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Nosema ceranae, a newly identified pathogen of *Apis mellifera* in the USA and Asia*

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Abstract – Globalization has provided opportunities for parasites/pathogens to cross geographic boundaries and expand to new hosts. *Nosema* disease is one of the most serious adult honey bee diseases and has high prevalence in honey bee colonies. For years, *Nosema apis* was thought to be the only microsporidian infecting domestic bee colonies. However, recently it was discovered that *N. ceranae* could cross the species barrier from Asian honey bees (*Apis cerana*) to European honey bees (*Apis mellifera*) that are widely used for crop pollination and honey production. Over the last few years, considerable progress has been made in our understanding of *Nosema* infections in honey bees. This review summarizes previous findings and recent progress in the understanding of *Nosema* infection of *A. mellifera* in the USA and Asia, with particular emphasis on the comparative epidemiological, morphological, pathological, and genomic organization of two *Nosema* species. The prospects of future research and remaining unresolved questions associated with the study of honey bee *Nosema* diseases are also discussed.

Nosema apis / *N. ceranae* / host range / distribution / morphology / pathology / genome

1. INTRODUCTION

Nosemosis (*Nosema* disease) is one of the most serious and prevalent adult honey bee diseases worldwide (Bailey, 1981; Matheson, 1993; Fries, 2010) and is caused by intracellular microsporidian parasites from genus of *Nosema*. For decades, *Nosema* disease was exclusively attributed to a single species of *Nosema*, *N. apis*, which was first described in European honey bees, *Apis mellifera* (Zander, 1909). In 1996, a new species of *Nosema* was first discovered in the Asian honey bee, *Apis cerana*, thus named *Nosema ceranae* (Fries et al., 1996). In 2005, a natural infection of *N. ceranae* was reported in *A. mellifera* colonies from Taiwan (Huang et al., 2005). Shortly thereafter, the infection of *N. ceranae*

to *A. mellifera* was reported in Europe (Higes et al., 2006; Paxton et al., 2007), United States (Chen et al., 2007), China (Liu et al., 2008), Vietnam and worldwide (Klee et al., 2007). Since its emergence as a potentially virulent pathogen of *A. mellifera*, *N. ceranae* has been associated with colony collapse of honey bees (Higes, et al., 2008; Paxton, 2010). A recent study showed that *N. ceranae* expanded its host range to South American native bumblebees (Plischuk et al., 2009) causing a new epidemiological concern for this pathogen. The present review summarizes recent findings on *Nosema ceranae* infection of *A. mellifera* in the USA and Asia, with particular emphasis on the comparative epidemiological, morphological, pathological, and genomic analysis of two *Nosema* species.

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2. THE PREVALENCE OF NOSEMA INFECTION IN THE UNITED STATES

A study for screening the prevalence of *Nosema* infections in the USA population of *A. mellifera* was conducted in 2007 (Chen et al., 2007). Bee samples collected between 1995 and 2007 from different geographic regions of the USA were examined individually for the presence of both *N. apis* and *N. ceranae* using the PCR method. The results showed that *N. ceranae* had a widespread infection of *A. mellifera* in the USA. *N. ceranae* infected bees were found in samples collected from each of 12 states including Oregon, California, Hawaii, Idaho, North Dakota, Minnesota, Texas, Ohio, Tennessee, Connecticut, Maryland and Florida, representing the Northeast, Southeast, Midwest, Southwest, and the West regions of the USA. Among the 180 bees examined for *Nosema*, 16% of the bees were positive for *N. ceranae*, while *N. apis* was not detected. The absence of *N. apis* may have been caused by inadequate sampling. The detection of *N. ceranae* in honey bees collected in 1995 indicated that *N. ceranae* is not a new emerging pathogen for *A. mellifera* in the USA and, in fact, had transferred from its presumed original host *A. cerana* at least a decade ago. Although the data presented in this study demonstrated that *N. ceranae* infection was widespread in the USA, the authors believed that distribution of *N. ceranae* infection of *A. mellifera* could be even more widespread than had been identified, if a more intensive epidemiological investigation was conducted. Later work by Williams et al. (2008) detected infection of *N. ceranae* in honey bees from the Maritime Provinces of Canada and Minnesota, USA and expanded the known distribution of this parasite.

While Chen et al. (2007) reported that PCR amplification using *N. apis* specific primers did not yield any positive results for bee samples tested, a study by the consortium scientists using a metagenomic approach to survey microflora in Colony Collapse Disorder (CCD) affected bee colonies and healthy colonies showed that co-infections of *N. apis* and *N. ceranae* were found in *A. mellifera*, and

that the infection rate of *N. ceranae* was significantly higher than that of *N. apis* in bees from both CCD affected colonies and normal healthy colonies (Cox-Foster et al., 2007). A similar result was obtained from a more recently conducted CCD descriptive epidemiological study (vanEngelsdorp et al., 2009). The studies showed that the infection rate of *N. ceranae* was 55% and 50% in CCD and control colonies, respectively, while the infection rate of *N. apis* was 29% and 18% in CCD and control colonies, respectively. All of these results were in line with a previous report that prior to 2003 most bee samples had *N. apis* infection but *N. ceranae* became a predominant infection after 2003 (Klee et al., 2007). The studies conducted in the USA confirm and extend early observations by Fries et al. (2006), Higes et al. (2006, 2007) and Huang et al. (2007) that *N. ceranae* was not restricted to its original host, but has established an infection in the European honey bee for some time, and that infection with *N. ceranae* is now more common than infection with *N. apis* in European honey bees.

3. THE PREVALENCE OF NOSEMA INFECTION IN EAST ASIA AND AUSTRALIA

A survey for the infection of *A. mellifera* with both *N. ceranae* and *N. apis* was performed in China (Liu et al., 2008). The samples of honey bees were collected from 12 different apiaries located in ten provinces and two municipalities in China. Thirty bees from each apiary were pooled together and examined for the presence of *N. ceranae* and *N. apis* using the PCR assay (Liu et al., 2008). *N. ceranae* were found to be present in every apiary examined. Sequence comparison of PCR fragments generated from the study with published sequences at the GenBank resulted in 99% sequence identity for *N. ceranae* and confirmed the specificity of the PCR assay. No *N. apis* was detected in any samples examined.

In contrast to the finding in the USA and China that *N. ceranae* was identified as the sole or predominant infection in *A. mellifera*, bee samples from Australia showed a notably

higher rate of *N. apis* infection (46.3%) than *N. ceranae* infection (15.3%) (by calculation from Table 2, Giersch et al., 2009). While *N. ceranae* was detected in samples collected from only four states (Queensland, New South Wales, Victoria, and South Australia), *N. apis* was found in samples collected from every state. Among the 307 bees examined for infection, only two bees had co-infection of both *Nosema* species. Further, the prevalence of *N. ceranae* infection varied considerably across states. While Western Australia and Tasmania were found to have no incidence of *N. ceranae* infection, *N. ceranae* was detected in 33.7%, 16%, 15.8%, and 4.5% of bees collected in Queensland, South Australia, New South Wales, and Victoria, respectively. The honey samples that originated from beekeepers in Queensland were also PCR positive for *N. ceranae* (Giersch et al., 2009). The infection of *N. ceranae* and *N. apis* in Australian population of *A. mellifera* obviously constitutes a unique case of *Nosema* prevalence compared to other reported cases from other regions of the world (Chen et al., 2007; Higes et al., 2007; Klee et al., 2007; Liu et al., 2008). One hypothesis is that *N. ceranae* in Australia may have a relatively recent introduction compared to other regions of the world. Queensland had the highest rate of *N. ceranae* infection among all the states and, therefore, may represent the region with the longest history of *N. ceranae* establishment. Alternatively, the variation in *Nosema* prevalence may also be due to different climate conditions in different geographical regions (Giersch et al., 2009).

Fries and Feng (1995) first reported that *N. apis* can infect *A. cerana* under laboratory conditions. A recent study conducted by Chen et al. (2009b) confirmed that this is also true under natural conditions. Samples of *A. cerana* collected from China, Japan and Taiwan showed that both *N. apis* and *N. ceranae* were present as single or as co-infections in Asian honey bees. However, *N. ceranae* was the significantly more common infection of the two *Nosema* species as *N. apis* was detected in 31% of examined bees while *N. ceranae* was detected in 71% of examined bees. Quantification of *Nosema* by real time quantitative PCR showed that the copy number of *N. ceranae*

was 100 times higher than the copy number of *N. apis* in coinfecting bees (Chen et al., 2009b). The study indicates that host shifting also occurred for *N. apis*, in that *N. apis* not only attacks European honey bees but also Asian honey bees and that *N. ceranae* is also the more common and predominant infection of the two *Nosema* species in Asian honey bees.

4. COMPARATIVE MORPHOLOGICAL, PATHOLOGICAL, AND GENOMIC ANALYSIS OF NOSEMA

4.1. Morphology

The morphological and developmental features of *N. ceranae* have been described by Fries et al. (1996, 2006), Higes et al. (2007) and Chen et al. (2009a) and the results from different work groups were generally similar. By light microscopy, fresh *N. ceranae* spores were oval or rod shaped, measuring $4.4 \pm 0.41 \mu\text{m}$ (mean \pm SD) in length and $2.2 \pm 0.09 \mu\text{m}$ (mean \pm SD) in width (Chen et al., 2009a). Compared to the spores of *N. apis* with $6.0 \mu\text{m}$ in length and $3.0 \mu\text{m}$ in width (Fries et al., 1996), the size of *N. ceranae* spores is smaller than that of *N. apis* spores. By electron microscopy, *N. ceranae* displayed all of the ultrastructural features of the genus *Nosema* including (1) diplokaryotic nuclei present in all developmental stages, (2) a long flexible polar filament that appears in the mature spores, (3) meronts, the earliest stages in the life cycle of the parasite, which are in direct contact with host cell cytoplasm, (4) mature spores that are bounded by a thickened wall consisting of electron-dense exospore and electron-lucent endospore layers, and (5) the thickness of exospore that is 48–52 nm, within the range of 40–60 nm in the genus *Nosema* (Larsson, 1986). The longitudinal section of a mature spore demonstrates the similarity of internal ultrastructures between *N. ceranae* and *N. apis* (de Graaf et al., 1994). The lamellate polaroplasts right below an anchoring disc and the posterior vacuole are located in the anterior and posterior ends of the spore, respectively. Each spore contains a coiled polar filament,

surrounding the diplokaryon. The number of coils of polar filament inside *N. ceranae* spores was 18 to 21 (Chen et al., 2009a). Compared to *N. apis* which has more than 30 coils (Fries, 1989; Liu, 1984), *N. ceranae* has a smaller number of coils in the polar filament. The difference in the size of spores and the number of polar filament coils provides evidence of morphological differences between *N. ceranae* and *N. apis*.

4.2. Tissue tropism and pathology

The tissue tropism (affinity to specific tissues) of a parasite is an important pathogenic factor. Infection of *Nosema* starts through ingestion of spores with food or water. Following ingestion, the spores develop in the site of the primary infection and multiplied parasites can spread to different tissues of the same host. A study conducted by Chen et al. (2009a) using PCR method showed that *N. ceranae* has a broad tissue tropism in the host of *A. mellifera*. The infection of *N. ceranae* was not restricted to the midgut tissue but spread to other tissues including the malpighian tubules, hypopharyngeal glands, salivary glands, and fat bodies (Fig. 1). Among bee tissues dissected and examined, *N. ceranae* was detected in 100% of alimentary canals, malpighian tubules, and hypopharyngeal glands, in 87% salivary glands, and in 20% of the fat bodies. No *N. ceranae*-specific PCR signal was detected in the muscle tissue. The infection of *Nosema* in European honey bees has often been reported to be associated with effects of reduced bee longevity, decreased population size, higher autumn/winter colony loss, reduced honey production and decreased brood production (Hassanein, 1953a, b; Rinderer and Sylvester, 1978; Anderson and Giacon, 1992; Goodwin et al., 1990; Malone et al., 1995). However, none of the disease symptoms such as dysentery and/or crawling behavior and/or milky white coloration of gut that are usually related with *N. apis* infection has been found in *N. ceranae* infected bees (Fries et al., 2006). It was shown recently that *N. ceranae* exerts a significant energy cost to infected bees and changes their feeding behavior (Mayack and

Naug, 2009; Naug and Gibbs, 2009). An early study by Bailey and Ball (1991) demonstrated that infection of hypopharyngeal glands by *N. apis* could lead to worker bees losing the ability to produce brood food and digest food. The absence of crawling behavior in *N. ceranae* infected bees might be the result of absence of *N. ceranae* infection in the muscles. Fat body is one of the primary sites of microsporidian infection in many insects. The infection of adipose tissue causes formation of whitish cysts and the infected gut becomes swollen and whitish as a result of impaired fat metabolism (Sokolova et al., 2006). The absence of milky white coloration of gut may reflect low infection of *N. ceranae* in the tissue of the fat body. Because all previous tissue tropism studies on *N. apis* were conducted using the presence of spores as a criterion (Hassanein, 1953a, b; Gilliam and Shimanuki, 1967; de Graaf and Jacobs, 1991), new efforts are under way as part of a recently funded USDA-CAP project to determine the tissue tropism of *N. apis* in the host of *A. mellifera* (Lee Solter, unpubl. data). While *N. apis* was known to cause earlier foraging in *A. mellifera* (Hassanein, 1953; Wang and Moeller, 1970), this behavioral change seems to be mediated by higher juvenile hormone titers in infected bees due to elevated juvenile hormone production (Huang, 2001), comparative data is lacking in *N. ceranae*. Further studies on the pathogenesis of both parasites will shed light on why *N. ceranae* has different pathological effects on the host of *A. mellifera* compared to *N. apis*.

4.3. Ribosomal RNA secondary structure models

Secondary structure refers to a folded, three-dimensional configuration of RNA based on the primary sequence of RNA. For RNA molecules, the secondary structure is more important for their biological functions than their primary sequences. Knowing the secondary structures can help to gain a deeper insight into the biological activities of the parasite in the host. A comparative sequence analysis was conducted to predict small

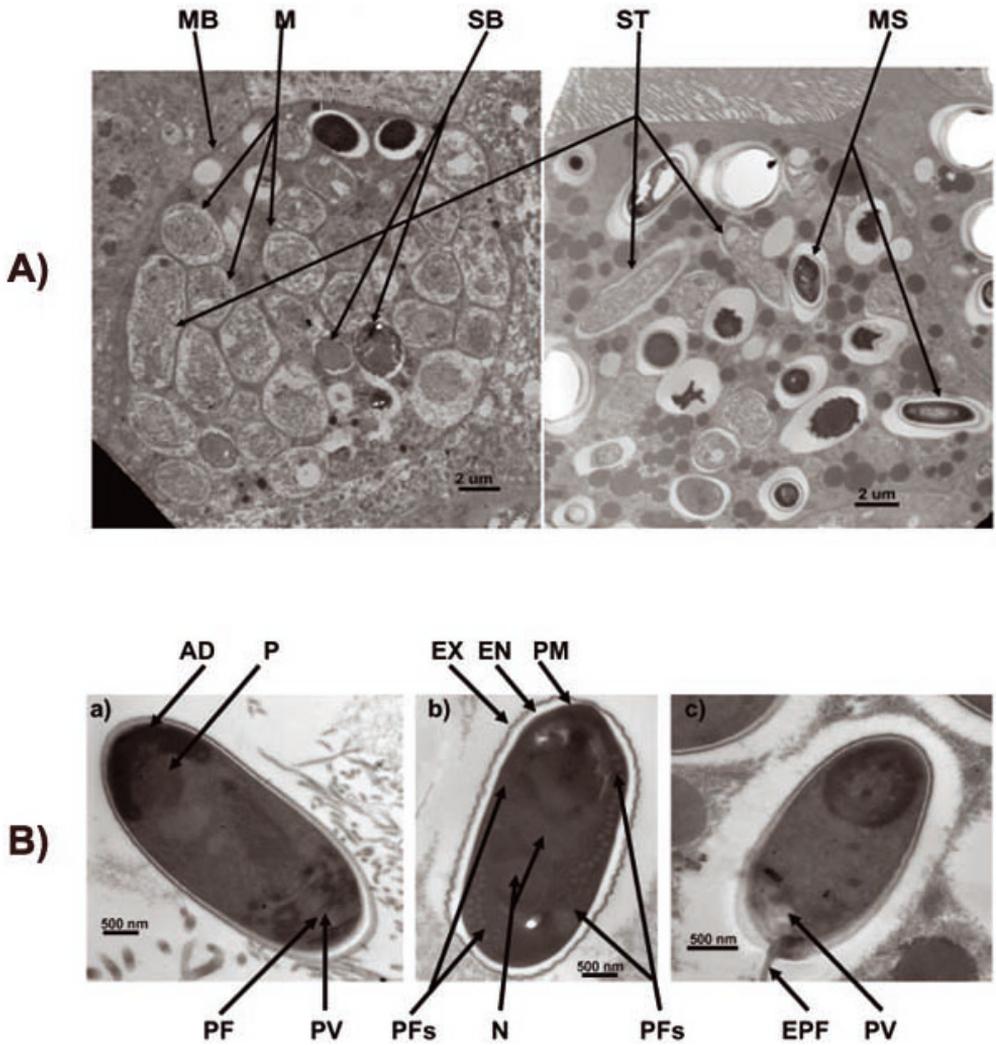


Figure 1. (A) The different developmental stages of *Nosema ceranae*. The developmental stages include meront (M), sporont (ST), sporoblast (SB), and mature spore (MS). MB = Membrane of the infected host cell. (B) Electron-micrograph of longitudinal section of *Nosema ceranae* spore showing (a) anchoring disk (AD), polaroplast (P), posterior vacuole (PV), polar filament (PF); (b) endospore (EN), exospore (EX), plasmamembrane (PM), nucleus (N), 20–22 isofilar coils of the polar filament (PFs); and (c) extruded polar filament (EPF) (From Chen et al., 2009a).

subunit ribosomal RNA (SSUrRNA) and large subunit rRNA (LSUrRNA) secondary structures for both *N. ceranae* and *N. apis* based on complete sequences of ribosomal genes of both species first deposited in GenBank. The complete DNA sequences of the ribosomal RNA gene of *N. ceranae* contained 4475 bp

(GenBank accession number DQ486027). The DNA sequence of the SSUrRNA cistron was located at the 5' end between nucleotide 1–1259. The G+C content of the SSUrRNA cistron was 36.46%. The internal transcribed space (ITS) region consisted of a 39 bp sequence and was located between nucleotides

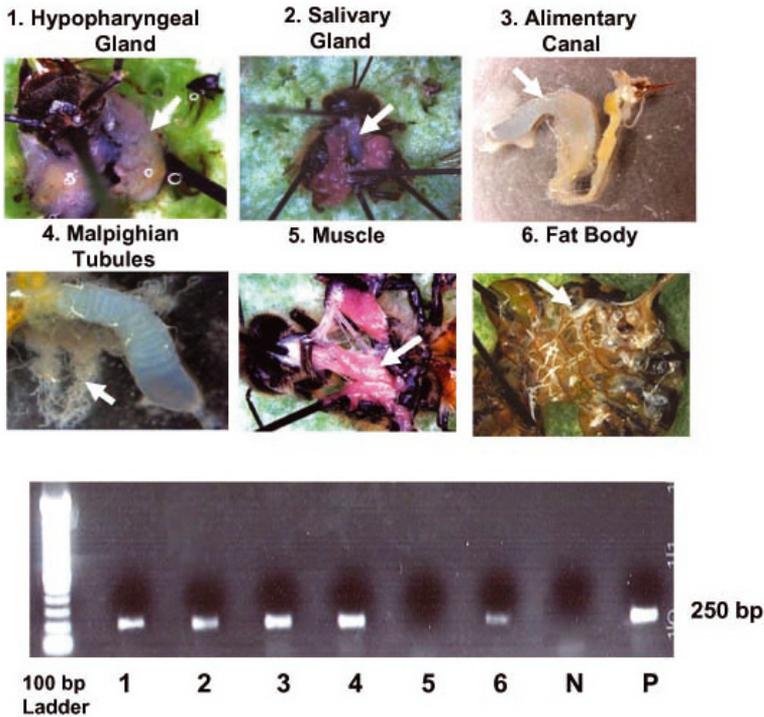


Figure 2. Tissue tropism of *Nosema ceranae*. Tissues such as hypopharyngeal gland, salivary gland, alimentary canal, malpighian tubules, muscle, and fat body were dissected and examined for the presence of *N. ceranae* by PCR method. For electrophoresis gel, numbers 1–6 indicate hypopharyngeal gland, salivary gland, alimentary canal, malpighian tubules, muscle, and fat body, respectively; letter N indicates negative control, and letter P indicates positive control. The size of PCR fragments is indicated on the right of the gel.

1260–1298. The DNA sequence of LSUrRNA contained 2530 bp and was located at the 3' end between nucleotide 1299–3828. The GC composition of the *N. ceranae* LSUrRNA sequences was 32.86% (Chen et al., 2009a). The complete DNA sequences of the rRNA gene of *N. apis* contained 3756 bp (GenBank accession number U97150). The DNA sequence of the SSUrRNA cistron was located at the 5' end (1242 bp) while the DNA sequence of the LSUrRNA was located at the 3' end (2481 bp). Both SSUrRNA and LSUrRNA were separated by an ITS (33bp). The DNA sequence is also presented for the regions flanking the 5' end of the small subunit gene and the 3' end of the large subunit gene (Gatehouse et al., 1998). As shown in Figure 2 and 3, comparative structural models of SSUrRNA and LSUrRNA indicate that ribosomal RNAs

of *N. ceranae* and *N. apis* are conserved and contain all of the structural features that are characteristic of known microsporidian rRNAs (Figs. 3 and 4) (Gutell et al., 1986a, b). While the microsporidian rRNAs contain some of the characteristic features found in the vast majority of the eukaryotic rRNAs, the SSUrRNA and LSUrRNA of *N. ceranae* and *N. apis* are very unusual. They lack many of the structural elements present in other nuclear-encoded eukaryotic rRNAs, and are significantly shorter in length. For example, the SSUrRNA and LSUrRNA of *Saccharomyces cerevisiae*, a species of budding yeast, are approximately 1800 and 3550 nucleotides in length respectively. The SSUrRNA of *N. ceranae* and *N. apis* are 1259 and 1242 bp nucleotide in length, respectively, while the LSUrRNA of *N. ceranae* and *N.*

Large Subunit Ribosomal RNA – 3' half

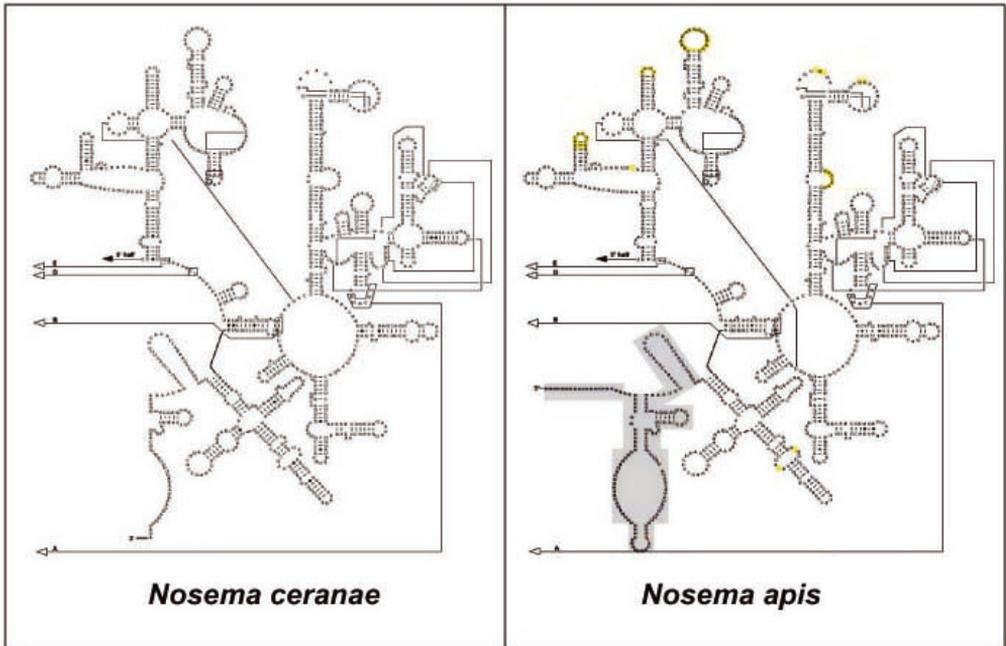


Figure 3. Secondary structure models for the large subunit ribosomal RNA (LSUrRNA) of *N. ceranae* and *N. apis*. The structure models of LSurRNA of *N. ceranae* and *N. apis* are identical.

apis are 2530 and 2481 nucleotides in length, respectively. Further studies are needed to determine how the reduction in size of rRNA contributes to the life cycle of the intracellular parasite in the host.

4.4. Phylogenetic analysis

A phylogenetic analysis of 20 species of microsporidia with highest BLAST score to *N. ceranae* was conducted with their sequences of SSUrRNA. Although *N. apis* and *N. ceranae* infect the same host and share similarities in sequences of rRNA gene, phylogenetic analysis showed that *N. apis* is not the closest relative of *N. ceranae*. Within the same clade, *N. ceranae* appears to be more closely related to *N. vespula*, a parasite infecting wasps. *N. apis* seems to have branched off earlier in evolution and is most closely linked to *N. bombi*, a parasite infecting bumble bees (Chen et al., 2009a) (Fig. 5). This result is in agreement with the earlier phylogenetic work by Fries

et al. (1996). The result obtained from *Nosema* phylogenetic analyses indicates that parasites from the same host species are not necessarily more closely related to each other and that evolutionary relationship is not always based on the host specificity of the taxa. The evolutionary distance between *N. ceranae* and *N. apis* may explain their difference in the morphological features and tissue specificities in the host.

4.5. Genome-wide sequencing and analysis

The complete genome of *N. ceranae* was recently sequenced using 454 sequencing approach (Cornman et al., 2009). The sequence information and annotations of *N. ceranae* are posted in GenBank under Genome Project ID32973. Pyrosequence data of *N. ceranae* lead to a draft assembly and annotated genome of 7.86 Mbp. *N. ceranae* has a strongly AT-biased genome, with 74% AT content and a

Small Subunit Ribosomal RNA

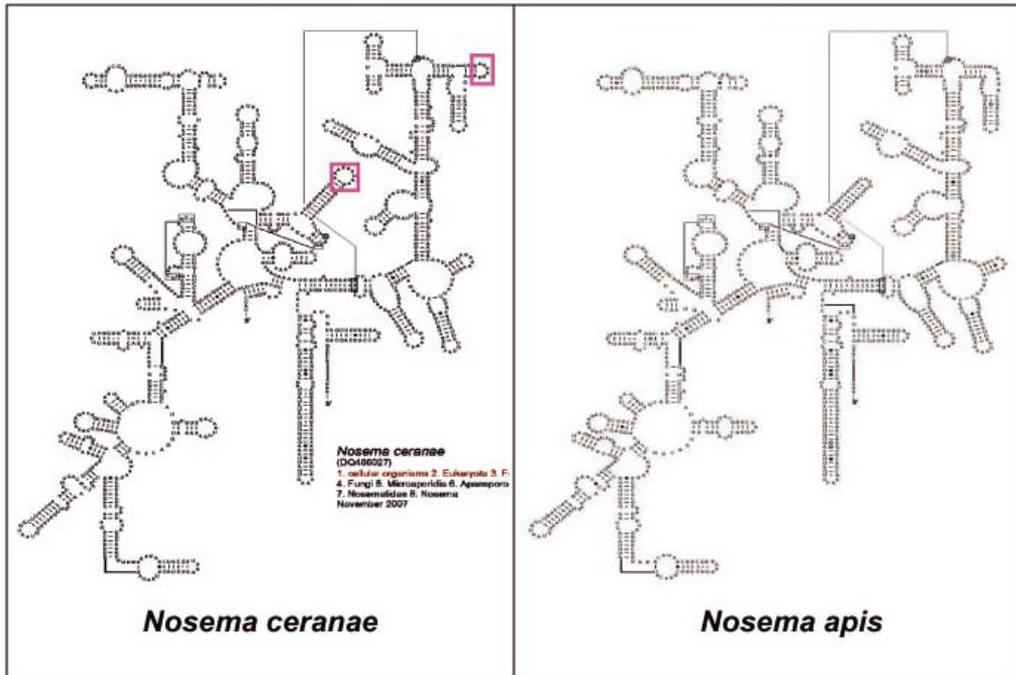


Figure 4. Secondary structure models for the SSUrRNA of *N. ceranae* and *N. apis*. The structure models of SSUrRNA of *N. ceranae* and *N. apis* are identical in general except there are two extra loops present in the secondary structure of SSUrRNA of *N. ceranae* (highlighted by boxes) compared with structure of *N. apis*.

diversity of repetitive elements. The initial sequencing and assembly of *N. apis* lead to a genome size of 6–9 Mbp with a GC content of less than 20%. Like *N. ceranae*, *N. apis* also has a strongly AT-rich genome (unpublished data). The genome sequence project of *N. apis* has just reached the stage of assembly and annotation.

The computational analysis of genomic sequence data of *N. ceranae* led to identification of 2641 putative protein-coding genes. A comparative genomics analysis of 2641 *N. ceranae* genes with those of another fully sequenced microsporidian, *Encephalitozoon cuniculi*, and with the yeast, *S. cerevisiae*, showed that *N. ceranae* has 1252 (48%) orthologous genes in *E. cuniculi* and 466 (18%) orthologous genes in *S. cerevisiae*. Of the 2614 predicted protein-coding sequences, there are only 11 genes that are both well-conserved and found only in microsporidia and lack clear homology outside this group. Future com-

parisons of the genes conserved among microsporidia in these two *Nosema* species will provide valuable insights and tools for identifying virulence factors in this group of the parasites. Mapping individual genes to standard metabolic pathways has provided important insights into the metabolic pathway in *N. ceranae*. A unique feature of microsporidia is that they do not have distinct mitochondria, a cell organell for generating energy, during the evolution and thus utilize the host ATP for their energy metabolism. The identification of metabolic 'chokepoints' of *N. ceranae* would be especially attractive targets for chemical or genetic control strategies.

5. CONCLUSION

The finding about the prevalence of *N. ceranae* in the USA and Asian bee populations in conjunction with previous findings in Europe

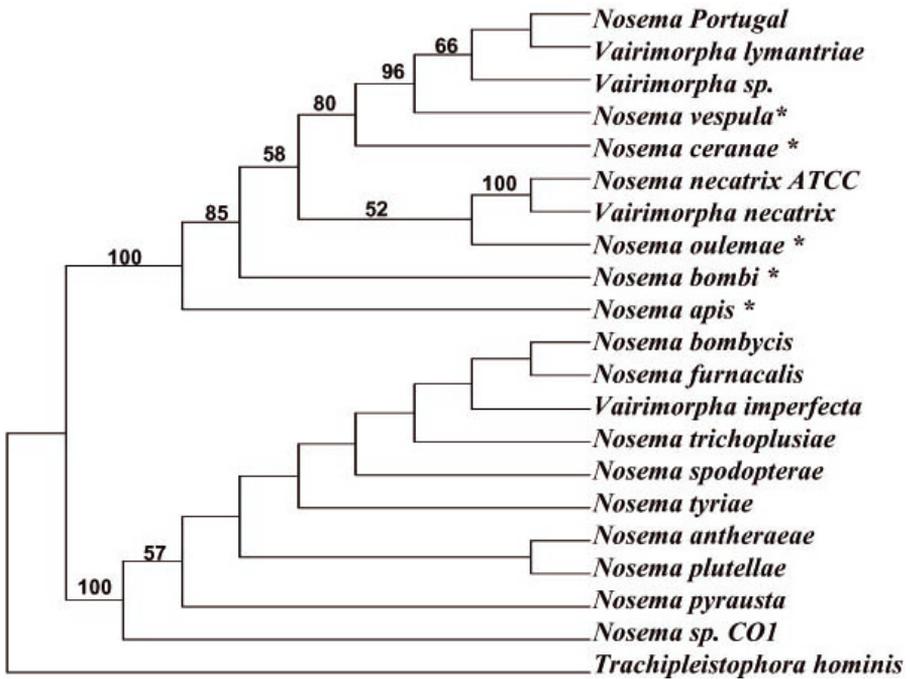


Figure 5. Phylogenetic tree of microsporidia infecting insects based on the sequences of the SSUrRNA gene. *Trachipleistophora hominis* infecting *Homo sapiens* was used as an outgroup. The tree was constructed by Maximum Parsimony analyses under a heuristic search. The reliability of the tree topology was determined by the bootstrap analysis (1000 replicates). The bootstrap values are located on the tree branches.

and other parts of the world raises several questions regarding *N. ceranae* infection in European honey bees. First, when was the exact time that *N. ceranae* expanded its host range from *A. cerana* to *A. mellifera*? Which transmission pathway(s) provided opportunities for *N. ceranae* to overcome the species barriers to expand its host range and establish infection in a new host? What mechanisms underlying virulence of *N. ceranae* led to *N. ceranae* becoming the more prevalent infection of the two *Nosema* species in *A. mellifera*? What physiologic and genetic characteristics of the host are favored by *N. ceranae* and contribute to determining host range expansion? All of these questions indicate a strong need for further investigation of the evolutionary history and molecular mechanisms of pathogenesis of *N. ceranae* in European honey bees. The availability of genomic information of two *Nosema* species will definitely enhance our understanding of the evolutionary history

and disease mechanism of *Nosema* in the host. The comparative genomic analysis of *N. ceranae* and *N. apis* will provide valuable insights and tools for identifying genes that are conserved between two *Nosema* species and genes that are responsible for the successful parasitism and major epidemics of *N. ceranae* in honey bees. The genomic information will also enable the researchers to develop and use genetic markers to seek a better understanding of the epidemiology of *Nosema* infections and pinpoint the signals that control gene function, which in turn should translate into new strategies for combating *Nosema* disease and improving honey bee health.

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***Nosema ceranae*, un agent pathogène d’*Apis mellifera* nouvellement identifié aux États-Unis et en Asie.**

***Nosema apis* / *Nosema ceranae* / spectre d’hôtes / distribution / morphologie / pathologie / génome**

Zusammenfassung – *Nosema ceranae*, ein neu entdecktes Pathogen von *Apis mellifera* in den USA und Asien. *Nosema* ist ein sporenbildender Parasit, der eine ernsthafte Erkrankung der erwachsenen Honigbienen verursacht und von einer Forschergruppe in Zusammenhang mit dem Colony Collapse Disorder (CCD) gebracht wurde. Die Erkrankung wird durch zwei verschiedene *Nosema*-Arten, *N. apis* und *N. ceranae* verursacht. Wir weisen nach, dass *N. ceranae* der für Bienen in den USA und Asien vorherrschende Erreger ist. Wir präsentieren auch die erste vollständige pathologische, genetische und genomische Analyse dieses Pathogens. Die Informationen aus dieser Arbeit können von anderen Forschern und Sachverständigen genutzt werden, um Bienenvölker auf die Krankheit hin zu untersuchen und um effektive Maßnahmen zu ihrer Bekämpfung zu entwickeln.

***Nosema apis* / *N. ceranae* / Wirtsspektrum / Verteilung / Morphologie / Pathologie / Genom**

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