8-70M ultracentrifuge. Spores became visible as a white band at the 75-100% interface, while some spores passed through the gradient and were visible as a pellet among the debris in the bottom of the ultracentrifuge tube. The band at the interface was removed with a Pasteur pipette, placed in a 50ml ultracentrifuge tube and diluted with 35ml of distilled water. The suspended spores were pelleted at 3,000 x g for 30 minutes and the supernatant removed. The spores were resuspended in 1ml of distilled water, transferred to an Eppendorf tube and washed three times with distilled water to remove Percoll. Washing of the spores was by centrifugation at 1,000 x g for 5 minutes, removal of the supernatant, and suspension in distilled water. At the third wash the spore concentration was determined using a Neubauer Counting Chamber (Cantwell, 1970). The spores were then pelleted once more, the supernatant removed, and the spores suspended in distilled water at concentrations of approximately 10^8 spores ml^{-1}. Each spore suspension prepared this way was stored at 4°C in 0.5ml aliquots pending the isolation of genomic DNA. The spore and debris sediment similarly washed by the above method. However, following the final wash, spores were suspended at concentrations of approximately 10^5 spores ml^{-1} for later use as inoculum for infecting honeybees or caterpillars.

1.1.3 Isolation of genomic DNA from purified *Nosema spp.* spores

1.1.3.1 Nosema apis

a. Isolation by spore germination

A 0.5ml aliquot of purified spores was pelleted at 1,000 x g for 5 minutes and the supernatant removed. The pellet of spores was resuspended in 200µl of freshly prepared germination buffer (0.5M sodium chloride, 0.5M sodium hydrogen carbonate, pH to 6.0 with 0.1M orthophosphoric acid; De Graaf *et al.*, 1993) and incubated at 37°C for 15 minutes allowing spores to germinate. One ml of DNA isolation buffer (0.5% sodium dodecyl sulfate (SDS), 124mM disodium ethylenediaminetetra-acetate (EDTA) pH 8.0, 250mM Tris-HCl pH 9.2, 0.2% 2-mercaptoethanol) was added to the germinating spore suspension, and the mixture shaken for 2 minutes. The mixture was incubated at 55°C for 1 hour and then chilled on ice. The SDS and proteins were precipitated by adding 300µl of 5M potassium acetate (pH 7.2) and incubating the mixture for 15 minutes on ice. The SDS-protein precipitate was pelleted by centrifugation at 15,000 x g for 20 minutes and the supernatant removed to a new Eppendorf tube. The DNA was precipitated from the supernatant by the addition of 950µl of absolute ethanol and incubation on ice for 15 minutes. The DNA precipitate was pelleted by centrifugation at 15,000 x g for 20 minutes, the supernatant removed, and the DNA pellet washed with 200µl of ice cold 70% ethanol. The pellet was then air dried at room temperature, suspended in 20µl of TE buffer pH 7.4 (10mM Tris-HCl pH 7.4, 1mM EDTA pH 8.0) and stored at -20°C.

b. Isolation by mechanical disruption of spores

This method was used to extract genomic DNA from the spores of *N. apis* isolates where there was insufficient material available to use the germination protocol. A 200µl aliquot of purified spores was placed in a 0.5ml Eppendorf tube and pelleted by centrifugation at 1,000 x g for 5 minutes. The supernatant was removed and the pellet suspended in 150µl of STE buffer (100mM sodium chloride, 1mM EDTA, and 10mM Tris-HCl, pH 8.0; Baker *et al.,* 1995). One hundred and fifty milligrams of 0.45mm glass beads (Sigma) was added and the tube vortexed at maximum speed for 20 seconds to disrupt the spore coat. Immediately, the tube was placed in a 95°C heating block for 5 minutes to denature proteins. The tube was then centrifuged at 15,000 x g for 3 minutes and the supernatant removed to a new tube and stored at -20°C.

1.1.3.2 Nosema vespula

The method of Crozier (1991) was devised for the isolation of genomic DNA from honeybees. The CTAB buffer used in Crozier's method was found to induce germination of *N. vespula* spores when the spores were incubated in the buffer at 37°C. A pellet of purified spores was suspended in 50µl of CTAB buffer (100mM Tris-HCl pH 8.0, 20mM EDTA pH 8.0, 1.4M sodium chloride, 2% Hexadecyltrimethylammonium bromide w/v) containing 0.2% 2-mercaptoethanol and incubated at 37°C for 30 minutes (to allow spores to germinate) and then at 65°C for 90 minutes. The mixture was extracted with phenol/chloroform twice (Sambrook *et al.,* 1989) and the DNA precipitated by adding of 750µl of isopropanol and incubating overnight at -20°C. The DNA precipitate was centrifuged at 15,000 x g for 20 minutes to pellet the DNA. The supernatant was removed and the pellet washed twice with 200µl of cold 70% ethanol. The pellet was air dried at room temperature and then suspended in 20µl of TE buffer and stored at -20°C.

1.1.4 Genomic Library replaced by PCR

Rapid advances in molecular techniques, namely polymerase chain reaction (PCR), made it possible to obtain complete and partial ribosomal RNA (rRNA) gene sequences from several *N. apis* isolates plus the complete rRNA sequence from the microsporidian *Nosema vespula* without the construction of genomic libraries. PCR allows for the enzymic amplification of specific regions of DNA sequence directly from the genomic DNA of the target organism. This proved to be a cost-effective and rapid technique that increased the number of populations sampled then would have otherwise been possible.

1.1.4.1 Selection of primers for PCR amplification

In designing the primers for this experiment, a target sequence was chosen that contained regions likely to be evolving at different rates, a phenomenon known to occur in the cytoplasmic rRNA genes (Van De Peer *et al.,* 1993; De Rijk *et al.,* 1995). Regions that are highly conserved aid in the correct alignment of sequences, while regions that evolve more quickly are likely to show genetic variation useful for comparative analysis.

The target sequence chosen encompasses the 3' end of the small subunit (SSU) rRNA gene, the internal transcribed spacer (ITS), and the 5' end of the large subunit (LSU) rRNA gene; a DNA sequence of approximately 665 base-pairs. Within the target sequence are three hypervariable regions: ITS, helices 15 to 18, and helix 25 (Figure 1.3). The target region is flanked by nucleotide sequences that are sufficiently conserved within the Microsporidia to allow Microsporidia-specific PCR amplification from heterogeneous genomic DNA.

The primers, used to amplify the target sequence, were based on sequence data obtained earlier from *N. vespula* sequence (Figure 1.2). Primers NV1161F (GTT GTG GAA TTC GTG CAA GCT ACT TGA ACA ATA TG) and NV1851R (GAA TGA GAA TTC GGA TCC AAT AAA CTG TTG CTT ATC) were used to amplify the target region. These primers also contained an *Eco*RI endonuclease restriction site (underlined) to facilitate cloning of PCR product. The primer identification code indicates three pieces of information. First, the 5' nucleotide position of the template sequence that corresponded to the complete *N. vespula* rRNA gene operon sequence. Second, whether the primer was complementary to the non-coding strand (F) or coding strand (R). Third, if the primer was used for direct sequencing (D). For example, NV765RD = *N. vespula*, sequence position 765, complementary to the coding strand, and used for direct sequencing from PCR product.

Primers for PCR amplification were synthesised with an Applied Biosystems model 381A DNA synthesiser. Primer concentration was calculated from the absorbancy measured at OD260 by the method of Sambrook *et al.* (1989).

1.1.4.2 Gene amplification using PCR

Target sequences were amplified in a GeneAmp PCR System 2400 thermal sequencer (Perkin Elmer). Thermal cycle amplifications were performed in 100µl final volume using rTth-XL DNA polymerase and 3.3x XL buffer (Perkin Elmer) containing dNTPs at 200µM, primers at 0.5 µM, sample DNA (100ng), and 2.5 units of rTth-XL DNA polymerase (Perkin Elmer). The "hot start" protocol for long PCR using paraffin wax beads was used according to the vendor's instructions (Perkin Elmer). Following an initial denaturation of 90 seconds at 94°C, the reaction cycles consisted of denaturation for 10 seconds at 94°C, primer annealing for 20 seconds at 55°C and extension for 3 minutes at 68°C. Cycles were repeated 30 times. The final cycle also included an extension of 10 minutes at 72°C. Negative controls, all the above reagents except template DNA, were included to screen for possible foreign DNA contamination. Positive controls were included to screen for host DNA contamination of the template.

1.1.4.3 Visualisation of PCR amplification products

PCR-amplified products (5µl aliquot) were separated and analysed by electrophoresis through a horizontal 1% agarose (Promega) gel containing 500ng/ml ethidium bromide. Molecular markers, either lambda/*Hind* III or lambda/*Spp* I/*Eco* RI (New England Biolabs), were included to assess the size of PCR-amplified fragments. Electrophoresed PCR-amplified fragments and molecular markers were visualised using a ultraviolet transilluminator (UVP Inc.), and the gels photographed using a Polaroid MP4 Landcamera and Polaroid 667 film (ISO 3000/36°).

1.1.4.4 Procedures for molecular cloning and sequencing

The vector pBluescript® SK^+ plasmid (Stratagene) was prepared by linearisation with *Eco*RI (Promega) and then dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim) according to the manufactures instruction. The PCR-amplified fragments were purified using the Wizard® PCR Preps system (Promega) and ligated into the prepared *Eco*RI cloning site of the Bluescript® SK^+. Plasmids were transformed into *E. coli* strain TG1 by the heat shock method (Sambrook *et al.*, 1989). Clones containing the PCR-amplified fragments were selected by their resistance to ampicillin and blue-white screening on 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) / isopropylthio-ß-D-galactoside (IPTG) plates. Plasmid DNA was prepared by the alkaline lysis method (Sambrook *et al.*, 1989). The sequence of the cloned, PCR-amplified fragments were determined by both dye-primer and dye-terminator sequencing methods (Applied Biosystems). Primers used for dye-terminator sequencing were NV1373 FD (GAT AAC CCT TTG AAC TTA AG), NV1629FD (ATG GTA TAC CGA TAG CAA AT), and NV1690RD (GGC TAA CAC CCA CAC ATT TTC AC). Sequencing was performed using an ABI 373A model sequencer (Applied

Biosystems). The accuracy of the sequence data was confirmed by sequencing both strands of each clone.

1.1.5 Sequence analyses

The sequence data obtained from the ABI sequencer was checked visually for sequence overlaps. Overlaps were marked on the chromatograms and the chromatograms ordered sequentially 5' to 3'. The completed sequences were entered manually into the sequence alignment program "DCSE" (Dedicated Comparative Sequence Editor version 2.54, De Rijk and Wachter, 1993). All sequences were manually aligned. A consensus sequence was generated from the aligned sequences for inclusion in the final alignment.

The *V. lymantriae* sequence data (accession number L13330) was obtained from the GenBank database using the 'ENTREZ' proprietary software (National Centre for Biotechnology Information). The *V. lymantriae* sequence data was aligned by eye to the *N. apis and N. vespula* sequences.

1.1.6 Phylogenetic analyses

The aligned sequence data stored in DCSE format was converted manually to "MASE+" format (Faulkner and Jurka, 1988) for analyses using the programs "SEAVIEW" and "PHYLO_WIN" (Galtier *et al.,* 1996). SEAVIEW is a sequence alignment program that also allows for the storage of additional information about the sequence alignment within the MASE+ data-file. In this regard, SEAVIEW is the precursor program for phylogenetic analysis using PHYLO_WIN. Unambiguous sequence positions for phylogenetic analysis were determined from secondary models of the SSU and LSU rRNAs (Gutell, 1994; Gutell *et al.*, 1992) and recorded in the MASE+ data file using SEAVIEW. Phylogenetic analyses were performed of the unambiguous rRNA alignment positions using the PHYLO_WIN program.

1.2 Results and Discussion

1.2.1 Sequence length and variation

A region of approximately 665 to 676 basepairs (bp) was amplified, cloned and sequenced. Figure 1.1 depicts the cloned fragment and the position and direction of the primers used to sequence the fragment. The aligned sequences from the nine *N. apis* isolates, one *N. vespula* (NV) and one *V. lymantriae* (VL) isolate are shown in figure 1.2. A consensus sequence containing the predominant nucleotide character-state at each alignment position was also included in the alignment. By comparison to the consensus sequence in figure 1.2, genetic variation is present in all taxa. These results reveal sequence length variation amongst the PCR fragments obtained from the nine *N. apis* isolates. This length variation ranges from 663 bp for Kangaroo Island (KI) to 666 bp for Canberra (CN). The PCR fragment length for NV is 675 bp. Only 519 nucleotides of sequence were available in Genbank for this region of VL. Among the nine isolates of *N. apis*, most of the length variation was restricted to a region within the ITS and the hypervariable region of helix 16 (Figure 1.2 and 1.3). The length variation between NV and the nine *N. apis* isolates occurs in the ITS and helices 10, 16, and 28. More specifically, two sets of three nucleotide insertions and three single nucleotide insertions are present. The three-nucleotide insertions form internal loops in helices 10 and 28, as indicated in figure 1.3. The single nucleotide insertions occur in helices 10 and 18, and the hairpin of helix 20 (Figure 1.3). As shown in figure 1.2, NV and VL share most of the insertion and deletion events.

1.2.2 Nucleotide variation including transition and transversion analysis

In addition to the insertion/deletion events discussed, other alignment positions differ from the consensus sequence. For example, amongst the *N. apis* isolates, this variation ranges from no

substitutions and two deletions in the Western Australian (WA) isolate to eight substitutions and three deletions in the *A. cerana* (AC) isolate. The variation between the consensus sequence and the sequences of NV and VL is far greater then that observed for the consensus sequence and the sequences of the nine *N. apis* isolates. For example, there are 50 positions (Figure 1.2) in which NV and VL share character-states but are different to the consensus sequence.

Amongst the sequences of the *N. apis* isolates there are 31 substitution events. Table 1.2 lists these events, table 1.3 summaries the character-state replacements, while figure 1.3 displays their location on the LSU rRNA secondary structure model for the region sequenced. Of these, 12 occur within secondary structure helices of the LSU, 14 occur in loops or hairpins of the LSU, and five occur in the ITS. Five of the 12 helical substitutions disrupt canonical bonds resulting in the formation of one A-A, one A-C, and three U-C base pairs. Four of the five disruptive substitutions are transversions (purine to pyrimidine and pyrimidine to purine). The remaining seven helical substitutions convert canonical bonds (3 C:G + 4 A:U) to either U:G or G:U bonds. These substitutions all represent transition events (pyrimidine to pyrimidine and purine to purine). The five substitutions within the ITS are three A to T transversions and two transitions, one C to T, and one A to G. The remaining 14 substitutions within loops and hairpins involve six transversion and eight transition events. In summary, these data demonstrate a 2:1 transition to transversion rate within the helices sequenced, a 4:3 transition to transversion rate within the loops and hairpins, and a 2:3 transition to transversion rate within the ITS. For character-state substitution possibilities in a 'random' system, transversions are twice as likely to occur as transitions. These data suggest that the process of substitution in the rRNA gene sequence may not be random. These data also suggest that the process of substitution may vary across the rRNA gene sequence. Finally, these data also indicate a possible preference of character-state for nucleotide replacement in the substitution process.

1.2.3 Phylogenetic analysis

Genetic variation, as demonstrated among the *N.* apis isolates, and the single NV and VL isolates, can be used as the basis for phylogenetic analysis. The phylogenetic analysis of these data is restricted to 501 positions because of the inclusion of VL data (Figure 1.2). Of the 501 positions, only 58 positions are phylogenetically informative of which 50 are shared exclusively by NV and VL. Therefore, the results obtained from the limited number of informative positions within the sequence data from the nine *N. apis* isolates should be interpreted with caution. It is difficult to determine which commonalities in the alignment data are due to homoplasy (convergence, parallelism, and reversals) or are plesiomorphs (ancestral character state). In these data, a sequence position is informative if two but not more than nine sequence positions share the same character-state, while among the remaining sequences, at least two share a different character-state.

The cladogram in figure 1.4a shows a consensus of the most parsimonious trees and requires 82 steps. Results of parsimony analysis suggest that the taxa can be divided into four distinct clades: 'Australia', consisting of KI, BB, CN, WA; 'Java', consisting of AC and JV; 'Vairimorpha', consisting of VL, NV and CA; 'International', consisting of SW and NZ. Bootstrap (Bs) analysis (1000 replicates) shows weak support for these clades. These grouping of taxa do however reflect some natural association.

The Australian clade for example consists of geographically distinct isolates from the mainland Australia and Kangaroo Island. Honeybees are an exotic species to Australia. The introduction of honeybees (and presumably *N. apis*) occurred about 1810 (Warhurst and Goebel, 1995). The number of introductions of bees since that time has been limited because of geographical isolation and quarantine regulations designed to keep Australia free of the many exotic pests and disease of bees. It is plausible (and most parsimonious) that all these *Nosema* isolates have descended from one original founder population of *N. apis*, although support for this is weak (Bs 7%). The few phylogenetic informative events that are seen would take place in a stepwise fashion: the divergence of the KI-BB group and the CN-WA groups from a common ancestor, and then the divergence of the sister taxa. The taxa of this group has since independently acquired lineage dependant substitutions.

Alternatively, all the informative position could be homoplasies caused by some common selective pressure exerted on the Australian population of *N. apis*.

Similarly, honeybees are only a relative recent introduction to New Zealand, with the first introduction occurring in 1839 (Matheson, 1993b). New Zealand also has very strict quarantine controls and so the number of likely introductions of honeybees, and hence *N. apis*, would have been limited. Therefore, given the geographic separation of Sweden and New Zealand, the sister relationship of SW and NZ in the International clade is almost certainly the results of homoplasy in the data. Their sister taxa relationship is supported only by the presence of a common T at position 142 (Table 1.2) that is not shared with any other taxa analysed.

The Java clade (AC and JV) indicates a true phylogenetic relationship between these two isolates despite only moderate support (Bs 75%). It is likely that the ancestor of these two isolates was a parasite of *A. mellifera* that subsequently switched species and is now a parasite of *A. cerana*. This is not surprising as *A. mellifera* and *A. cerana* are very closely related and are believed to be in an immature stage of speciation (Ruttner and Maul, 1983). There are reports of *N. apis* being found in *A. cerana* (Singh, 1975; Yakobson *et al.*, 1992) but the accuracy of these reports, which were based on light microscopy, has been questioned as it may have been *N. ceranae* (Fries *et al.*, 1996). This is the first report based on molecular data that proves the presence of *N. apis* as a parasite of *A. cerana.*

The remaining clade presents two interesting results. First, there is an apparent close taxonomic relationship (Bs 100%) between NV and VL instead of NV and *N. apis.* The sister-taxa relationship has been observed in phylogenetic trees constructed from SSU rRNA sequence data (Baker *et al.*, 1995; Malone and McIvor, 1996; Rice, 1999). Therefore, it is not surprising then that the sequence data from the LSU rRNA also supports this relationship. The data in figure 1.2 demonstrates 50 phylogenetically informative positions exclusive to NV and VL. Second, and somewhat more puzzling, is the closer association of CA to the NV-VL group (Bs 65%). This is likely to be the consequence of homoplasy as evident by the substitution events relative to the consensus sequence (plesiomorphic state) as shown in table 1.2. In this table, CA, NV and VL share two informative positions (Figure 1.2, positions 343 and 460) plus a deletion (position 92). This compares to AC, NV and VL that share two informative positions (positions 153, 168) and BB, NV and VL that share one informative position (position 70). Other than position 360, that involve the transition from a C:G bond to a U:G bond, all the remaining positions are unpaired in the secondary structure and are in regions that appear to be evolving more quickly than adjacent sequence. For example, nucleotides at sequence positions 142 to 171 (Figure 1.3 helices 6 and 7) in these taxa have undergone a number of deletion events relative to the eukaryotic standard model.

Analysis of these data by neighbor joining of a Jukes and Cantor (1969) distance measure (Figure 1.4b) and maximum likelihood (Figure 1.4c) demonstrates the same branching pattern as seen in the parsimony analysis (Figure 1.4a) including similar bootstrap support. In particular, both of these analyses provide statistical support for the sister branching of NV and VL (100%). Additionally, these analyses indicate the relative distance of the NV-VL group from *N. apis* (maximum likelihood 0.1188 and jukes cantor 0.0566). This result supports other findings based on SSU rRNA sequence comparisons that NV is more closely related to the genus *Vairimorpha* than *Nosema*.

1.3 Summary and Recommendations

The first object of creating genomic libraries of several *N. apis* isolates was replaced by the more expedient and cost effective use of PCR. To this end, nine geographically distinct isolates of *N. apis* were studied. A region encompassing the 3' end of the SSU rRNA sequence, ITS and the 5' end of the LSU rRNA sequence was determined for each isolate. These sequences and those of *N. vespula* and *V. lymenteria* for this region were aligned for comparison.

The second objective used the aligned sequence data. The data presented demonstrates the presence of intraspecific variation in *N. apis* isolates and between *N. apis, N. vespula,* and *V. lymantriae*. Moreover, excluding the Swedish and New Zealand isolates, phylogenetic analysis grouped the remaining *N. apis* isolates and other taxa in apparently natural groupings based on their origin. It is also apparent that the *N. apis* isolates formed a coherent grouping distinct from *N. vespula* and *V. lymantriae*.

The phylogenetic analysis supported findings by others that *N. vespula* is more closely related to the genus *Vairimorpha* than *Nosema.* This result was also strongly supported by comparisons in the sequence alignments. The Canadian isolate contained sufficient phylogenetically informative positions to cause it to group with the NV-VL group during tree construction. This result, based on sequence alignment comparisons, is an artefact caused by the limited number of phylogenetic informative positions. The sequence alignments and tree constructions support previous findings that *N. apis* has switched species from *A. mellifera* to *A. cerana* (Singh, 1975; Yakobson *et al.*, 1992).

Excluding the NV-VL grouping the observed bootstrap values for the maximum parsimony, neighbor joining and maximum likelihood analyses are low. These low bootstrap values are indicative of the small number of phylogenetically informative positions among the *N. apis* isolates. This region is not evolving at a rate that provides enough discriminatory information to allow for the positive identification of strains or isolates within *N. apis*. Therefore, other molecular markers are required. Current DNA technologies such as restriction length polymorphism (RFLP) may be more informative. The rapidly evolving non-transcribed spacer situated between the LSU and SSU rRNA genes may be a suitable target for study. Alternatively, mini- or micro-satellites (small repetitive sequences $\leq$ 20 base pairs) may prove to be more informative and reliable as molecular markers.

At this point there is no obvious intellectual property associated with these results. It is recommended that this research be made available to industry through a suitable refereed journal.

Table 1.1

Details of the *N. apis* isolates used for this research.

Isolate	Origin	Supplier
CN	Canberra, ACT, Australia	Dr. Denis Anderson CSIRO, Division of Entomology, Black Mountain, Canberra, ACT, Australia.
BB	Bateman's Bay, NSW, Australia	Mr Noel Bingley Sutton, NSW, Australia.
WA	Perth, WA, Australia	Mr Jeff Beard Western Australia Department of Agriculture, Perth, WA, Australia.
KI	Kangaroo Island, WA, Australia	Mr Bruce White Western Australia Department of Agriculture, Perth, WA, Australia.
NZ	Auckland, New Zealand	Dr. Louise Malone Hort+Research, Mount Albert, Research Centre, Auckland, New Zealand.
SW	Uppsala, Sweden	Dr. Ingemar Fries Swedish University of Agricultural Science, Bee Division, Uppsala, Sweden.
CA	Dawson Creek, BC, Canada	Mr John Gates Agriculture Canada, Dawson Creek, BC, Canada.
AC	Bogor, Java, Indonesia	Didik Bumi Ciluar Indah AI/1, Ciluar, Bogor, Java, Indonesia.
JV	Semarang, Java, Indonesia	Ministry of Forestry, Semarang, Java, Indonesia.

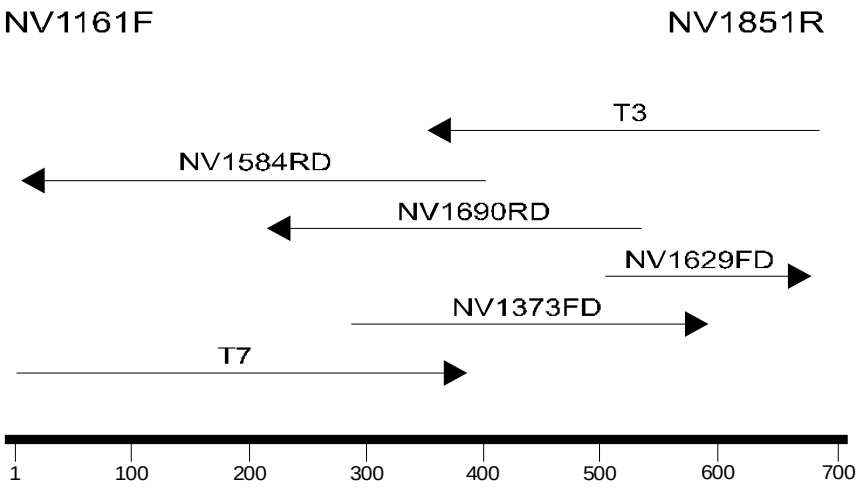


Figure 1.1

A map showing a typical clone used to determine the partial sequence of the SSU rRNA gene, the ITS and the LSU rRNA gene of nine isolates of *N. apis* and one isolate of *N. vespula*. The primers used to amplify the region (Section 1.1.4.1) are shown above the map. The scale bar indicates the position of each primer. Each region determined by direct sequencing (Section 1.1.4.4) is identified by the code of primer used (Section 1.1.4.1). The regions determined by dye primer sequencing (Section 1.1.4.4) of the parent clone (pBSII SK^+ plus insert) are indicated by their respective primer, either T7 or T3. Each arrow and its direction represent the size and the direction of the region sequenced. Position 1 indicates the first nucleotide position of the sequence in figure 1.2.

Figure 1.2

Comparative sequence alignments of a 676 base pair region from the rRNA gene operon of nine *N. apis* isolates, one *N. vespula* (NV) isolate, and one *V. lymantriae* (VL) isolate (*Vossbrinck* et. al., 1993). The first nucleotide position shown corresponds to the 1175th nucleotide position of the SSU of *V. necatrix* (Vossbrinck *et al.,* 1987). The alignments cover a region from the 3' end of the SSU rRNA gene, the ITS, and the 5' end of the LSU rRNA gene. The primers (Section 1.1.4.1), and their positions used to amplify and sequence the region, are also shown. Note, the region shown for primer NV1161F indicates the 3' half of the primer only. The region for the ITS, helices 10, 16 and 28, and their compliments helices 10', 16' and 28' are shown by the letter H.

The abbreviations for the isolates are: AC - Bogor, Java, Indonesia; JV - Semarang, Java, Indonesia; KI - Kangaroo Island, Australia; BB - Batemans Bay, Australia; CN - Canberra, Australia; WA - Perth, Western Australia; SW - Uppsala, Sweden; NZ - Auckland, New Zealand; CA - Dawson Creek, Canada; NV - *N. vespula* (ex D. Anderson)*;* and VL - *V. lymantriae* (Acc. No. L13330). *Note that:* the JV, KI, BB, CN, WA, SW, NZ, and CA isolates were obtained from European honeybee colonies (*A. mellifera*), while the AC isolate was obtained from the Asian hive bee (*A. cerana*). The consensus sequence was obtained as described in the text (Section 1.1.5).

```
AC        :  .......... .......... .......... .......... ..........50
JV        :  .......... .......... .......... .......... ..........
KI        :  .......... .......... .......... .......... ..........
BB        :  .......... .......... .......... .......... ..........
CN        :  .......... .......... .......... .......... ..........
WA        :  .......... .......... .......... .......... ..........
SW        :  .......... .......... .......... .......... ..........
NZ        :  .......... .......... .......... .......... ..........
CA        :  .......... .......... .......... .......... ..........
NV        :  .......... .......... .......... .......... ..........
VL        :  .......... .......... .......... .......... ..........
Consensus :  ACAATATGTA TTAGATCTGA TATAAGTCGT AACATGGTTG CTGTTGGAGA
             NV1161F

AC        :  .......... .......... .......... ..-....... ..........100
JV        :  .......... .......... .......... ..-....... ..........
KI        :  .......... .......... .......... ..-....... ..........
BB        :  .......... .........T .......... ..-....... ..........
CN        :  .......... .......... .......... ..T......G ..........
WA        :  .......... .......... .......... ..-....... ..........
SW        :  .......... .......... .......... ..-....... ..........
NZ        :  .......... .......... .......... ..-....... ..........
CA        :  .......... .......... .......... .TT....... .-........
NV        :  .......... .........T ...T..AA.A .C.....G.. .-.....A..
VL        :  .......... .........T ...T..AA.A .T.....G.. .-.....A..
Consensus :  ACCATTAGCA GGATCATAAC GAAGAATTAC AAATTTTTTA GAATTAGTTT
                3' end of SSU ←  Internal Transcribed Spacer

AC        :  .......... .......... .......... .......... ..........150
JV        :  .G........ .......... .......... .......... ..........
KI        :  .......... .......... .......... .......... ..........
BB        :  .......... .......... .......... .......... ..........
CN        :  .......... .......... .......... .......... ..........
WA        :  .......... .......... .......... .......... ..........
SW        :  .......... .......... .......... .......... .T........
NZ        :  .......... .......... .......... .......... .T........
CA        :  .G........ .......... .......... .......... ..........
NV        :  A.AT...... .......... .........A .......... .T....G...
VL        :  -..T...... .......... .........A .......... .T....G...
Consensus :  TATATTTGCC CACACATGGG ATCAATAGGG TACCATAACG AGGAAGATCG
             → 5' end of LSU

AC        :  ..A....... .......... .......... .......... .---......200
JV        :  .G........ .......G.G A......... .......... .---......
KI        :  .......... .......... .......... .......... .---......
BB        :  .......... .......... .......... .......... .---......
CN        :  .......... .......... .......... .......... .---......
WA        :  .......... .......... .......... .......... .---......
SW        :  .......... .......... .......... .......... .---......
NZ        :  .......... .......... .......... .......... .---......
CA        :  .......... .......... .......... .......... .---......
NV        :  ..AAA..... .......G.. ..T.C..... TA..AT..T. ......T...
VL        :  ..ATA..... ...NN..G.. ..T.C....N TA..AT.NT. ......T...
Consensus :  TAGCGGAATA CGAAAGATTA TTGATCGAAT ATATTAATAT ATATATAGAT
                                               H10       H10'
```

```
AC          :  .......... .......... .......... .......... ..........250
JV          :  .......... .......... .......... .......... ..........
KI          :  .......... .......... .......... .......... ..........
BB          :  .......... .......... .........G .......... ..........
CN          :  .......... .......... .......... .......... ..........
WA          :  .......... .......... .......... .......... ..........
SW          :  .......... .......... .......... .......... ..........
NZ          :  .......... .......... .......... .......... ..........
CA          :  .......... .......... .......... .......... ..........
NV          :  A......... .......... .......... ......A... ..........
VL          :  A......... .......... .......... ......A... ..........
Consensus   :  TACCCTTTGA ACTTAAGCAT ATCATTAAAA GGAGGAGAAG AAACTAACTA
                    NV1373FD

AC          :  .......... .......... .......... .......... ..........300
JV          :  .......... .......... .......... .......... ..........
KI          :  .......... .......... .......... .......... ..........
BB          :  .......... .......... .......... .......... .A........
CN          :  .......... .......... .......... .......... ..........
WA          :  .......... .......... .......... .......... ..........
SW          :  .......... .......... .......... .......... ..........
NZ          :  .......... .......... .......... .......... ..........
CA          :  .......... .......... .......... .......... ..........
NV          :  .......... ......G... .......... ..T....... ..........
VL          :  .......... ......G... .......... ..T....... .........C
Consensus   :  GGATTTCTTT AGTAGCAGCG AGTGAACAAG AAACAACCCT TGATTGTAAT


AC          :  .T....-..- .......... .......... .......... ..........350
JV          :  .T....-..- .......... .......... .......... ..........
KI          :  ....-.-... .......... .......C.. .......... ..........
BB          :  ....-.-... .......... .......C.. .......... ..........
CN          :  ....-.-... .......... .......... .......... ..........
WA          :  ....-.-... .......... .......... .......... ..........
SW          :  .-....-... .......... .......... .......... ..........
NZ          :  .......-... .......... .......... .......... ..........
CA          :  ....-.-... .......... .......... .......... ..A.......
NV          :  ....A..... ..A....... ...T....C. ...AT....A ..A.....A.
VL          :  ....A..... ..A......C ...T....C. ...AT....A ..A.....A.
Consensus   :  CCTTTACTGG AGCTGTAAAT CATATATTTT ATTTCTTATT TCGTAGAGGA
                  H16

AC          :  ...-...... .......... ...-...... .......... ..........400
JV          :  ...-...... .......... ...-...... .......... ..........
KI          :  ...-...... .......... ...-...... .......... ..........
BB          :  ...-...... .......... ...-...... .......... ..........
CN          :  ...-...... .......... ...-...... .......... ..........
WA          :  ...-...... .......... ...-...... .......... ..........
SW          :  ...-...... .......... ...-...... .......... ..........
NZ          :  ...-...... .......... ...-...... .......... ..........
CA          :  ...-...... .......... ...-...... .......... ..........
NV          :  .T...A..T. ....GTTGAT ......G.AT ..TT.CT... ..........
VL          :  .T......T. ....CTTGAT ......G.AT ..TT.CT... ..........
Consensus   :  TGTTATATCC GTTATAAATG AGAATATATA AAAGTAATTG AGTAGGGCTG
                                              H16'       NV1584RD
```

```
AC          :  .......... .......... .......... ....G..... ..........450
JV          :  .......... .......... .......... .......... ..........
KI          :  .......... .......... .......... .......... ..........
BB          :  .......... .......... .......... .......... ..........
CN          :  .......... .......... .......... .......... ..........
WA          :  .......... .......... .......... .......... ..........
SW          :  .......... .......... .......... .......... ..........
NZ          :  .......... .......... .......... .......... ..........
CA          :  .......... .......... .......... .......... ..........
NV          :  .......... ..........C......... .......... ..........
VL          :  .......... .........N .C........ .......... ..........
Consensus   :  CTTGGTAGTG CAGTTTGAAT ATAGGTAGAA TGAGATATCT AAGGTTAAAT

AC          :  .......... .......... .......... .......... ..........500
JV          :  .......... .......... .......... .......... ..........
KI          :  .......... ...-...... .......... .......... ..........
BB          :  .......... .......... .......... .......... ..........
CN          :  .......... .......... .......... .......... ..........
WA          :  .......... .......... .......... .......... ..........
SW          :  .......... .......... .......... .......... ..........
NZ          :  .......... .......... .......... .......... ..........
CA          :  .........T .......... .......... .......... ..........
NV          :  .........T .......... .......... .........T ..........
VL          :  .........T .......... .......... .........N ..........
Consensus   :  ATAATGGTAC ACCGATAGCA AATAAGTACT GCGAAGGAAC TTGTGAAAAT
                          NV1629FD

AC          :  .....T.... .......... .......... .......... ..........550
JV          :  .....T.... .......... .......... .......... ..........
KI          :  .AT....... .......... .......... .......... ..........
BB          :  .......... .......... .......... .......... ..........
CN          :  .......... .......... .......... .......... ..........
WA          :  .......... .......... .......... .......... ..........
SW          :  .......... .......... .......... .......... ..........
NZ          :  .......... .......... .......... .......... ..........
CA          :  .......... .......... .......... .......... ..........
NV          :  .........GT .......... .......... .........G ..........
VL          :  .........GT ........C. .
Consensus   :  GTGTGGGTTA TAGCCTTATT TTTAAGGACC CGTCTTGAAA CACGGACCAA

AC          :  .......... .......... ...---.... .......... ..........600
JV          :  .......... .......... ...---.... .......... ..........
KI          :  .......... .......... ...---.... .......... ..........
BB          :  .......... .......... ...---.... .......... ..........
CN          :  .......... .......... ...---.... .......... ..........
WA          :  .......... .......... ...---.... .......... ..........
SW          :  .......... .......... ...---.... .......... ..........
NZ          :  .......... .......... ...---.... .......... ..........
CA          :  .......... .......... ...---.... .......... ..........
NV          :  .......... ........A. .........T ..T..A.... .....AT...
VL          :
Consensus   :  GGAGATTATA ATTATAGCGA GATAAAAACA ATGTAGTCGT TATTAGCTTG
                                       H28        H28'
```

```
AC         :  .......... .......... .......... .......... ..........650
JV         :  .......... .......... .......... .......... ..........
KI         :  .......... .......... .......... .......... .........A
BB         :  .......... .......... .......... .......... ..........
CN         :  .......... .......... .......... .......... ..........
WA         :  .......... .......... .......... .......... ..........
SW         :  .......... .......... .......... .......... ..........
NZ         :  .......... .......... .......... .......... ..........
CA         :  .......... .......... .......... .......... ..........
NV         :  .......... .......... .......... .......... ..........
VL         :
Consensus  :  ATAAGTTATA ATTATAAGAC CCGAAACACA GTGAACTATA CATGTTCTGG


AC         :  .......... .......... ......676
JV         :  ....G..... .......... ......
KI         :  .......... .......... ......
BB         :  .......... .......... ......
CN         :  .......... .......... ......
WA         :  .......... .......... ......
SW         :  .......... .......... ......
NZ         :  .......... .......... ......
CA         :  .......... .......... ......
NV         :  .......... .......... ......
VL         :
Consensus  :  TTGAAGATAA GCAACAGTTT ATTGGA
                        NV1851R
```

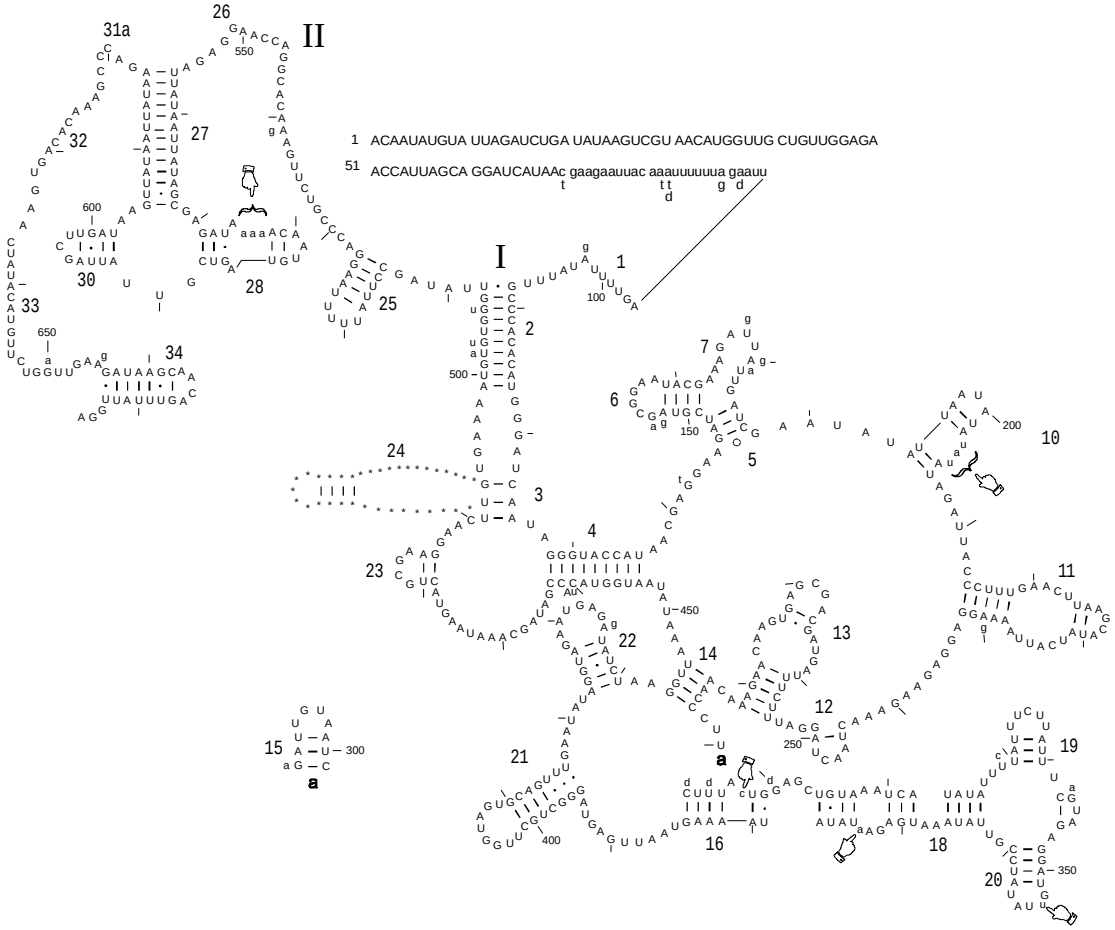


Figure 1.3

A consensus partial secondary structure of the microsporidian LSU rRNA based on the alignment of nine *N. apis* isolates, one *N. vespula* isolate, and one *V. lymantriae* isolate. Nucleotide insertions (pointer), base substitutions (lower case), and deletions (d) are shown. Numbering of nucleotide positions is as for figure 1.1. Every 50th nucleotide is numbered while every 10th nucleotide has a stroke mark. Helix 15 (indicated by **a**) is drawn separate to the remaining consensus secondary model. The actual position of helix 15 within the secondary structure model is also indicated by **a**. Helix 24 is highly conserved in the eubacteria, archaebacteria and eukaryotes, and absent in these Microsporidia. In this diagram, the nucleotide positions normally occurring in helix 24 of eukaryotes are marked by *****.

Table 1.2

Nucleotide positions within a region of 676 nucleotides of the rDNA operon from nine *N. apis* isolates, one *N. vespula* isolate (NV), and one *V. lymantriae* isolate (VL) are compared. Only nucleotide positions that have undergone a substitution event or loss within at least one of the nine isolates are considered in conjunction with the other two species. The abbreviations are: 'Seq Pos' - sequence position relative to figure 1.2; 'consensus nucleotide' - indicates dominant nucleotide character state at a particular position; 'paired nucleotide' - indicates a bonded nucleotide within secondary model; 'M' - indicates an absent nucleotide relative to the consensus nucleotide; and A, C, T, G - indicates nucleotide character state. Abbreviations for the isolates as are as shown in figure 1.2.

Seq	Consensus	Paired	*Nosema apis* Isolate										
Pos	Nucleotide	Nucleotide	AC	JV	KI	BB	CN	WA	SW	NZ	CA	NV	VL
70	C					T						T	T
82	A										T	C	T
83	A		M	M	M	M	T				T		
84	T		M	M	M	M		M	M	M			
90	A						G						
92	A										M	M	M
102	A	U		G							G		
142	G								T	T			
152	A	U		G									
153	G		A									A	A
168	T		G									G	G
170	A		G										
171	T		A										
230	A	U				G							
292	G	C				A							
302	C	G	T	T					M				
305	T		A	A	M	M	M	M			M		
310	G		M	M									
328	T				C	C							
343	G										A	A	A
435	A		G										
460	C	G									T	T	T
501	T	A			A								
502	G	C			T								
505	G	C	T	T									
650	G				A								
655	A			G									

Table 1.3

A comparison of nucleotide substitution events based on the consensus sequence of figure 1.2.

Consensus Nucleotide	Replacement Nucleotide	Occurrence
A	G	8
	T	3
	C	0
G	T	5
	A	4
	C	0
C	T	4
	A	0
	G	0
T	A	4
	C	2
	G	1

Figures 1.4a to c

Phylogenetic trees constructed from a partial rRNA gene operon sequence alignment (Figure 1.2) of nine isolates of *N. apis*, one *N. vespula* (NV) isolate and one *V. lymantriae* (VL).

a. Maximum parsimony consensus tree.
   501 sites (58 informative), 82 steps, 1000 bootstrap replicates
b. Neighbor joining tree using Jukes and Cantor distance measure.
   501 site, 1000 bootstraps
c. Maximum likelihood tree.
   501 sites, 200 bootstraps, maximum likelihood ln(L) -1084.573

Abbreviations for the isolates are as shown in figure 1.2.

Figure 1.4a

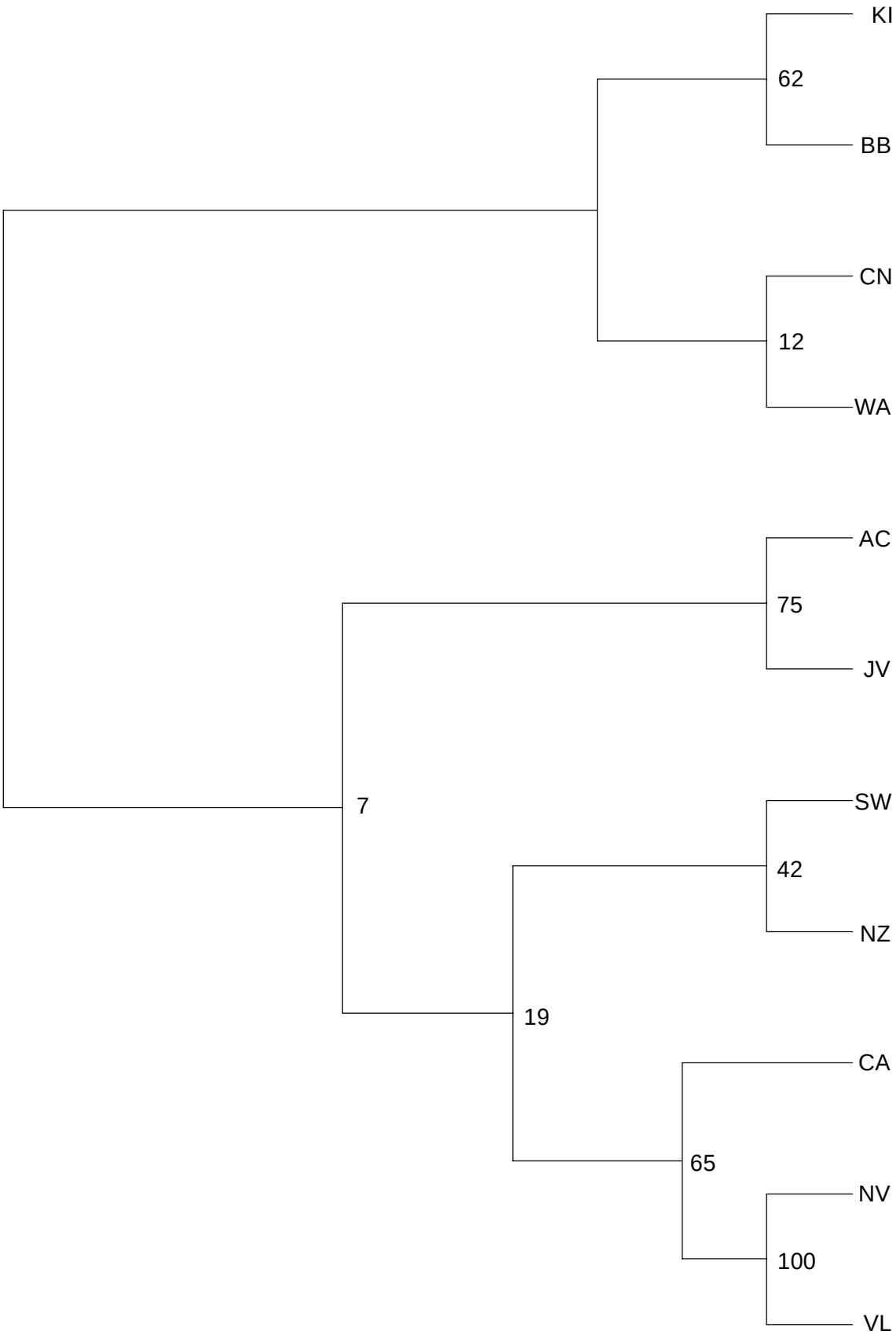


Figure 1.4b

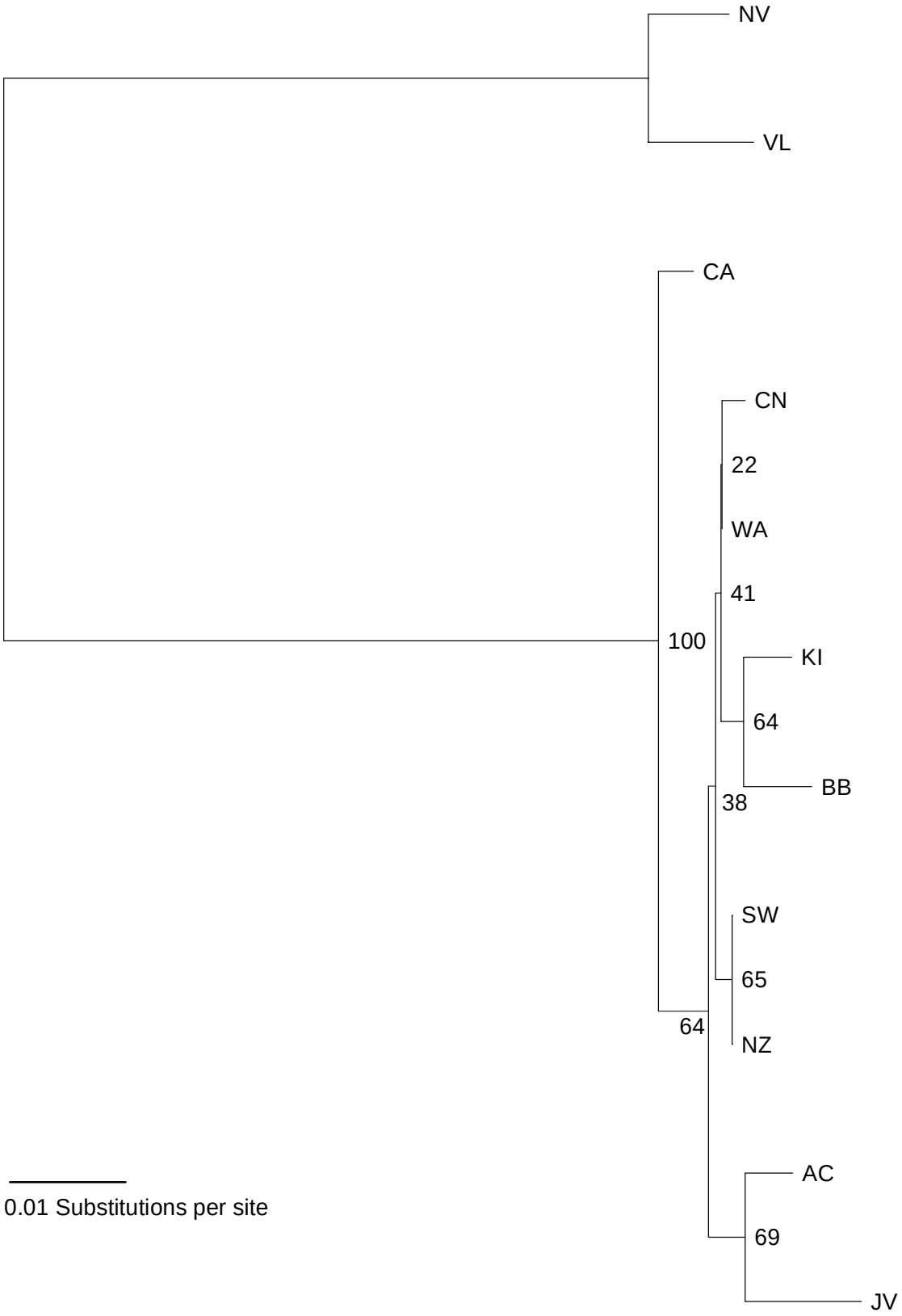
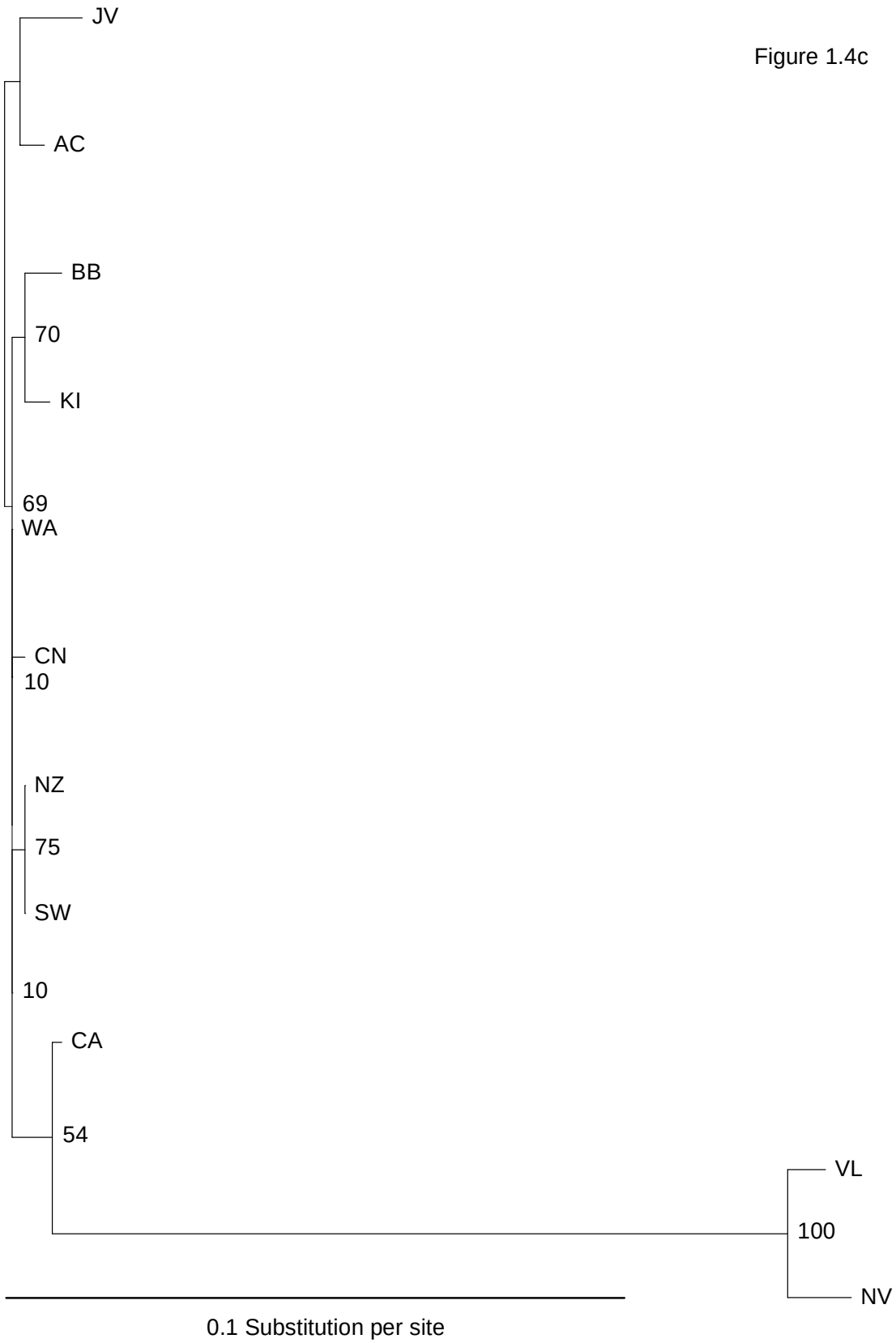

NV
VL
CA
CN
22
WA
41
100
KI
64
BB
38
SW
65
64
NZ
AC
69
JV
0.01 Substitutions per site

Figure 1.4c


JV
AC
BB
70
KI
69
WA
CN
10
NZ
75
SW
10
CA
54
VL
100
NV
0.1 Substitution per site

2. The Effects of Thymol on *Nosema*

2.0 Introduction

Thymol (3-Hydroxy-*p*-cymene) is a constituent of the essential oil derived from thyme and many other plant species. Thymol is also made synthetically. In its pure form, thymol is a colourless crystal with a pleasant yet strong smell. Thymol is effective in suppressing the growth of pathogenic bacteria and fungi. For example, thymol has been shown to suppress the growth of bacteria such as *Salmonella typhimurium* and *Staphylococcus aureus* (Juven *et al.,* 1994), and fungi such as *Aspergillus flavus* (Mahmoud, 1994) and *Cryptococcus neoformans* (Viollon and Chaumont, 1994). Thymol's action against bacteria results in the disintegration of the outer and cytoplasmic membranes releasing the cellular components (Helander *et al.,* 1998), and similarly, disintegrates the fungal hyphae (Zambonelli *et al.,* 1996).

More recently, the suppressive effects of thymol have been demonstrated against parasitic mites of European honeybees (*Apis mellifera*) including *Varroa jacobsoni* (Calderone, 1999; Sammataro *et al.,* 1998) and *Acarapis woodi* (Calderone *et al.,* 1997). Honeybees are tolerant to the use of thymol (Imdorf *et al.,* 1995). The action of thymol as a miticide is not clear, but it is known to be most effective in the absence of honeybee brood (Calderone *et al.,* 1997). Thymol is not detectable by taste in honey at concentrations less than 1.1 mg/kg (Bogdanov *et al.,* 1998). The toxicity of thymol (LD50), as determined by oral dose in rats, is 980 milligrams (mg) per kilogram (kg) of body weight (Lenga, 1988).

Anecdotal evidence suggests that thymol may suppress nosema disease in honeybees (Brown, personal communication). Over a period of 20 years, wintering honeybees were fed sugar syrup containing 0.44mM thymol as a preservative. During this period of time, no incidence of nosema disease was recorded in these honeybee colonies.

Nosema disease is of economic importance because the disease can substantially reduce honey yields (Fries *et al.,* 1984) and pollen collection by bees (Anderson and Giacon, 1992). The causative agent of nosema disease is the microsporidian *Nosema apis.* Spores of *N. apis* are ingested by adult honeybees during activities such as hive cleaning. The ingested spores germinate and inject their infective sporoplasm into the epithelial gut cells where they multiply. The infected gut cells rupture, releasing new spores that may re-infect the host, or are passed out in faeces to infect other honeybees (Bailey and Ball, 1991).

Incidents of nosema disease usually occur in the autumn and spring. The severity and duration of the disease appear to be linked inversely to the quantity and quality of available pollen and nectar sources. Apparently, increased nutrition and brood rearing leads to a decline in the level of the disease during the summer months. At the height of summer, the presence of *N. apis* within the beehive is almost undetectable.

Nutrition alone may not be entirely responsibly for the suppression of nosema disease. Perhaps in addition to nutrition, pollen and nectar may contain other substances such as thymol that act in a synergistic way to suppress nosema disease. Many Australian species including eucalypts (Boland *et. al.,* 1991) contain thymol as a constituent of their oils. Thymol may also be contained in the pollen and nectars of these species.

In this section, I report on thymol as a potential control agent for nosema disease in honeybees.

2.1 Methodology

This research was undertaken as a pilot-study to investigate the effectiveness of thymol in suppressing nosema disease. This study was not conducted using honeybees and *N. apis.* Rather, a model system was used. This model system eliminated many of the problems associated with nutritional studies in honeybees. For example, control over diet of foraging bees or providing a nutritionally balanced diet for caged bees. The data from this model system can be extrapolated to honeybees and form the basis of further studies.

The lepidopteran *Helicoverpa armigera* and the microsporidian *Nosema vespula* were chosen for this study. The caterpillar of the moth *H. armigera* is a pest species of cotton and is used extensively as a test animal in laboratory trials; their dietary requirements and rearing methods are well known. *N. vespula* was chosen for three reasons: 1) it has an extensive host range including *H. armigera* (Anderson, personal communication), 2) it is closely related to *N. apis* (Section 1) and, 3) its ease-of-culture under laboratory conditions.

At the time of inoculation with *N. vespula* spores, thymol was included in the caterpillar diet at concentrations of 30mM and 15mM. The use of thymol as a prophylactic was also investigated. An additional group of caterpillars received the 30mM thymol diet commencing one day before inoculation. The study included two controls. The first control tested for the presence of a natural microsporidial infection in the laboratory population of *H. armigera*. The second control tested the suppressive effects of ethanol against microsporidial infection. Absolute ethanol was used as the carrier-agent to dissolve thymol crystals before its addition to the diet.

Ten days post-inoculation, the numbers of mature *N. vespula* spores per caterpillar was determined by microscopic examination. This data were statistically analysed using the one-way analysis of variance (ANOVA) and student's t test. The null hypothesis was assumed: addition of thymol to the diet of *H. armigera* does not suppress infection by *N. vespula*.

2.1.1 Host Animals

Third instar *H. armigera* caterpillars were a gift from Dr Peter Christian, CSIRO Division of Entomology, Canberra, Australia.

2.1.2 Parasite Spores

Spores of the *N. vespula* isolate were a gift from Dr Denis Anderson, CSIRO Division of Entomology, Canberra, Australia. This species of microsporidia was originally isolated from the infected larvae of the European wasp *Vespula germanica* (Vespoidea: Vespidae) by Dr Anderson. Experimental research has shown that this isolate infects an extensive host range including hymenopterans, dipterans, and lepidopterans. Since discovery, this species has been maintained in the laboratory using *H. armigera* caterpillars. *The host ingests N. vespula spores*. The spores germinate in the gut lumen in response to external stimuli and infect the host's epithelial gut cells. Here minor replication occurs. The parasite then moves from the gut cells to the host's fat bodies and replicates. The host eventually dies from the infection, releasing mature spores from the decaying corpse (Dr Denis Anderson, personal communication).

The *N. vespula* inoculum was recovered from frozen, infected caterpillars. *N. vespula* spores were liberated from the host tissue by macerating five entire caterpillars in 30ml of distilled water using a mortar and pestle. Large particulate matter was removed from the spore suspension by filtration through four layers of Kimwipe (Kimberly-Clark, Australia). The filtrate was transferred to an Oakridge tube and centrifuged at 1000 x g for 20 minutes using a Beckman Ti 20 rotor and Beckman J2-MI centrifuge. The supernatant was removed and the remaining pellet of spores suspended in 1ml

of distilled water. A discontinuous gradient of neutralised Percoll (Sigma) was constructed in a 50ml ultracentrifuge tube (25 x 89 mm) by the sequential layering of 7ml 100%, 8ml 75%, 8ml 50% and 8ml 25% Percoll (Sato and Watanabe, 1980). The 1ml aliquot of spore suspension was immediately overlayed onto the gradient and centrifuged at 3,000 x g for 90 minutes using a Beckman 28S rotor and L8-70M ultracentrifuge. Most spores settled as a white band at the 75-100% interface, while some spores passed through the gradient and were visible as a pellet among the debris in the bottom of the ultracentrifuge tube. The band at the interface was removed with a Pasteur pipette, placed in an Oakridge tube and diluted with 35ml of distilled water. The suspended spores were pelleted at 3,000 x g for 30 minutes and the supernatant removed. The spores were suspended in 1ml of distilled water, transferred to an Eppendorf tube and washed three times with distilled water to remove Percoll. Washing of the spores was by centrifugation at 1,000 x g for 5 minutes, removal of the supernatant, and suspension in distilled water. At the third wash the spore concentration was determined using a Neubauer Counting Chamber (Cantwell, 1970). The spores were then pelleted once more, the supernatant removed, and the spores suspended in distilled water at concentrations of approximately 10^6 spores ml^{-1}. The purified spore suspension was stored in 0.5ml aliquots at 4°C.

2.1.3 Dietary Media

Thymol is not directly soluble in water; thus absolute ethanol was used as a carrier agent. A 1.5M thymol stock in absolute ethanol was prepared. A standard caterpillar diet containing soybean flour, wheat germ, agar and water was prepared (Teakle and Jensen, 1985). The ingredients were combined and the mixture brought to the boil. The mixture was cooled to 50°C and thymol added to a final concentration of either 30mM or 15mM. The mixture was poured into 50mm petrie dishes and refrigerated to set. An ethanol-only control was also included in the experiment.

2.1.4 Experimental design

Nuclon™ Delta Multidishes (Nunc) containing 12 wells were used to house the caterpillars for the experiment. On the first day, a 0.5gm block of standard diet was placed in each well of 25 multidishes. In addition, a 0.5gm block of 30mM thymol diet was placed in each well of 5 multidishes. A third instar caterpillar was placed on the diet in each well of all the multidishes. The caterpillars were maintained at room temperature overnight (approximately 23°C to 25°C).

On the second day, all unconsumed diet was removed from the wells of all multidishes and the caterpillars starved for three hours. The group of 25 multidishes were allocated randomly into 5 groups of 5 multidishes and labelled treatment 1 to treatment 5. The remaining 5 multidishes of caterpillars pre-fed 30mM thymol diet were labelled treatment 6. Each caterpillar in each treatment was given a 0.25gm block of one of the following diets:

- Treatment 1, standard diet (60 caterpillars);
- Treatment 2, standard diet (60 caterpillars);
- Treatment 3, standard diet containing ethanol only (60 caterpillars);
- Treatment 4, standard diet containing 15mM thymol (60 caterpillars);
- Treatment 5, standard diet containing 30mM thymol (60 caterpillars); and
- Treatment 6, standard diet containing 30mM thymol (60 caterpillars).

The block of diet for each caterpillar in treatments 2 to 6 was also inoculated with 10µl (~10,000 spores) of *N. vespula* spore suspension. The caterpillars were fed their respective diets on demand for 9 days. The caterpillars were maintained at room temperature.

On the tenth day, all surviving caterpillars were stored at -20°C pending the estimation of the total number of mature *N. vespula* spores per caterpillar (spore load). Each caterpillar was homogenised

with a micro-pestle in an Eppendorf tube and suspended in water to a final volume of 1ml. The spore load of each caterpillar was determined microscopically using a Neubauer counting chamber.

The data was statistically analysed, first by a one-way analysis of variance (Anova) and then by student's t test for each treatment pair.

2.2 Results and Discussion

Premature deaths occurred within each treatment. The number of surviving caterpillars ranged from 42 in treatment 5 to 55 in treatment 1 (Table 2.1). The causes of death were not determined, although most deaths were accompanied by the rapid onset of a fungal infection as evidenced by the hyphae that engulfed the corpse. In some instances though, deaths were associated with the rapid onset of a bacterial infection. The addition of an antibiotic and fungicide to the diets may have prevented the deaths. However, these chemicals may have also contributed to the suppression of the microsporidial infection.

2.2.1 Natural Infection

All surviving caterpillars from treatment 1 were found by microscopic examination to be free of microsporidial spores. Therefore, it was assumed that the entire laboratory population of *H. armigera* caterpillars was free of microsporidial infections.

2.2.2 Inoculated Caterpillars

All caterpillars from treatments 2 and 3 contained mature *N. vespula* spores (Table 2.1). Conversely, less than half of the caterpillars from treatments 3 to 6 contained mature *N. vespula* spores. The mean number of spores per caterpillar ranged from 2×10^8 for treatment 4 to 2×10^9 for treatments 2 and 3 (Table 2.1). The highest individual spore count was observed in treatment 2 and was estimated at 1.12×10^{10} spores. Treatment 2 served as a minus thymol control and as such demonstrated the extent to which the disease would progress.

2.2.3 Statistical Analysis

The total number of mature *N. vespula* spore was determined for each surviving caterpillar ten days post-infection. This data was statistically analysed by one-way analysis of variance (ANOVA) and the student's t test. The null hypothesis was assumed: the inclusion of thymol in diet does not suppress *N. vespula* in *H. armigera* caterpillars. ANOVA analysis rejected the null hypothesis (alpha 0.05, pValue 0.000, Table 2.2). The addition of thymol to the diet of inoculated *H. armigera* caterpillars does suppress infection by *N. vespula*.

The student's t test (alpha 0.05) was used to test for statistical significance between pairs of treatments (Table 2.3). Treatments 2 and 3 (no thymol) are not significantly different. Similarly, treatments 4, 5, and 6 (thymol) are not significantly different. However, as depicted in figure 2.1, these two treatment groups are significantly different in their response to the inclusion of thymol in the diet.

Treatments 4 (15mM thymol) and 5 (30mM thymol) are not significantly different. There are equivalent numbers of healthy and infected caterpillars, the infected caterpillars having equivalent spore loads (Table 2.1). These data indicate that the inclusion of 15mM thymol in the diet should be sufficient to prevent infection.

The response to treatment 6 was not significantly different to that of treatments 4 and 5. Therefore, it is unlikely that thymol (when pre-fed) provides or induces immunity in *H. armigera* to *N. vespula*.

Furthermore, the spore counts per caterpillar overlaps between treatments 2 to 6 (Table 2.1). This overlap suggests that thymol has little or no effect post-infection on *N. vespula*.

Approximately 45% of the caterpillars from treatments 4, 5, and 6 became infected with *N. vespula*. Observations of *H. armigera* feeding behavior may provide an explanation for the phenomena. All animals were starved for 3 hours before being provided with their respective inoculated diets. The time lapse between the provision of diet and the commencement of feeding was observed to differ between caterpillars. Simply, those caterpillars that began feeding at a later time consumed spores that had been exposed to thymol for a longer period and hence were potentially at a reduced risk of infection.

These data suggest that thymol's acts directly on the spore prior to germination and that the length of exposure is a factor.

2.2.4 Mode of action

Microsporidian spores are known to germinate in response to five main factors, namely pH, osmotic pressure, the type and concentration of cations in the media (Dall, 1983; Whitlock and Johnson, 1990), and desiccation followed by hydration (Olsen *et al.*, 1986). The response of different species of microsporidia to these factors varies considerably (Whitlock and Johnson, 1990).

One possible mode of action for thymol is to simply bind to the spore coat of *N. vespula* and block recognition by the spore of the factors required for germination. The data suggest that this mode of action is unlikely. The inclusion of 15mM thymol in the diet prevented infection in 53.8% of caterpillars, while 30mM prevented infection in 59.2% of caterpillars. These percentages are not significantly different. The concentration of thymol in the diet was not rate limiting with regard to a simple process of spore-coat binding, that in turn, prevented germination in response to environmental stimuli. Thymol must exert its effect by some other mode of action.

Helander *et al.* (1998) found that thymol inhibited the growth of *Escherichia coli* and *Salmonella typhimurium*at at growth-media concentrations of 3mM and 1mM respectively. The inclusion of thymol resulted in the uptake of fluorescent dye, and lipopolysaccharide and protein release indicative of outer membrane disintegration. Additionally, it was found that the inclusion of thymol in the growth media increased the permeability of the cytoplasmic membrane to ATP.

Zambonelli *et al*. (1996) demonstrated that the inclusion of *Thymus vulgaris* L. oil (thyme oil) in growth-media at concentrations of 400-800 ppm containing 50.06% thymol inhibited the growth of several species of pathogenic fungi. They observed that the inclusion of thymol led to morphological changes; the hyphae appeared to have collapsed and fewer conidia were produced.

The mature microsporidial spore consists of three outer layers that envelop the cytoplasm containing the cell organelles: the exospore, the endospore, and the plasma membrane. The exospore is a proteinaceous coat containing electron dense layers, while the endospore is electron transparent layer consisting mostly of α-chitin. The inner surface of the endospore is lined with the plasma membrane that separates the cytoplasm and the endospore (reviewed in Vávra and Larsson, 1999). It is the plasma membrane that is likely to be susceptible to the disintegrating properties of thymol.

In response to specific external stimuli, a sequence of events occurs within the spore that increases the internal hydrostatic pressure. In consequence, the polar filament everts and the sporoplasm is injected into a neighbouring host cell. The primary events associated with spore germination are a calcium ion flux and activation of the enzyme trehalase. Trehalase cleaves the disaccharide trehalose into smaller molecules, increasing the osmotic pressure potential. Water enters the spore through transmembrane pathways and interacts with these molecules. The pressure within the spore increases dramatically

causing the spore coat to rupture and the polar filament to evert (reviewed in Cali and Takvorian, 1999).

It is possible that thymol enters the spore through the same transmembrane pathways that allow the penetration of water during germination. Penetration through these pathways may occur prior to germination by passive diffusion or during the germination process prior to polar filament eversion. Alternatively, thymol may transverse the spore coat by some other pathway.

2.3 Thymol and Honeybees

2.3.1 Possible mode of action

Two critical differences exist between *N. vespula* and *N. apis* that may be important regarding the effectiveness of thymol as a control for nosema disease. First, unlike *N. vespula* that infects the digestive tract epithelium and then moves to the fat bodies to replicate, N. *apis* undergoes replication entirely within the digestive tract epithelium. Second, *N. vespula* is released from the host after the host dies, whereas *N. apis* spores are continually being released from infected honeybees. Mature spores are found to contaminate regurgitated brood-food (nurse honeybees), regurgitated nectar (forager honeybees) and faeces.

It is likely that thymol enters the spore, disrupts the plasma membrane and thus prevents germination. For *N. vespula*, there was only one opportunity for thymol contained in the diet to come in contact with the spore, at the time of inoculation of the diet with spores. The life cycle of *N. apis* presents several opportunities for thymol contained in sugar syrup to come in contact with *N. apis* spores.

Brown fed wintering honeybees sugar syrup containing 0.44mM thymol. The syrup was fed to the honeybees in a container within the hive. One possible model to explain the suppressive effects of thymol on nosema disease relates to normal honeybee activities following the provision of sugar syrup. Honeybees draw the syrup into their honey sack before transferring the syrup to the honeycomb for storage. During this process some of the syrup is consumed. Mature *N. apis* spores contaminating the mandibles, the honey sack and digestive tract are exposed to thymol during the transfer process. Additionally, *N. apis* spores contaminating honeycombs in which the syrup is stored are also exposed to thymol. During the storage process, honeybees condense the syrup by evaporating off excess moisture and so increase the concentration of thymol per volume of syrup. At this point, mature spores within the digestive tract of honeybees and within the comb have been ‘killed’. However, spores continue to develop within the digestive tract epithelium and are released into the digestive tract. The honeybees continue to consume the sugar syrup as a source of energy during wintering. Maturing spores being released from the digestive tract epithelium are continually exposed to thymol. Over a period of time, the level of infective *N. apis* spores in the hive declines to a point below that which can cause nosema disease.

2.3.2 Nectar, pollen, and thymol

Many plant species have thymol as a constituent of their essential oils. Furthermore, numerous other compounds are also found in the essential oils of various plant species (eg. *Trans*-cinnamaldehyde, Limonene, and eucalyptol) (Boland *et. al.*, 1991). These are biologically active against bacteria (Helander *et al.*, 1998) and pathogenic fungi (Zambonelli *et al*. 1996).

Many of these species produce pollen and nectar that is collected by honeybees. Possibly, the decline of nosema disease observed to occurs in late spring and summer may in part result from the consumption of pollen and nectar containing thymol or other substances that are biologically active against *N. apis*.

2.4 Summary and Recommendations

To address the third objective of the research proposal a model system was used. It was demonstrated that thymol is affective in suppressing *N. vespula* infections in *H. armigera* caterpillars under laboratory conditions. From evidence of the action of thymol on bacteria and fungi, it is likely that thymol acts directly on the spore, penetrating the spore coat and disrupting the plasma membrane. This action prevents germination of the spore and the subsequent disease caused by the replicating sporoplasm. Brown observed that sugar syrup containing 0.44mM thymol was effective in controlling nosema disease in over wintering honeybees. The data for *N. vespula* supports this claim. The life cycle of *N. apis* presents several opportunities for the thymol contained in sugar syrup to come in contact with *N. apis* spores, and as such, thymol is likely to be a highly effective control for nosema disease. Australian honeybee colonies may also benefit substantially from the inclusion of thymol in the sugar syrup fed.

It is recommended that three lines of research be followed. First, determine the suitability of thymol as an additive to dietary supplements used in commercial beekeeping. Second, examine the effectiveness of other organic substances derived from essential oils for their biological activity against *N. apis.* Third, test the pollen and nectar of a range of economically important floral species for the presence of thymol and other substances that are shown to be biologically active against *N. apis.*

Intellectual property pertaining to this research relates to the possible commercialisation of dietary supplements containing thymol. The availability of essential oils, although more expensive than synthetic thymol, most likely precluded the patenting of thymol as a control for *N. apis* on economic grounds. The information is not considered commercial sensitive. Therefore, it is recommended that the details of this research be made available to industry through refereed, scientific journals.

Table 2.1

A comparison by treatment of the number of surviving caterpillars, their mean infection levels, the range of infections observed, and the percentage of caterpillars containing mature *N. vespula* spores on the tenth day post-inoculation.

TREATMENT	DIET	SURVIVORS	MEAN OF SPORE LOAD	RANGE OF SPORE LOAD	PERCENTAGE INFECTED
1	Standard	55	0	0	0.0%
2	Standard	48	2×10^9	5.0×10^7 - 1.12×10^{10}	100%
3	Ethanol	53	2×10^9	1×10^8 - 5.75×10^9	100%
4	15mM Thymol	52	2×10^8	0 – 2.78×10^9	46.2%
5	30mM Thymol	42	3×10^8	0 – 3.37×10^9	40.8%
6	30mM Thymol	53	5×10^8	0 – 7.31×10^9	47.2%

Table 2.2

ANOVA calculated from the infection levels of mature *N. vespula* spores in surviving *H. armigera* caterpillars on the tenth day post-infection.

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	1689.1569	422.289	39.8093
Error	243	2577.6991	10.608	***Prob>F***
C Total	247	4266.8560		0.0000

Table 2.3

Comparisons for each pair of Treatments using the Student's t (t = 1.96980). Negative values are not significantly different.

Abs(Dif)-LSD	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Treatment 2	-1.30957	-1.16978	3.99535	4.15661	3.71968
Treatment 3	-1.16978	-1.24627	4.13578	4.29532	3.86026
Treatment 4	3.99535	4.13578	-1.25820	-1.09832	-0.97075
Treatment 5	4.15661	4.29532	-1.25820	-1.09832	-0.81120
Treatment 6	3.71968	3.86026	-0.97075	-0.81120	-1.24627

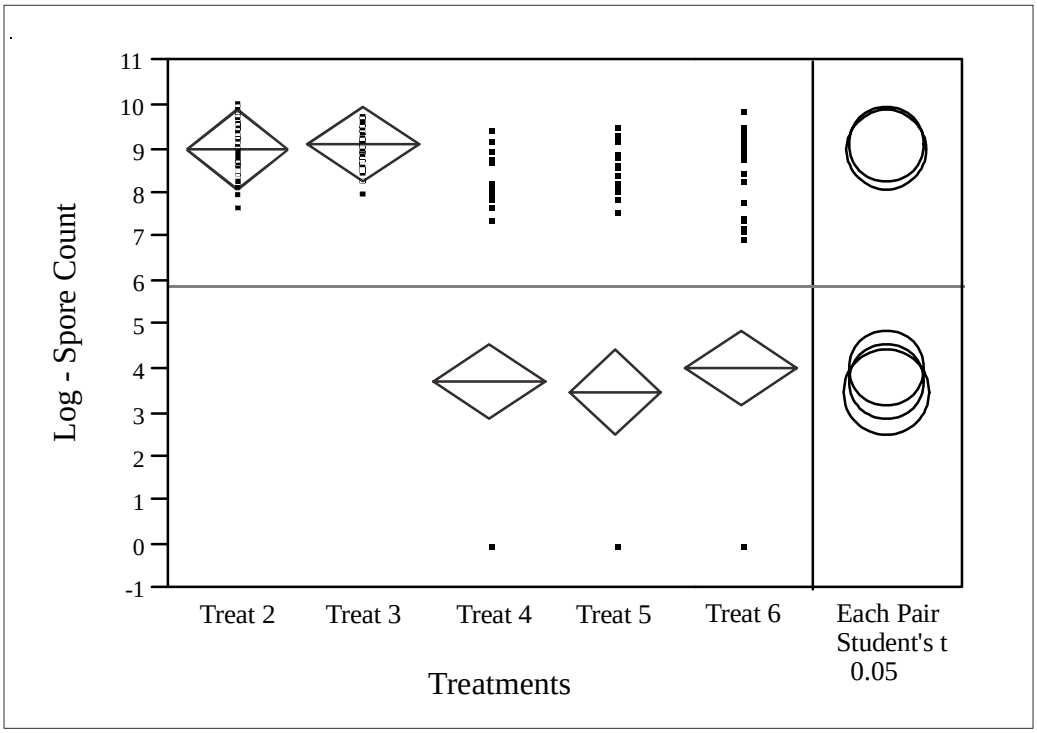


Figure 2.1

A chart depicting spore count per animal at day 10 post-inoculation as a function of diet consumed. Diamonds indicate mean and standard error for each treatment. Analysis using the student's t test is depicted as circles corresponding to the diamonds. Overlapping circles indicate mean levels of infection by *N. vespula* in *H. armigera* that are not significantly different.

3. References

Anderson, D.L. CSIRO, Division of Entomology, Black Mountain, Canberra, Australian Capital Territory, Australia.

Anderson, D. L., and Giacon, H. 1992. Reduced pollen collection by honey bee (Hymenoptera: Apidae) colonies infected with *Nosema apis* and sacbrood virus. *J. Econ. Entomol.* 85(1), 47-51.

Bailey, L. and Ball, B.V. 1991. *Honey Bee Pathology* (2nd edition). pp 62 - 74. Academic Press, London.

Baker, M.D., Vossbrinck, C.R., Maddox, J.V. and Undeen, A.H. 1994. Phylogenetic relationships among *Vairimorpha* and *Nosema* species (Microspora) based on ribosomal RNA sequence data. *J. Invertebr. Pathol.*, 64: 100-106.

Baker, M.D., Vossbrinck, C.R., Dider, E.S., Maddox, J.V. and Shadduck, J.A. 1995. Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. *J. Euk. Microbiol.*, 42(5): 564-570.

Bogdanov, S., Kilchenmann, V., Imdorf, A., and Fluri, P. 1998. Residues in honey after application of thymol against varroa using the franko thymol frame. *American Bee Journal*, Vol 133(8): 610-611.

Boland, D.J., Brophy, J.J., and House, A.P.N. 1991. Eucalyptus leaf oil: use, chemistry, distillation and marketing. Inkata Press Proprietary Limited, Melbourne, Australia.

Brown, R., 1993. Personal Communication

Bulla, Jr., L.A. and Cheng, T.C. 1977. Systematics of the Microsporidia. In L. A. Bulla Jr. and T. C. Cheng (eds.) and V. Sprague and Jírí Vávra Contributing (eds.). *Comparative Pathobiology*. Vol. 2. Plenum Press, New York.

Buys, B. 1972. Nosema in brood. *South African Bee J.*, 44: 2-4.

Buys, B. 1977. A nosema disease affecting honeybee brood. In S. Colibaba (ed.). *Symposium of Bee Biology and Pathology*. pp. 73-76. Apimondia Publishing House, Bucharest, Romania.

Calderone, N.W., Wilson, W.T., and Spivak, M. 1997. Plant extracts used for control of the parasitic mites Varroa jacobsoni (Acari: Varroidae) and Acarapis woodi (Acari: Tarsonemidae) in colonies of Apis mellifera (Hymenoptera: Apidae). *J. Econ. Entomol.*, 90 (5): 1080-1086.

Calderone, N.W. 1999. Evaluation of formic acid and a thymol-based blend of natural products for the fall control of *Varroa jacobsoni* (Acari : Varroidae) in colonies of *Apis mellifera* (Hymenoptera : Apidae). *J. Econ. Entomol.*, 92(2): 253-260.

Cali A. and Takvorian P.M. 1999. Developmental morphology and life cycles of the microsporidia. In Murray Wittner and Louis M. Weiss (eds.) *The Microsporidia and Microsporidiosis*, ASM Press, Washington, D.C.

Cantwell, G.E. 1970. Standard methods for counting nosema spores. *Amer. Bee. J.*, June: 22-23.

Clark, T.B. 1980. A second microsporidian in the honeybee. *J. Invertbr. Pathol.*, 35: 290-294.

Croizer, Y.C., Koulianos, S. and Croizer, R.H. 1991. Am improved test for africanized honeybee mitochondrial DNA. *Experientia*, 47: 968-969.

Dall, D.J. 1983. A theory for the mechanism of polar filament extrusion in the microspora. *J. Theor. Biol* **105,** 646-659.

De Graaf, D.C., Masschelein, G., Vandergeynst, F., De Brabander, H.F. and Jacobs, F.J. 1993. *In vitro* germination of *Nosema apis*(Microspora: Nosematidae) spores and its effect on their alpha,alpha-trehalose/D-glucose ratio. *J. Invertbr. Pathol.,* 62: 220-225.

De Rijk, P. and De Wachter, R. 1993. DCSE v2.54, an interactive tool for sequence alignment and secondary structure research. *Comput. Applic. Biosci.*, 9: 735-740.

De Rijk, P., Van de Peer, Y., Van Den Broeck, I. and De Wachter, R. 1995. Evolution according to large ribosomal subunit RNA. *J. Mol. Evol.*, 41: 366-375.

Faulkner, D.V. and Jurka, J. 1988. Multiple alignment sequence editor (MASE). *Trends In Biochem. Sci*., 13: 321.

Fries, I., Ekbohm, G., and Villumstad, E., 1984. *Nosema apis*, samping techniques and honey yield [Apis mellifera, Sweden]. *J. Apic. Res.,* 23 (2): 102-105.

Fries. I. 1993. *Nosema apis*—A parasite in the honey bee colony. *Bee World,* 74: 5-19.

Fries, I., Feng, F., da Silva, A., Slemenda, S.B. and Pieniazek, N.J. 1996. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterisation of a microsporidian parasite of the asian honey bee *Apis cerana* (Hymenoptera, Apidae). *Europ. J. Protistol*., 32: 356-365.

Galtier, N., Gouy, M. and Gautier, C. 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *CABIOS,* 12: 543-548.

Gutell, R.R., Gray, M.W. and Schnare, M.N. 1992. A compilation of large subunit (23S- and 23S-like) ribosomal RNA structures. *Nucl. Acids Res.,* 20 : 2095-2109.

Gutell, R.R. 1994. Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucl. Acids Res.,* 22: 3502-3507.

Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., and Wright, A.-von. 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. *J. Agric. Food. Chem.,* 46 (9): 3590-3595.

Imdorf, A., Kilchenmann, V., Bogdanov, S., Bachofen, B., and Beretta, C. 1995. Toxic effects of thymol, camphor, menthol and eucalyptol on *Varroa Jacobsoni* Oud and *Apis Mellifera* L in a laboratory test. *Apidologie*., 26 (1): 27-31.

Jukes, T.H. and Cantor, C.R. 1969. Evolution of protein molecules. In H.H. Munro (ed.). *Manual of Protein Metabolism.* pp. 21-32. Academic Press, New York.

Juven, B.J., Kanner, J., Schved, F., and Weisslowicz, H. 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *J. Appl. Bacteriol.,* 76 (6): 626-631.

Lenga, R.E. 1988. The Sigma-Aldrich Library of Chemical Safety Data. Edition 2, Volume 2, Sigma-Aldrich Corporation.

Mahmoud, A.L.E., 1999. Inhibition of growth and aflatoxin biosynthesis of Aspergillus flavus by extracts of some Egyptian plants. *L. Appl. Microbiol*, 29 (5): 334-336.

Malone, L.A. and McIvor, C.A. 1996. Use of nucleotide sequence data to identify a microsporidian pathogen of *Pieris rapae* (Lepidoptera, Pieridae).

Matheson, A. 1993a. World bee health report. *Bee World*, 74: 176-212.

Matheson, A. 1993b. Practical Beekeeping in New Zealand. GP Publications Ltd, Wellington, New Zealand.

Molan P.C. 1992. The antibacterial activity of honey. 1. The nature of the antibacterial activity. *Bee World*, 73(2) 5-28.

Olsen, P.E., Rice, W.A., and Liu, T.P. 1986. In vitro germination of Nosema apis spores under conditions favourable for the generation and maintenance of sporoplasms. *J. Invertebr. Pathol* 47, 65-73.

Pomport-Castillon, C., Romestand, B. and De Jonckheere, J. 1997. Identification and phylogenetic relationships of microsporidia by riboprinting. *J. Euk. Microbiol.*, 44: 540-544.

Rice, R.N. 1999. An evolutionary study of two microsporidia. A thesis submitted for the degree of Doctor of Philosophy, The Australian National University, Canberra, ACT, Australia.

Ruttner, F. and Maul, V. 1983. Experimental analysis of the reproductive interspecific isolation of *Apis mellifera* L. and *Apis cerana* Fabr. *Apidologie*, 14: 309-327.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning. A laboratory Manual.* Second edition. Cold Spring Harbour Press. Cold Spring Harbour, New York. USA.

Sammataro, D., Degrandi-Hoffman. G., Needham, G., and Wardell, G. 1998. Some volatile plant oils as potential control agents for varroa mites (Acari: Varroidae) in honey bee colonies (Hymenoptera: Apidae). *American Bee Journal*. 138(9): 681-685.

Sato, R. and Watanabe, H. 1980. Purification of mature microsporidian spores by iso-density equilibrium centrifugation. *J. Seric. Sci. Japan*, (in Japanese with English summary). 49: 512-516.

Singh, Y. 1975. Nosema in indian honey bee (*Apis cerana* indica*). Amer. Bee J.*, 115: 59.

Teakle, R.E. and Jensen, J.M. 1985. *Heliothis punctigera.* In P. Singh and R.F. More (eds.). *Handbook of Insect Rearing.* Vol. 2 pp. 312-322., Elsevier, Amsterdam.

Van De Peer, Y., J. -M. Neefs, P. De Rijk and R. De Wachter. 1993. Reconstructing evolution from eukaryotic small-ribosomal-subunit RNA sequences: calibration of the molecular clock. *J. Mol. Evol.*, 37: 221-232.

Viollon, C. and Chaumont, J.P. 1994. Antifungal properties of essential oils and their main components upon *Cryptococcus neoformans*. *Mycopathologia*., 128 (3): 151-153.

Vossbrinck, C.R., Maddox, J.V., Friedman, S., Debrunner-Vossbrinck, B.A. and Woese, C.R. 1987. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature*, 326: 411-414.

Vossbrinck,C.R., Baker,M.D., Didier,E.S., DeBrunner-Vossbrinck,B.A. and Shadduck,J.A. 1993. Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: species identification and phylogenetic construction. *J. Euk.. Microbiol.*, 40, 354-362.

Warhurst, P and Goebel, R. 1995. The Bee Book: beekeeping in the warmer areas of Australia. Information Series Q194060, Department of Primary Industries, Queensland, Australia.

Whitlock, V.H. and Johnson, S. 1990. Stimuli for the in vitro germination and inhibition of Nosema locusta (Microspora: Nosematidae) spores. *J. Invertebr. Pathol.*, 56 (1): 57-62.

Yakobson, B., Pothichot, S. and Wongsiri, S. 1992. Possible transfer of *Nosema apis* from *Apis mellifera* to *Apis cerana*. In: *Abstracts of papers of Int. Conf. Asian honey bees and bee mites,* Bangkok, 9-14 February, 1992, p. 97. Bee Biol. Res. Unit, Dept. Biology, Chulalong Univ., Bangkok, Thaliand.

Zambonelli, A., Daulerio, A.Z., Bianchi, A., and Albasini, A. 1996. Effects of essential oils on phytopathogenic fungi in vitro. *J. Phytopatholo.*, 144(9-10): 491-494.