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The comparison of rDNA spacer regions of *Nosema ceranae* isolates from different hosts and locations ☆

Wei-Fone Huang [a], Michel Bocquet [b], Ker-Chang Lee [a], I-Hsin Sung [c], Jing-Hao Jiang [a], Yue-Wen Chen [d], Chung-Hsiung Wang [a,*]

[a] *Department of Entomology, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., 106 Taipei, Taiwan*
[b] *Individual Researcher, BP 22, 74371 PRINGY Cedex, France*
[c] *Tainan District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, 712 Tainan, Taiwan*
[d] *Department of Animal Science, National I-Lan University, I-Lan 260, Taiwan*

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Abstract

Nosema ceranae is a common microsporidian pathogen, one of two *Nosema* species that cause "nosema disease" in honeybees, *Apis cerana* and *Apis mellifera*. Samples of *N. ceranae* rDNA from isolates collected in different locations were sequenced and one 5S rRNA was found to be upstream of SSUrRNA. The rDNA arrangement, 5′-5S rRNA–IGS–SSUrRNA–ITS–LSUrRNA-3′, was found in all isolates. In order to better understand the distribution relationship between *N. ceranae* isolates from *A. cerana* and *A. mellifera*, their rRNA spacer regions were also sequenced for analysis. Results showed that there are no significant differences between the IGS sequences of the isolates and no difference in the ITS sequence with the exception of one transition found in an isolate from Martinique. These isolates showed consistency in the IGS phylogenic analysis suggesting that no transmission barrier exists between *A. mellifera* and *A. cerana* and there is no difference between isolates from geography separated areas.

Keywords: *Nosema ceranae*; Nosema disease; rDNA; IGS; ITS; France; Martinique; Spain; Taiwan

1. Introduction

Nosema ceranae is one of two microsporidian pathogens causing "nosema diseases" in honeybees, *Apis cerana* and *Apis mellifera*. Nosema disease is not uncommon in temperate climates and could be devastating to honey production (Fries and Ekbom, 1984). *Nosema apis* was first observed by Zander (1909) in *A. mellifera* and can now be found worldwide (Matheson, 1996) in *A. mellifera* and in *A. cerana* (Singh, 1975). *N. ceranae* was first recovered from *A. cerana* by Fries et al. (1996). The authors compared the morphological and ultrastructure characters between *N. apis* and *N. ceranae* and found that the SSUrRNA sequences differed between the two species. The spores of *N. apis* and *N. ceranae* are also somewhat different. The spore of *N. ceranae* is shorter on average and ultrastructural studies revealed fewer polar filament coils in *N. ceranae*, 20–23 compared to 30–44 in *N. apis* (Fries, 1989). Lower identity of SSUrRNA sequences from *N. apis* and *N. ceranae* has been reported compared to the sequences from other *Nosema* species utilizing the same host (Huang et al., 2007). Recently, *N. ceranae* was found in cultivated *A. mellifera* colonies (Huang et al., 2005; Higes et al., 2006; Huang et al., 2007). It is not known whether *N. ceranae* is a new, emerging honeybee parasite or is a naturally occurring species that had been overlooked due to its morphological similarity to *N. apis*, but its success in two closely related hosts and close molecular relationship suggest an historic presence in at least one species of honeybees.

☆ rDNA of *Nosema ceranae* isolates from different hosts and locations.
* Corresponding author. Fax: +886 2 27364329.
E-mail address: wangch@ntu.edu.tw (C.-H. Wang).

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New findings enable us to examine the details of *N. ceranae*'s rDNA arrangement. The SSUrRNA gene of *N. cerana* was sequenced to confirm the transcript initiation site and the reversed orientation of 5S rRNA in *N. ceranae* was found to be upstream of SSUrRNA. This gene arrangement seems to be unique to *N. ceranae* because no such arrangement was found in *N. apis*, *N. bombycis* and other closely related species (Huang et al., 2004; Tsai et al., 2005; Ku et al., 2007).

To further study *N. ceranae* distribution, we obtained the *N. ceranae* isolates from *A. mellifera* collected from several different geographic locations and *A. cerana* in Taiwan. We report here the intergenic spacer region, IGS (between 5SrRNA and SSUrRNA), and the ITS regions of all isolates. We use comparisons and analyses of these sequences to establish the relationship among these *N. ceranae* isolates.

2. Materials and methods

2.1. N. ceranae and N. apis isolates

Samples of *N. ceranae* and *N. apis* were recovered from honeybees collected from Taiwan and from several overseas sites. All microsporidium samples from Taiwan were identified as *N. ceranae* and were separated into three groups: those recovered from *A. mellifera* colonies in Taipei (Group 1) (Huang et al., 2007); Group 2 recovered from *A. cerana* in an apiary in I-Lan, Taiwan; and Group 3 recovered from a wild *A. cerana* colony collected at an altitude of 800 m in a southern Taiwan mountain area. The overseas samples included both *N. ceranae* and *N. apis*. Spain's isolates of *N. ceranae* and *N. apis* from *A. mellifera* were gifts from Dr. Mariano Higes. The *N. ceranae* samples from Martinique Island and France were isolated by Michel Bocquet from infected *A. mellifera* colonies. All isolates were collected in the years 2004–2006 (Table 1).

2.2. DNA purification

The DNA of the isolates from Spain was purified according to a method reported by Higes et al. in 2006. The DNA of Taiwan Group 1 was previously isolated (Huang et al., 2007). The remaining DNA samples were each isolated from one ventriculus of *A. cerana* or *A. mellifera*. The ventriculus was ground in TE buffer (0.1 M Tris, 0.01 M EDTA, pH 9.0) followed by Proteinase K treatment. The phenol/chloroform extraction method was then used to remove most protein and was followed by alcohol precipitation. The DNA pellets were dissolved in ddH_2O and stored at −20 °C.

2.3. PCR amplification strategy

The upstream region of SSUrRNA was sequenced by SSP-PCR (single-specific-primer PCR; Shyamala and Ames, 1989). A specific primer 5SF (ACA AAA ACT ACA GCA CCT AGC) was then designed based on the 5S rRNA sequence. The SSUrRNA gene of each sample was sequenced as described previously (Huang et al., 2007). Fig. 1 depicts the primer annealing sites for spacer region amplification. The IGS region of the ribosomal gene repeat unit was amplified through the primer set 5SF and SSU5r (ACG TCA GGC AGA ATC AAC). The ITS region was amplified using the primer set, S1129f (TGA ATG TGT CCC TGT TCT TTG) and L1328r (GGT ATC CTA TTG ATC CCA TGT G). The PCR mixture was prepared according to the manufacturer's instructions (HiFi DNA polymerase, Yeastern Biotech) and 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. These products were then eluted using a DNA Clean/Extraction Kit (Genemark) and cloned into a T&A cloning vector (Real Biotech Corp.). The inserted DNA fragments were sequenced on an automated DNA Sequencer (DNA Sequencer 377, Applied Biosystems).

2.4. Phylogenetic analyses

Because the intra-isolate variations of the isolate from Spain and two isolates (wild *A. cerana* and *A. mellifera*) from Taiwan are high (more than 3%), two IGS sequences from these isolates were added to the phylogenetic analysis.

Table 1
Spacer region sequences of *Nosema ceranae* and *Nosema apis* isolates

Species	Isolated region	Host	Sequences			Reference
			ITS[a]	IGS	IGS GenBank Accession No.	
N. ceranae	Taiwan, Asia	*A. mellifera*	39 bp	606 bp	EF091879	
	Taiwan, Asia	Domestic *A. cerana*	39 bp	606 bp	EF091883	
	Taiwan, Asia	Wild *A. cerana*	39 bp	614 bp	EF091884	
	France, Europe	*A. mellifera*	39 bp	606 bp	EF091881	
	Spain, Europe	*A. mellifera*	39 bp	607 bp	EF091880	
	Martinique, America	*A. mellifera*	39 bp	606 bp	EF091882	
N. apis	Spain, Europe	*A. mellifera*	33 bp	N/A	N/A	
	New Zealand, Oceania	*A. mellifera*	33 bp	N/A	N/A	Gatehouse and Malone (1998)
Nosema sp.	France, Europe	*A. mellifera*	28 bp	N/A	N/A	

[a] The ITS sequences alignments are shown in Fig. 3.

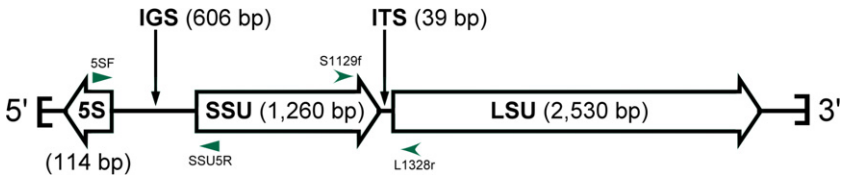

Fig. 1. Schematic diagram of the *Nosema ceranae* rRNA genes. The rRNA domains are indicated by hollow arrows and the direction of arrow indicates the direction of transcription. The small arrowheads represent primers used to amplify the IGS and ITS regions.

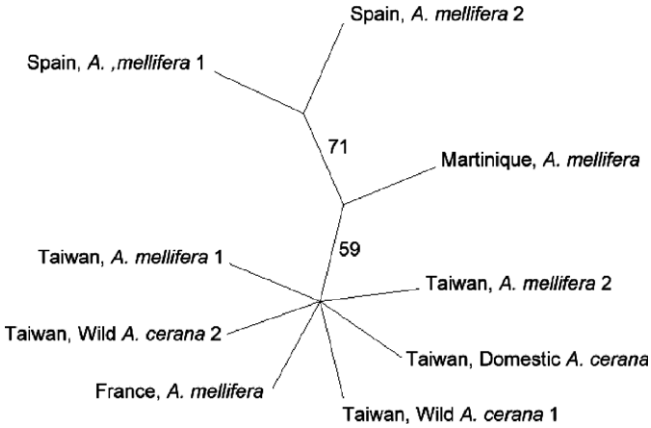

Fig. 2. Unrooted phylogenetic tree based on IGS sequences. Nine taxa were analyzed based on a maximum likelihood approach using PAUP 4.0 b10 (Swofford, 2003), run with 100 bootstrap replications. Numbers indicate branch support statistics.

One is the consensus sequence (those with number 1 on the phylogenetic tree; Fig. 2) and the other is the sequence with variation (number 2 on the phylogenetic tree). In the phylogenetic analysis, we used the consensus sequences for the IGS sequences of the samples from Martinique, France, and domestic *A. cerana* in Taiwan, because their intra-isolate variations are lower. Sequences were aligned using ClustalX (Thompson et al., 1997). Poorly aligned regions were excluded manually. Phylogenetic analyses were conducted based on maximum likelihood (ML) with PAUP 4.0 b10 (Swofford, 2003). The substitution model of the ML analysis was set according the HKY model and the variable sites of among site rate variation followed gamma distribution. It was set according to the Modeltest (Posada and Crandall, 1998) result. The rate matrix, base frequency, proportion of invariable sites, and shape parameter were estimated. Node support of ML analyses was assessed with 100 bootstrap replicates.

3. Results

The SSUrRNA of each of the *N. ceranae* isolates was sequenced and confirmed to be *N. ceranae*. The SSUrRNA of all *N. ceranae* isolates found to be polymorphic, including the isolates from a single ventriculus. The polymorphic sites are similar to those previous published (Huang et al., 2007). The 5′ region of SSUrRNA was sequenced and found to be 5′-5S rRNA–IGS–SSUrRNA–ITS–LSUrRNA-3′ (Fig. 1). The orientation of 5S rRNA in *N. ceranae* is the reverse of SSUrRNA and LSUrRNA, and the length of the 5S rRNA is 114 bp. The 5S rRNA in *N. ceranae* shares 84–93% similarity with other known microsporidia. The IGS is AT rich (about 83%) and polymorphic, and it is approximately 606 bp long. The IGS regions of all *N. ceranae* isolate from different locations were sequenced and submitted to GenBank (accession numbers are listed in Table 1), and the similarity among isolates was 94–97%. The phylogenetic tree of the IGS region (Fig. 2) included more than one sequence from some isolates in the analysis that showed the polymorphism effect. The phylogenetic tree indicates that the isolates from Spain were closely related to their counterparts from Martinique.

The ITS sequence of all isolates consists of only 39 base pairs (Table 1) and the alignment of these sequences is shown (Fig. 3). The ITS sequences were highly conserved in both *N. ceranae* and *N. apis*. Only one transition was found in the *N. ceranae* samples collected from Martinique. A similar result was found in *N. apis*, with only one transition between the ITS sequence of isolate from Spain and the ITS sequence (U97150) of the *N. apis* collected in New Zealand.

4. Discussion

The SSUrRNA sequences of *N. ceranae* samples collected in Spain, Martinique, and Taiwan all proved to be polymorphic, and the similarity was 98–100%. However, the ITS is conserved within the *Nosema* species infest honeybee, and it is only 50–57% identical among them. The ITS sequence seems to be a better marker than the SSUrRNA.

The discovery of the unique configuration of the 5S rRNA in *N. ceranae* was unanticipated. The SSP PCR was performed to sequence the 5′ uncoded region of SSUrRNA and certify the transcription initiation site. The 5S rRNA was found in the upstream sequence of SSUrRNA, and its orientation was reversed compared to SSUrRNA and LSUrRNA in the rDNA repeat unit. This reversed orientation has never been reported in other known microsporidian 5S rRNA located near the rDNA repeat unit. The microsporidian 5S rRNA is usually located downstream of the rDNA repeat unit. Moreover, rDNA head-to-tail or palindromic repeats may not explain the reverse orientation of 5S rRNA, and the 5S rRNA may not be located downstream of LSUrRNA. (664 bp

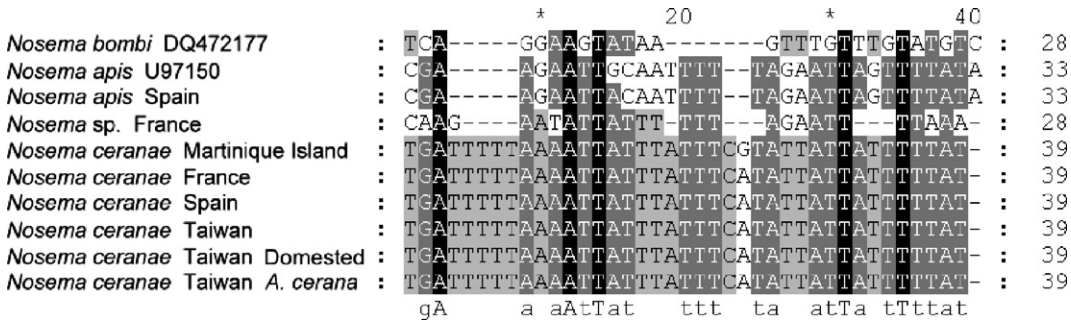

Fig. 3. Alignment of the ITS from *N. cerana* isolates, *N. apis*, and *N. bombi*. The isolate, *Nosema ceranae* Taiwan domested, was collected from the apiary which kept *A. cerana* and *A. mellifera* in the same environment. The isolate, *Nosema ceranae* Taiwan *A. cerana*, was collected from the wild colony of *A. cerana*. Other isolates were all collected from *A. mellifera* colonies.

downstream of the putative stop site were sequenced, Huang et al., 2007.) We speculate that the 5S rRNA of *N. ceranae* might be dispersed randomly between genes as is the 5S rRNA gene in the *Encephalitozoon cuniculi* genome (Katinka et al., 2001) and is fortuitously inserted upstream of an rDNA repeat. This arrangement may not exist in every rDNA repeat unit, because the IGS region seems to be more difficult to amplify through PCR. Further research will be needed to clarify this situation.

The IGS is polymorphic, and the consensus sequences of the IGS from different isolates share 94–97% similarity. The IGS is an uncoded region and more variable than the SSUrRNA of *N. ceranae*. We attempted to establish the relationship of the isolates from France, Martinique, Spain, and Taiwan via phylogenic tree analysis of the IGS sequences. However, the IGS sequences showed consistency. The isolates cannot be definitely differentiated in the analysis, except the isolates from Spain and Martinique show a close relationship. This result is unexpected as Martinique is French Caribbean Island but there may be frequent interaction among honeybee colonies from the nearby islands. The IGS sequences from *A. cerana* isolate showed little difference from those isolated from *A. mellifera* suggesting that there might be no barrier for transmission between these two hosts. However, the precise means by which *N. ceranae* is transmitted is not clear, nor is means for transmission between *A. mellifera* and *A. cerana* known. Water and food contamination and interactions among bees were considered transmission pathways between colonies for nosema disease, but *A. mellifera* and *A. cerana* differ in behavior and flower preference. We suggest that robbing behavior between the two species sometimes observed in apiaries could be a possible transmission pathway between *A. mellifera* and *A. cerana*.

The ratio of *N. ceranae* to *N. apis* or other microsporidia causing nosema disease may vary in different geographic locations. In Spain, the ratio of *N. ceranae* infection in colonies infected with nosema disease was reported to be as high as 90% (Higes et al., 2006). In the samples from Taiwan and Martinique, only *N. ceranae* was recovered. No co-infections were found in these samples. The SSUrRNA (GenBank Accession No. EF585399) of the unidentified microsporidium from France shares 96% identity with *N. ceranae* and 94% identity with *N. apis*. Moreover, the ITS region was sequenced and aligned with the other ITS sequences (Fig. 3); there were noticeable differences from both *N. apis* and *N. ceranae*. The unique rDNA arrangement of *N. ceranae* was not found in this species.

The results of these studies confirmed that *N. ceranae* has already spread in many areas of the world (Klee et al., 2007). The rDNA arrangement in every *N. ceranae* isolate we tested was unique in its location and reverse arrangement. Although the IGS region of *N. ceranae* is polymorphic, the ITS region is conserved. The ITS of *N. apis* may be also quite conserved unlike the polymorphic ITS found in *N. bombycis* (Huang et al., 2004). The unidentified species found in France suggested that in addition to *N. ceranae* and *N. apis*, other *Nosema* species might be infecting *A. mellifera*. The rDNA sequence (including SSUrRNA and ITS) of the unidentified species is an intermediate between those of *N. apis* and *N. ceranae*, generating interesting questions about in nosema disease research.

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