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Original article

A *Nosema ceranae* isolate from the honeybee *Apis mellifera**

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Abstract – Microsporidiosis (nosema disease) of the honeybee, *Apis mellifera*, has spread worldwide and caused heavy economic losses in apiculture. We obtained a spore isolate from worker ventriculi of *A. mellifera* colonies kept on the campus of National Taiwan University and sequenced the ribosomal genes. The entire length of the ribosomal DNA is about 3828 bp and the organization is similar to that of *Nosema apis*. However, the SSUrRNA, ITS, and LSUrRNA sequences have comparatively low identities with those of *N. apis* (92, 52, and 89%, respectively) and the SSUrRNA has a 99% identity with *Nosema ceranae*. These results indicate that this isolate is not *N. apis*, but *N. ceranae*. Moreover, the morphological characteristics are identical to those of *N. ceranae*. These results show that nosema disease of the honeybee, *A. mellifera*, may not be caused solely by the infection of *N. apis*.

***Nosema ceranae* / rRNA / nosema disease / Microsporidia**

1. INTRODUCTION

Microsporidiosis (nosema disease) is a frequently encountered and important honeybee disease that has many negative effects on production capacity, especially in temperate climates (Fries and Ekbom, 1984). The identified pathogen from the European honeybee, *Apis mellifera* L., is *Nosema apis* Zander which is found worldwide (Matheson, 1996) and may also afflict the Asian honeybee, *A. cerana* Fabricius (Singh, 1975). However, another pathogen found in *A. cerana,* namely *N. ceranae*, raised the questions about the accuracy of records that attribute the infection of *A. cerana* exclusively to *N. apis* (Fries et al., 1996). *N. ceranae* is still found exclusively in *A. cerana,* although *N. ceranae* can artificially infect *A. mellifera* (Fries, 1997). *N. apis* and *N. ceranae* have similar morphologies under an optic microscope and SSUrRNA sequence identity is 92% (Fries et al., 1996). However, the identity is low compared to other lepidopteran *Nosema* and *Vairimorpha* species that infect the same or similar hosts (Canning et al., 1999; Tsai et al., 2003, 2005). Moreover, by the analyses of SSUrRNA phylogenetic trees, *N. ceranae* turned out to be more closely related to *Vairimorpha* species and other *Nosema* species than to *N. apis* (Fries et al., 1996; Keeling and Macfadden, 1998; Slamovits et al., 2004).

The microsporidiosis of honeybee is not considered a serious problem of apiculture in Taiwan, maybe because Taiwan extends from tropical into subtropical regions. However, this disease spread widely throughout Taiwan after 1972 and was consistently detected each spring and autumn (An and Ho, 1980). The pathogen of microsporidiosis in Taiwanese apiaries was assumed to be *N. apis* based on optic microscopic observations[1].

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[1] Some preliminary data of our spore isolate from *A. mellifera* colonies kept on the National Taiwan University (NTU) campus were published as a note in the 38th Annual Meeting of the Society of Invertebrate Pathology (2005). The article dated 2005-11-25, has been posted on the NTU website as an informal record and is not cited here.

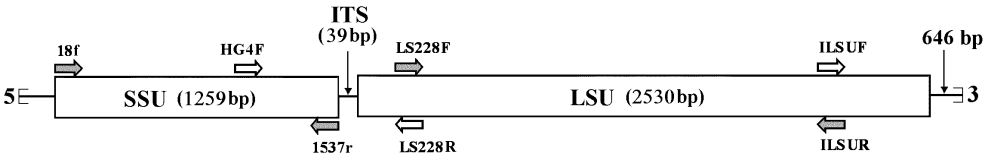

Figure 1. Schematic diagram of the *Nosema ceranae* isolate rRNA gene. The rRNA domains are indicated by boxes. The grey arrows represent primers used to amplify the major coding regions of rRNA while the white arrows represent primers for the ITS and the SSP-PCR.

2. MATERIALS AND METHODS

2.1. Spore purification and nucleic acid preparation

Microsporidian spores were isolated from diseased workers of the honeybee, *Apis mellifera,* from colonies kept on the NTU campus. The alimentary tracts were removed by crushing the thorax with tweezers and removing the terminal sclerites from the abdomen. The hindguts and sclerites were also removed and the remaining midguts were homogenized with a pestle. This crude tissue homogenate was filtered through nylon filters and centrifuged (1000 *g*, 10 min, 4 °C). The pellets were then resuspened with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and centrifuged again before being collected and centrifuged in 90% Percoll (Amersham Biosciences) at 30 000 *g* (30 min, 10 °C). The purified spores were then collected.

DNA purification was carried out as described previously (Huang et al., 1998; Tsai et al., 2002; Huang et al., 2004). Briefly, a suspension of purified spores (2×10^7 spores in 0.25 mL TE buffer) was mixed with an equal volume of zirconia/silica beads (0.1 mm diameter) in a 10×75 mm glass tube and shaken at maximum speed using a vortex mixer for 1 min (Undeen and Cockburn, 1989). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The DNA was eluted in TE buffer and then stored at −20 °C. The DNA concentration was then measured with a GeneQuant II RNA/DNA Calculator (Amersham Biosciences).

2.2. Amplification and sequencing of rRNA genes

Figure 1 shows the primer annealing sites for rRNA gene amplification. The coding region of SSUrRNA was amplified using the primer set: 18f and 1537r (Vossbrinck et al., 1993). Additionally, the major coding region of LSUrRNA was amplified using the primer set: LS228F and ILSUR (Tsai et al., 2002). The 3′ end region of LSUrRNA was amplified using SSP-PCR (single-specific-primer PCR, Shyamala and Ames, 1989). The internal primers for sequencing are not shown.

For accurate sequencing and finding the variation in the rRNA sequences, Platinum Pfx DNA polymerase (Invitrogen) with proofreading activity was used. The PCR mixture was prepared according to the manufacturer's instructions. Amplifications were performed in an AG-9600 Thermal Station (Biotronics Corp.). A 10 μL aliquot from each reaction was run on a 1.0% agarose gel to visualize the PCR products. The PCR products were eluted using a DNA Clean/Extraction Kit (Genemark) and then treated with Taq polymerase for another 15 min to add a 3′ terminal thymidine. The treated PCR products were eluted again as previously and then cloned into T&A cloning vector (Real Biotech Corp.). The inserted DNA fragments were sequenced on an automated DNA Sequencer (DNA Sequencer 377, Applied Biosystems).

2.3. Fixation method and TEM analysis

The fixation procedure followed the protocol described by Larsson (2005). The isolated spores were prefixed in 4% paraformaldehyde at 60 °C for 2 h, followed by washing in 0.1M phosphate buffer (pH 7.2). The fixed spores were then postfixed in 1% osmium tetroxide in 0.1M phosphate buffer for 2 h at 4 °C and washed in the same buffer. After dehydration in a gradated series of ethanol, the specimens were transferred to propylene oxide. The specimens were then infiltrated and embedded in Epon. Ultra-thin sections were taken and stained with uranyl acetate and lead citrate (Reynolds, 1963).

2.4. Phylogenic tree construction

Tree construction for seven complete LSUrRNA sequences followed the method of Slamovits et al. (2004). Sequences were aligned using ClustalX (Thompson, 1997). Poorly aligned regions were excluded manually. Phylogenetic analyses were conducted based on maximum likelihood (ML) with PAUP 4.0 b10 (Swofford, 2003). ML analyses followed the GTR model with site-to-site rate variation modeled on a discrete gamma distribution with four variable rate categories plus invariable sites. The shape parameter alpha and proportion of invariable sites were estimated based on maximum likelihood via a distance tree topology using PAUP 4.0 b10. Node support was assessed with 100 replicates for the ML analysis.

3. RESULTS

3.1. rDNA sequence

The entire rDNA length of the isolate (Fig. 1) from *A. mellifera* varied between 3828 and 3834 bp due to variation in the SSUrRNA region. The overall organization of the rDNA (5′-SSUrRNA-ITS-LSUrRNA-3′) was similar to that of *N. apis* (Gatehouse and Malone, 1998). This sequence has been deposited in the GenBank database (GenBank accession number DQ486027). In the process of SSUrRNA sequencing we found several interesting variations. Thirteen clones of SSUrRNA were sequenced to verify the variable sites. The most obvious variation was an insertion after the 116th bp of the SSUrRNA sequence. The length of the SSUrRNA sequence is 1259 to 1265 bp, and the GC content is about 36%, which is similar to that of *N. ceranae* (GenBank accession number U26533) and in the range of the GC content of SSUrRNAs determined for *Nosema* spp. (33.9 to 38.6%, Fries et al., 1996). The SSUrRNA sequences of the clones share more than 99% (99.1 to 99.6%) nucleotides with *N. ceranae,* but have only about 92% identity with *N. apis* (GenBank accession number U97150, Gatehouse and Malone, 1998). The 1265 bp SSUrRNA sequence, which contains all the insertions, has also been deposited in GenBank (GenBank accession number DQ486028).

The sequence of the ITS (internal transcribed spacer) region is 39 bp (Fig. 1), and no variation has been found within it. The GC content of the ITS is 5.1%, which is much lower than that of other *Nosema* species (18.2 to 20%) and probably the lowest in microsporidia. The ITS region shows only 52% identity with that of *N. apis* (Gatehouse and Malone, 1998). The LSUrRNA sequence is 2530 bp long (Fig. 1) and the GC content is 32.9%, similar to that of *Nosema* spp. (31.8 to 35.8%). Very little single base variation was found in this sequence. The putative start and termination points were determined by comparison to the *N. apis* LSUrRNA sequence (Gatehouse and Malone, 1998). The LSUrRNA sequence shares 89% and 75% identity, respectively, with that of *N. apis* (GenBank accession No. U97150) and the type species of *Nosema*, *N. bombycis* (GenBank accession No. AY259631). The LSUrRNA sequence of our isolate is 49 bp longer than that of *N. apis* LSUrRNA, and this is due to several insertions (Fig. 2). The sequence of the 3′ end was also obtained by SSP-PCR and we also sequenced a 646 bp stretch after the LSUrRNA putative terminal end. Three domains of the 3′ untranscribed region share 84, 85, and 87% identities with those of the *N. apis* 3′ untranscribed region (Gatehouse and Malone, 1998).

3.2. The morphological characters

The living spore size was 4.5 × 2.4 μm on average when measured under a light microscope (400 ×). The TEM observations revealed that the number of polar filament coils was between 20 to 23, and most spores contained 22 polar filament coils and showed a two-layer arrangements of polar filament coils in the posterior part. Transversal sections of polar filaments showed four layers and we also observed a posterosome (posterior body) (Fig. 3).

3.3. Phylogenic tree constructed from LSUrRNA sequences

The maximum likelihood phylogenetic tree (Fig. 4) obtained from the complete LSUrRNA

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Nosema apis      TTTGCCCACACATGGGATCAATAGGGTACCATAACGAGGAAGATCGTAGCGGAATACGAAAG------ATTATTGATCGAAT--ATATT- :   81
Nosema ceranae   TTTGCCCACACATGGGATCAATAGGATACCATAACGATGAAGGTCGTAATAGAATACGAAAGTATTTTAATATTTACCGAATTAATATTT :   90

Nosema apis      AGAAACAACCCTTGATTGTAATCCTTA--TGGAGCTGTAAATCA----TATATTTTATTTCTTATTTCGTAGAGGATGTATATCCGTTAT : 255
Nosema ceranae   AGAAATAACCCTTGATTGTAATCCTTAATTGGAGATGTAAATCAATTTTATTTTTATTTAATTATATCATAGAGAATATTTATTATTCGT : 270

Nosema apis      ACGGACCAAGGAGATTATAATTATAGCGAGATAAC------AATGTAGTCGTTATTAGCTTGATAAGTTATAATTATAAGACCCGAAACA : 514
Nosema ceranae   --CGACCA-GGAGATTATATTTA-ACCGAGATAATTTTTTTATTTTAGCCGTTATTAATTTG-TAAGTTATA-TTATAAGACCCGAAACA : 533

Nosema apis      TGTATAGTGGCGAAAGACCAATCGAACTGTGTGGTAGCTGGTTCACAGCGAAATGTCTCTCAGGACAGCAGTCATTTTTT---------A :  685
Nosema ceranae   TGTATAGTGGCGAAAGACCAATCGAACTGTGTGGTAGCTGGTTCACAGCGAAATGTCTCTCAGGACAGCAGTCATTATTTTTTTTATATA :  713

Nosema apis      ACCTACCGAATGTATTATTGTATAAAATGGAAGAAGATTACTACTTTT-ATGAGATG-TTTCTGTATAGTAT-----TCAGGTAGCTGTG : 1035
Nosema ceranae   ATCTACCGAATGTATTATTGTATAAAATGGAAGAAGATTACTACTTTTTATGAGATGGTTTCTGTTNTATTTATTTATCAGGTAGCTGTG : 1073

Nosema apis      CAAGGCGACTTAATTATGACGGTATATTTTTTG-CATAAGAAAAATGAATTATGTGATGT-----TTAGCTATGGATTGTCATGATAAGA : 1297
Nosema ceranae   TAAGGCGACTTGATGATGACAGTATATTTATTAATCTAAGATAAATGAATTAGATTATTTATTTATTAACTATGGATTGTCATGATAAGA : 1343
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Figure 2. Discontinuous alignment of the LSUrRNA fragment. The upper sequence is the *N. apis* LSUrRNA sequence and below is the *N. ceranae* LSUrRNA sequence. Underlined are insertions that are longer than 3 bp.

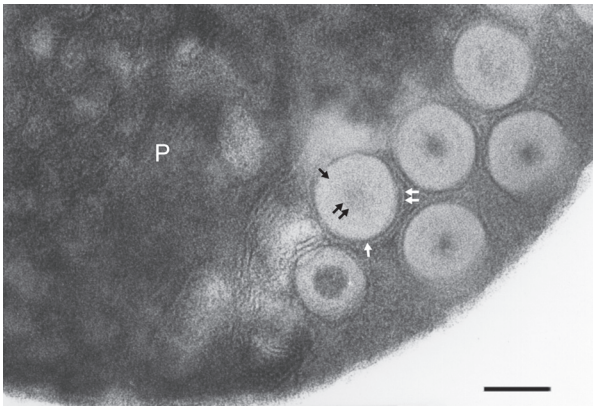

Figure 3. TEM sections of mature spores. The polar filament appears as a four layered structure. The single white arrow indicates layer 1, the double white arrow indicates layer 2, the single black arrow indicates layer 3 and the double black arrow indicates layer 4 of the longitudinally sectioned polar tube. Posterosome (P). Scale bar = 50 nm.

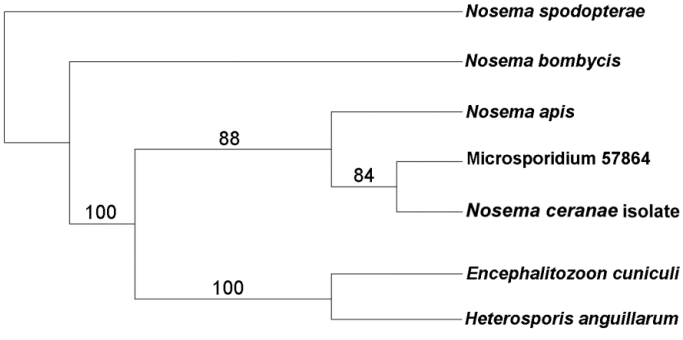

Figure 4. Phylogenetic tree based on LSUrRNA sequences. Seven microsporidian species were analyzed based on a maximum likelihood approach using PAUP 4.0 b10 (Swofford, 2003), run with 100 bootstrap replications. Numbers indicate branch support statistics.

sequences shows that *N. apis* (Gatehouse and Malone, 1998), this isolate and Microsporidium 57864 (GenBank accession number U90885) form a clade wherein this isolate is more closely related to the species Microsporidium 57864 than to *N. apis*. In addition, the *Nosema* type species, *N. bombycis* (Huang et al., 2004), and *N. spodopterae* (Tsai et al., 2003) are located in another clade. The topology of the LSUrRNA tree (Fig. 4) is congruent with that of the SSUrRNA phylogenetic trees (Keeling and Macfadden, 1998; Slamovits et al., 2004), but it shows a greater distance when the tree is presented in a phylogram. Although the *Vairimorpha* and *Nosema* species were grouped in the same clade in the SSUrRNA phylogenetic tree, no complete LSUrRNA sequences of *Vairimorpha* species are available so far, and thus, the LSUrRNA tree shown in this present study does not include a *Vairimorpha* species.

4. DISCUSSION

This isolate from *Apis mellifera* colonies kept in the NTU campus was assumed to be *N. apis* (An and Ho, 1980). However, the SSUrRNA sequence of this isolate showed higher identity with that of *N. ceranae* than with that of *N. apis*. Since comparison of rRNA sequence data should have greater reliability than comparison based on electron micrographs (Weiss and Vossbrinck, 1999), our isolate from *A. mellifera* colonies in Taiwan was therefore considered to be *N. ceranae* or a subspecies of it. In the morphological observations, the living spores were shorter than those of *N. apis* and similar to those of *N. ceranae*. They have 20 to 23 polar filament coils, which is the same number as that for *N. ceranae* (Fries et al., 1996) and fewer than *N. apis*, which usually contains 30 to 44 polar filament coils (Lui, 1973; Lui and Lui, 1974; Fries, 1989). The number of polar filament coils is one criterion used in discriminating *Nosema* species (Burges et al., 1974). In addition, the four-layer structure of the polar filament and posterosome in the longitudinal sections of spores (Fig. 3) are both characteristics of mature spores of *N. ceranae* (Fries et al., 1996).

The rDNA organization (Fig. 1) of this isolate was the same as that of *N. apis* (Gatehouse and Malone, 1998) and of other microsporidian species for which complete rDNA gene sequences are available (Tsai et al., 2002; Peyretaillade et al., 1998), but differs from that of *N. bombycis* (Huang et al., 2004) and closely related species (Tsai et al., 2005). The rDNA organization of *Nosema* species turned out to be heterogeneous and coincident with the topology of the LSUrRNA tree (Fig. 4), and this further confirmed the assumption that the rDNA organization may have implications for the taxonomy of the *Nosema* group (Huang et al., 2004).

The SSUrRNA sequence showed minor variations: six of thirteen clones had an insertion, GATT, after the 116th base of the SSUrRNA sequence; eight clones had a single T insertion after the 470th base; four clones had a single A insertion after the 661st base; and three clones had a single T insertion after the 934th base. Although some intraspecific single base variation may be seen in the SSUrRNA sequences of microsporidia, the insertion after the 116th base is quite unusual, and these insertions caused errors in the SSUrRNA sequence (GenBank accession number DQ078785) that was sequenced directly from the PCR product.

The insertion after the 116th base should not affect SSUrRNA secondary structure since it is located in the variable region, the loop between helix eight and nine (Van de Peer et al., 2000). The insertion after the 471st base has also been noted in isolates from Europe (GenBank accession number DQ329034, DQ374655), but the insertion after the 116th base and other insertions were not found in the European isolates. The insertion after the 661st base is found in the SSUrRNA sequence published by Fries et al. (1996), but most clones did not have this insertion. None of the insertions and variable bases of the SSUrRNA sequence are related to the differences between *N. apis* and *N. ceranae*.

The LSUrRNA of our isolate has 87% identity with that of *N. apis* and is 49 bp longer than that of *N. apis* LSUrRNA. In

addition, most insertion sequences were A/T only (Fig. 2) and should did not affect the secondary structure, except for a few changes in the D14 helix. Moreover, most insertions were in the loops and not in the helices of the secondary structure. Three domains of the 3′ untranscribed region showed high identity to those of *N. apis*, but the role of these regions needs to be clarified in further studies.

In the SSUrRNA tree constructed using neighbor-joining algorithm (Slamovits et al., 2004) and the SSUrRNA tree constructed using maximum likelihood algorithm (Keeling and Macfadden, 1998), *Vairimorpha* and *Nosema* were grouped together and could be further sub-grouped into a pure *Nosema* group, including the type species of *Nosema* and the *Nosema*/*Vairimorpha* group. *N. ceranae* was grouped in the *Nosema*/*Vairimorpha* group. The LSUrRNA phylogenetic tree (Fig. 4) had fewer taxa, and only *E. cuniculi*, *N. apis*, *N. bombycis*, and *N. ceranae* were included in both SSUrRNA trees. *H. anguillarum* was included in the SSUrRNA tree published by Slamovits. The LSUrRNA phylogenetic tree has a similar topology and supports the notion that the genus *Nosema* should be separated into two groups (Baker et al., 1994; Muller et al., 2000; Tsai et al., 2003, 2005), although the complete LSUrRNA sequence of *Vairimorpha* is not available. In the LSUrRNA tree, *N. ceranae* is grouped with other species instead of *N. apis*, and the same situation is found in both SSUrRNA trees mentioned above and the SSUrRNA tree published by Fries et al. (1996). These results suggest that *N. ceranae* and *N. apis* are phylogenetically quite distinct.

The present study identified a microsporidia isolate from *A. mellifera* to be the species *Nosema ceranae*. Furthermore, it revealed that under normal beekeeping practices, nosema disease in *A. mellifera* may not be exclusively caused by *N. apis* and that species certification of the pathogen in nosema disease research is important. It is possible that the *N. ceranae* isolate found in *A. mellifera* colonies kept on the NTU campus was transmitted by *A. cerana*. Not only are native *A. cerana* present throughout Taiwan, but they were also near the *A. mellifera* colonies several years ago on the NTU campus. Thus, our observation may not be a rare case and *N. ceranae* and *N. apis* may have both existed in *A. mellifera* for quite a long period. The morphological records of *N. apis* show high variability and *N. ceranae* was also been found in Europe (Higes et al., 2006) and on Martinique Island (unpublished data). The origin of *N. ceranae* will have to be elucidated by further research.

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Un isolat de *Nosema ceranae* provenant d'*Apis mellifera*.

***Nosema ceranae* / nosémose / ARNr / Taïwan / microsporidie**

Zusammenfassung – Ein Isolat von *Nosema ceranae* aus der Honigbiene *Apis mellifera*. Nosema-Erkrankungen (Mikrosporidien) bei Honigbienen, *Apis mellifera*, sind weltweit verbreitet und verursachen erhebliche wirtschaftliche Schäden in der Imkerei. Die Nosemose verbreitete sich nach 1972 in ganz Taiwan und konnte in einer bis 1980 dauernden Untersuchung in sämtlichen Frühjahrs- und Herbstuntersuchungen nachgewiesen werden. Aufbauend auf diesen Untersuchungen amplifizierten und sequenzierten wir die ribosomalen Gene von Sporen aus dem Verdauungstrakt von Arbeiterinnen, die aus Bienenvölkern auf dem Campus der National Taiwan Universität stammten. Die vollständige Sequenz der ribosomalen DNA beträgt 3.828 Basenpaare und ähnelt der von *Nosema apis* (Abb. 1). Allerdings weist die SSUrRNA-Sequenz eine 99 %ige Übereinstimmung mit der von *N. ceranae* auf und die SSUrRNA-, ITS- und LSUrRNA-Sequenzen haben eine verhältnismäßig geringe Ähnlichkeit mit denen von *N. apis* (92, 52 bzw. 89 %). Zudem stimmen bei den untersuchten Sporenisolaten auch die morphologischen Charakteristika, die Größe der lebenden Sporen sowie die Struktur des Polfadens im Längsschnitt (Abb. 3) mit denen von *N. ceranae* überein. All diese Ergebnisse zeigen, dass diese Sporen nicht von *N. apis* sondern von *N. ceranae* stammen. Bei der phylogenetischen Analyse zeigen sowohl der SSUrRNA- als auch der LSUrRNA- Stammbaum (Abb. 4), dass *N. ceranae* und *N. apis* phylogenetisch gut zu trennen sind. Daher muss für Nosema-Erkrankungen

bei Honigbienen (*A. mellifera*) nicht ausschließlich *N. apis* verantwortlich sein. Der Erreger der Nosemose sollte in zukünftigen Untersuchungen daher eindeutig bestimmt werden.

***Nosema ceranae* / Nosemose / Mikrosporidie / Taiwan**

REFERENCES

An J.K., Ho K.K. (1980) The seasonal variation of *Nosema apis* Zander in Taiwan, Honeybee Sci. 1, 157–158.

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

Burges H.D., Canning E.U., Hulls I.K. (1974) Ultrastructure of *Nosema oryzaephili* and the taxanomic value of the polar filament, J. Invertebr. Pathol. 23, 135–139.

Canning E.U., Curry A., Cheney S., Lafranchi-Tristem N.J., Haque M.A. (1999) *Vairimorpha imperfecta* n. sp., a microsporidian exhibiting an abortive octosporous sporogony in *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), Parasitology 119, 273–286.

Fries I. (1989) Observation on the development and transmission of *Nosema apis* Z. in the ventriculus of the honeybee, J. Apic. Res. 28, 107–117.

Fries I. (1997) Protozoa, in: Morse R.A. (Ed.), Honey bee pests, predators, and diseases, 3rd ed., A.I Root Company, Medina, Ohio, USA, pp. 57–76.

Fries I., Ekbom G. (1984) *Nosema apis*, sampling techniques and honey yield, J. Apic. Res. 23, 102–105.

Fries I., Feng F., da Silva A., Slemenda S.B., Pieniazek N.J. (1996) *Nosema ceranae* n. sp. (Microspora, Nosematidae), Morphological and Molecular Characterization of a Microsporidian Parasite of the Asian Honey bee *Apis cerana* (Hymenoptera, Apidae), Eur. J. Protistol. 32, 356–365.

Gatehouse H.S., Malone L.A. (1998) The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size, J. Invertebr. Pathol. 71, 97–105.

Higes M., Martín R., Meana A. (2006) *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe, J. Invertebr. Pathol. 92, 93–95.

Huang H.W., Lo C.F., Tseng C.C., Peng S.E., Chou C.M., Kou C.H. (1998) The small subunit ribosomal RNA gene sequence of *Pleistophora anguillarum* and the use of PCR primers of diagnostic detection of the parasite, J. Eukaryot. Microbiol. 45, 556–560.

Huang W.F., Tsai S.J., Lo C.F., Soichi Y., Wang C.H. (2004) The novel organization and complete sequence of the ribosomal gene of *Nosema bombycis*, Fung. Genet. Biol. 41, 473–481.

Keeling P.J., Macfadden G.I. (1998) Origins of microsporidia, Trends Microbiol. 6, 19–23.

Larsson J.I.R. (2005) Fixation of microsporidian spores for electron microscopy, J. Invertebr. Pathol. 90, 47–50.

Lui T.P. (1973) The fine structure of frozen-etched spore of *Nosema apis* Zander, Tissue and Cell 5, 315–322.

Lui T.P., Lui H.J. (1974) Evaluation of some morphological characteristics of spore from two species of microsporidia by scanning electron microscope and frozen-etching techniques, J. Morphol. 143, 337–339.

Matheson A. (1996) World bee health update 1996, Bee World 77, 45–51.

Müller A., Trammer T., Ghioralia G., Seitz H.M., Diehl V., Franzen E. (2000) Ribosomal RNA of *Nosema algerae* and phylogenetic relationship to other microsporidia, Parasitol. Res. 86, 18–23.

Peyretaillade E., Biderre C., Peyret P., Duffieux F., Metenier G., Gouy M., Michot B., Vivares C.P. (1998) Microsporidian *Encephalitozoon cuniculi*, a unicellular eukaryote with an unusual chromosomal dispersion of ribosomal genes and a LSUrRNA reduced to the universal core, Nucleic Acids Res. 26, 3513–3520.

Reynolds E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, J. Cell Biol. 17, 208–212.

Shyamala H., Ames G.F. (1989) Genome walking by single-specific-primer polymerase chain reaction: SSP-PCR, Gene 84, 1–8.

Singh Y. (1975) Nosema in Indian honey bee (*Apis cerana* indica), Am. Bee J. 115, 59.

Slamovits C.H., Willams B.A.P., Keeling P.J. (2004) Transfer of *Nosema locustae* (Microsporidia) to *Antonospora locustae* n. comb. Based on molecular and ultrastructure data, J. Eukaryot. Microbiol. 51, 207–213.

Swofford D.L. (2003) PAUP*, Phylogenetic analysis using parasimony (* and other methods), Sinauer Associates, Sunderland, MA.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 25, 4876–4882.

Tsai S.J., Kou G.H., Lo C.F., Wang C.H. (2002) Complete sequence and structure of ribosomal RNA gene of *Heterosporis anguillarum*, Dis. Aquat. Org. 49, 199–206.

Tsai S.J., Lo C.F., Soichi Y., Wang C.H. (2003) The characterization of Microsporidian isolates (Nosematidae: *Nosema*) from five important lepidopteran pests in Taiwan, J. Invertebr. Pathol. 83, 51–59.

Tsai S.J., Huang W.F., Wang C.H. (2005) Complete sequence and gene organization of *Nosema spodopterae* rRNA gene, J. Eukaryot. Microbiol. 52, 52–54.

Undeen A.H., Cockburn A.F. (1989) The extraction of DNA from microsporidia spores, J. Invertebr. Pathol. 54, 132–133.

Van de Peer Y., De Rijk P., Wuyts J., Winkelmans T., De Wachter R. (2000) The Europen small subunit ribosomal RNA database, Nucleic Acids Res. 28, 175–176.

Vossbrinck C.R., Baker M.D., Didier E.S., Debrunner-Vossbrinck B.A., Shadduck J.A. (1993) Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: species identification and phylogentic construction, J. Eukaryot. Microbiol. 40, 354–362.

Weiss L.M., Vossbrinck C.R. (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia, in: Wittner M., Weiss L.M. (Eds.), The Microsporidia and Microsporidiosis, American Society for Microbiology, Washington, DC, pp. 129–171.