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Original article

Molecular detection of *Nosema ceranae* and *N. apis* from Turkish honey bees*

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Abstract – Polymerase chain reaction specific for the rDNA marker for *Nosema ceranae* and *Nosema apis* was conducted on 84 *Apis mellifera* samples collected from 20 provinces in Turkey. *N. ceranae* was detected from three samples from the provinces of Artvin, Hatay, and Muğla. *N. apis* was detected in samples from the provinces of Sivas, Izmir, Bitlis and Gaziantep. All of the positive samples were from honey bees belonging to the 'C' lineage of *A. mellifera*. DNA sequencing analysis of the *N. ceranae* samples revealed that there was no intraspecific variation in the 208 bp of the 16S SSU of *N. ceranae* from Turkey. A TCS analysis revealed that the 16S SSU genotype from Turkey is identical to *N. ceranae* DNA sequences from Europe, Australia, and the United States. TCS analysis also revealed that this genotype is the basal ancestral genotype among six *N. ceranae* genotypes. This is the first study to confirm that *N. ceranae* is present in honey bees from Turkey.

***Apis mellifera* / *Nosema ceranae* / *Nosema apis* / molecular diagnostics / Turkey**

1. INTRODUCTION

The microsporidia species *Nosema apis* and *N. ceranae* cause a destructive disease of honey bees, *Apis mellifera* L., worldwide (Bailey, 1991; Higes et al., 2006; Huang et al., 2007). *Nosema* affects adults only, infecting epithelial cells lining the midgut. *Nosema* often escapes notice due to its frequent lack of outward symptoms. The organism can cause digestive disorders, shorten bee life spans, reduce pollen collection, induce queen supercedure, decrease colony population, reduce honey production, and cause greater colony mortality in the winter. Efforts to control *Nosema* include removing older combs and treating hives with fumagillan. Observations indicate that *Nosema* is distributed by mailing and transportation of honey bees (Jay, 1966) and infective beekeeping materials (Klee et al., 2007) combined with migratory beekeeping practices (Giersch et al., 2009). Environmental reservoirs and non-honey bee vectors of *Nosema* spores may contribute to its epidemiology. For example, the range of the migratory bee-eating bird *Merops apiaster* includes Western Asia, and its regurgitated pellets have been shown to contain infective *Nosema* spores (Higes et al., 2008a). In 1913, Fantham and Porter demonstrated that *N. apis* is capable of infecting other insect species, though some of their results have been called into question through subsequent experiments (Bailey, 1991).

Recently an illness of *A. mellifera*, called "Colony Collapse Disorder" (CCD) or "Colony Loss" has been causing large-scale losses of honey bees in the United States (Chen et al., 2007) and in Europe (Topolska et al., 2008; Higes et al., 2008b). A possible contributor to CCD may be *N. ceranae*, a species of *Nosema* from *Apis cerana* that has

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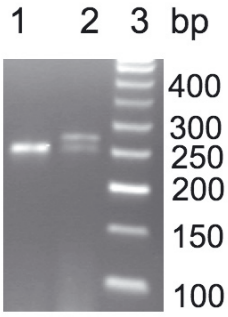

Figure 1. PCR products of *N. ceranae* and *N. apis*: Lane 1: *N. ceranae* positive sample; Lane 2: *N. apis* (269 bp) and *N. ceranae* (250 bp) control; Lane 3: 50 bp DNA ladder.

been recently found in *A. mellifera* (Higes et al., 2006; Topolska et al., 2008; Vejsnaes et al., 2010; Paxton, 2010). Recent studies by Chauzat et al. (2007), Williams et al. (2008), Klee et al. (2007), Martín-Hernández et al. (2007), Paxton et al. (2007), Higes et al. (2009), Tapaszti et al. (2009), and Giersch et al. (2009) have revealed that *N. ceranae* is widespread in Africa, Europe, Australia, and North America. To date, *N. ceranae* has not been observed in Turkey. A previous study by Aydin et al. (2005) identified the presence of *N. apis* in honey bees from Turkey. However, this study did not attempt to distinguish *N. apis* from *N. ceranae* using molecular diagnostic methods.

The objectives of this study were to detect *N. ceranae* and *N. apis* from Turkish honey bees using PCR and to determine the genetic relationship of *N. ceranae* from Turkey relative to *N. ceranae* from other countries.

2. MATERIALS AND METHODS

2.1. Sample collection

Samples were collected by one of the authors (M.K.) from beekeepers in 2005 and 2006 from 20 provinces in Turkey (Tab. I). Five to 20 worker honey bees were placed in a plastic liquid scintillation vial containing 70% ethanol.

2.2. DNA extraction

The Puregene DNA extraction kit (Gentra, Minneapolis, MN) was used to obtain DNA from honey bee abdomens using a procedure similar to Magnus and Szalanski (2010). Abdomens from two bees from each sample were allowed to air dry before adding the samples to cell lysis buffer. Extracted DNA was resuspended in 50 ul of Tris: EDTA buffer (pH 8.0), and stored at –20 °C until PCR.

2.3. PCR amplification

Polymerase chain reaction specific for the rRNA marker for *N. ceranae* and *N. apis* was conducted using the primers *N. ceranae* F and *N. ceranae* R and *N. apis* sense and antisense per Chen et al. (2008, 2009). These primers were used together for a multiplex PCR reaction and resulted in a 250 bp amplicon of the small subunit (16S) ribosomal RNA gene for *N. ceranae* and a 269 bp amplicon for *N. apis* (Fig. 1). Positive controls consisted of *N. ceranae* from honey bees collected from Nebraska, USA, and *N. apis* from honey bees collected from Beaverlodge, Alberta, Canada. In addition, a negative control was included for most PCR runs. For PCR 2 μL of DNA template was used for the 50 μL multiplex PCR. Concentration of reagents, other than PCR primers, is provided in Szalanski and McKern (2007). The PCR thermocycler profile consisted of 40 cycles of a 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 60 s, with a final extension of 5 min at 72 °C. Amplicons were run on a 2% agarose gel electrophoresis, and products were visualized under UV.

2.4. DNA sequencing

Three positive *N. ceranae* PCR samples were subjected to DNA sequencing. For these samples a 208 bp region of the 16S gene was subjected to PCR using the primers *Nosema* F/*Nosema* R (Chen et al., 2008) using the thermocycler profile outlined above. Once PCR products were purified using Microcon-PCR Filter Units (Millipore, Bedford, MA), they were sent to the University of Arkansas Medical Sciences DNA Sequencing Core Facility (Little Rock, AR) for direct sequencing in both directions. CLUSTAL W (Thompson et al., 1994) and Bioedit v5.0.7 software (Hall, 1999) were used to align DNA sequences.

Table I. Prevalence of *N. ceranae* and *N. apis* in different regions of Turkey.

Region (n)	Province (n)	*N. ceranae*	*N. apis*
Marmara (42)	Gökçeada (6)	0	0
	Kirklareli (5)	0	0
	Bursa (15)	0	0
	Sakarya (16)	0	0
Aegean (4)	Izmir (2)	0	1
	Muğla (2)	1	0
Black Sea (15)	Düzce (2)	0	0
	Giresun (5)	0	0
	Artvin (8)	1	0
Central Anatolia (3)	Ankara (1)	0	0
	Sivas (2)	0	1
South East Anatolia (11)	Gaziantep (2)	0	1
	Adiyaman (2)	0	0
	Diyarkakir (3)	0	0
	Batman (1)	0	0
	Bitlis (2)	0	1
	Sirnak (1)	0	0
Medditerranean (7)	Hatay (7)	1	0
Eastern Anatolia (2)	Erzincan (1)	0	0
	Hakkari (1)	0	0

A BLAST search (http://blast.ncbi.nlm.nih.gov) was used to compare genetic variation of the 208 bp amplicons from Turkey with those available on GenBank. Genealogy of *N. ceranae* 16S genotypes from our lab and from GenBank was determined using the method of Templeton et al. (1992), which represent the evolutionary steps between genotypes, using TCS v 1.21 (Clement et al., 2000).

3. RESULTS

From the 84 samples subjected to PCR analysis, *N. ceranae* was amplified one time in each from samples from the provinces of Artvin, Hatay, and Muğla (Tab. I, Fig. 2). *Nosema apis* was detected one time each from the provinces of Sivas, Izmir, Bitlis and Gaziantep (Tab. I, Fig. 2). These positive samples were from beekeepers located at widely dispersed geographical regions in Turkey, including the Aegean, Mediterranean, South East Anatolia, Central Anatolia, and the Black Sea. The only region we found to have *A. mellifera* positive for both *N. apis* and *N. ceranae* infection was the Aegean region.

From the three positive *N. ceranae* samples from Turkey, no intraspecific DNA sequence variation was observed among the 208 bp 16S sequences. Using the *N. ceranae* 16S DNA sequences available on GenBank, a total of 6 different genotypes, designated as genotypes G1-G6, were found using the same 208 bp region used in this study (Tab. II, Fig. 3). A BLAST search revealed that the *N. ceranae* DNA 208 bp sequence from Turkey, designated as genotype 2 (G2), was identical to *N. ceranae* DNA sequences from the United States, Europe, and Australia. TCS spanning network of the *N. ceranae* genotypes revealed that genotype G2 was the basal type, which was one base pair different with other genotypes, except for G3 and G6 (Fig. 3). Genotype G2 can be considered as the basal ancestral genotype because it is located at the base of the genealogy. Genotype G3 was the most distantly related to G2, with three base pair differences, and only occurred in Australia (GenBank accession number FJ789791).

4. DISCUSSION

This study demonstrated that both *N. apis* and *N. ceranae* are present in honey bees

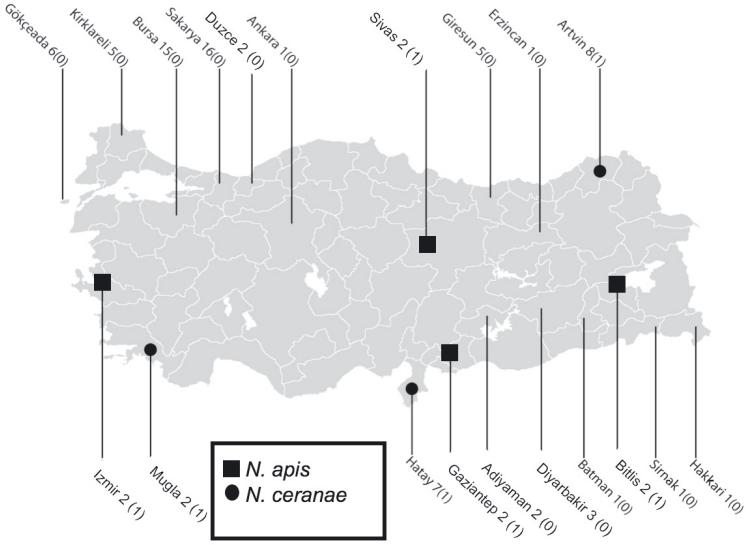

Figure 2. Map of Turkey showing provinces from which *A. mellifera* were collected and tested for *N. ceranae* and *N. apis*. The number of samples screened is provided along with the numbers positive for *N. ceranae* and *N. apis*, respectively, in parentheses.

Table II. Mitochondrial DNA haplotypes of *Nosema ceranae* using a 208 bp region of the rDNA 16S gene.

Haplotype	Location	GenBank Accession Number	Source
G1	Unknown	U26533	Fries et al. (1996)
	Korea	FJ481912	Unpublished
G2	Switzerland	DQ673615	Unpublished
	Australia	FJ789797	Unpublished
	Turkey	–	This Study
	USA	–	JW unpublished
	Austria	EU045844	Unpublished
	France	DQ374655	Unpublished
	Germany	DQ374656	Martín-Hernández et al. (2007)
G3	Australia	FJ789791	Unpublished
G4	Argentina	EU025027	Unpublished
G5	Australia	FJ789795	Unpublished
G6	Taiwan	DQ486028	Unpublished

from the Republic of Turkey. *N. ceranae* was observed in geographically distant regions of Turkey, including the Aegean, Mediterranean and Black Sea. Aydin et al. (2005) found *Nosema* sp. from six regions in Turkey, with the majority occurring in Marmara and the Black Sea. We did not find *N. apis* or *N. ceranae* in Marmara, Central Anatolia, or East Anatolia, but we did find it in Southeast Anatolia, a region where *Nosema* sp. was not detected by Aydin et al. (2005).

Our finding of three positive *N. ceranae* samples out of 84 (3.6%) is on the low end of the spectrum, relative to the frequency of *N. ceranae* (65.6%) in samples from France in 2002–2005 (Chauzat et al., 2007), Minnesota in the United States (40%), three Canadian provinces (19.6%) (Williams et al., 2008), and in Australia in 2008 (4.5–33.7%) (Giersch et al., 2009). This number is provided for comparison only and should be interpreted with caution. The goal of our study was only to

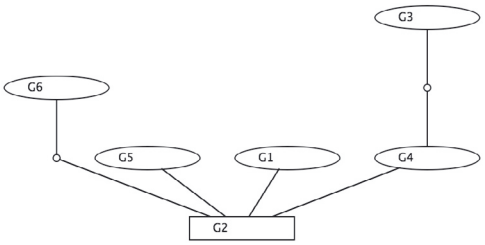

Figure 3. Genealogical relationships among 16S genotypes of *Nosema ceranae* estimated by TCS (Clement et al. 2000). A unit branch represents one mutation and small ovals indicate genotypes that were not observed.

detect the presence or absence of *N. ceranae* and *N. apis* in Turkey. Our sample size of 2 bees per colony is insufficient to provide a statistically significant measurement of *Nosema* prevalence.

Some of the Turkish provinces are over-represented as compared to others, but the samples are representative of a wide number of subclimates and are from locations spanning the entire country. Samples used for our *N. apis*, *N. ceranae* PCR study were previously used for a population genetics study of *A. mellifera* in Turkey (Solorzano et al., 2009). The *N. ceranae* positive honey bee samples belonged to mtDNA COI-COII 'C' lineage mitotypes, C11 (Artvin), and C12 (Hatay and Muğla), and the *N. apis* positive samples belonged to the mitotypes C12 (Izmir and Bitlis), C13 (Sivas) and C19 (Gaziantep). To our knowledge, no other studies on molecular detection of *N. ceranae* or *N. apis* have documented the genetic lineage or mitotype of the host bees, so the relationship of *N. ceranae* infection and different host lineages is unknown. All of the three provinces (Artvin, Hatay, and Muğla) where *N. cerenae* were detected also showed high rates of colony losses in 2006/2007 (Giray et al., 2010). For Artvin province, 50% of 2505 colonies maintained by 44 beekeepers were lost, while 39.4% of 7628 colonies maintained by 36 beekeepers in Hatay province were lost, and 22.7% of 6516 colonies maintained by 36 beekeepers were lost in Muğla province. Though the cause of the colony losses cannot be conclusively attributed to *N. ceranae* infection, there exists a potentially significant relationship between its presence and the occurrence of the losses. Similar colony loss reports that may be attributed to *N. ceranae* have been documented in Poland (Topolska et al., 2008) and Denmark (Vejsnaes et al., 2010).

The high level of genetic similarity of 16S rDNA sequences of *N. ceranae* from Turkey relative to those found elsewhere complicates efforts to determine a geographic origin for *N. ceranae*, as well as efforts to identify strains with varying degrees of virulence. DNA sequencing analysis of a larger region of 16S rDNA, or the use of additional genetic markers may provide additional insight. It is perhaps not surprising that *N. ceranae* should be found in Turkey, as it is close to the native range of the original host of *N. ceranae,* the Asiatic honey bee *A. cerana* F., which is found from Western Afghanistan to Japan (Dietz, 1992). The minor variation found between strains of *N. ceranae* encourages shifts in future emphases on research from finding differences in parasitic virulence perhaps to evaluating different *N. ceranae* genes for variation and to finding differences in honey bee lineage susceptibility, and examining climatic and environmental variables involved in *N. ceranae* prevalence and transmission. It may be that certain bee lineages are more or less able to cope with *N. ceranae* infection, or simply that an increase in their genetic diversity through breeding programs or interbreeding with feral "survivor" populations will provide them with more mechanisms to adapt. In addition, the potential role of climatic and environmental variables should not be ignored.

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Détection moléculaire de *Nosema ceranae* et *N. apis* chez des abeilles de Turquie.

Diagonostic moléculaire / Turquie / *Apis mellifera* / *Nosema ceranae* / *Nosema apis*

Zusammenfassung – Molekulare Detektion von *Nosema ceranae* und *N. apis* in Honigbienen aus der Türkei. Das Mikrosporidium *Nosema ceranae* verursacht weltweit eine ernsthafte Krankheit der Honigbiene *Apis mellifera* L. (Higes et al., 2006). Eine vor kurzem aufgetretene Krankheit unter der Bezeichnung „Colony Collapse Disorder“ (CCD) oder „Völkerverlust“ führt ebenfalls zu hohen Verlusten bei Honigbienen in den Vereinigten Staaten (Chen et al., 2007) und in Europa (Higes et al., 2008b). Als ein möglicher Mitverursacher der CCD kommt *N. ceranae* in Frage, eine *Nosema*-Art von *Apis cerana,* die vor kurzem auch bei *A. mellifera* nachgewiesen wurde (Higes et al., 2006). Über das Vorkommen von *N. ceranae* in der Türkei gab es bisher noch keine Berichte. Eine frühere Studie von Aydin et al. (2005) lieferte allerdings Hinweise auf das Vorkommen von *N. apis* in türkischen Honigbienen. Diese Studie wurde jedoch nicht mit molekularen Diagnostikmethoden durchgeführt, die eine Unterscheidung von *N. apis* und *N. ceranae* erlaubt hätten. Ziel der vorliegenden Studie war es deshalb, zum einen das Vorkommen von *N. ceranae* und *N. apis* in türkischen Honigbienen mittels einer Polymerasekettenreaktions(PCR)-Methode zu überprüfen und andererseits *N. ceranae* aus der Türkei mit *N. ceranae* aus anderen Ländern genetisch zu vergleichen.
Hierzu wurde DNA aus Abdomina von Bienen mittels einer auf der von Magnus und Szalanski (2010) entwickelten Methode extrahiert. Für PCR-Analysen von *N. ceranae*- und *N. apis*-spezifischen rRNA Markern bei 84 *Apis mellifera* Proben aus 20 Provinzen der Türkei (Abb. 1 und 2) benutzten wir die von Chen et al. (2008, 2009) entwickelten Primer *N. ceranae* F und *N. ceranae* R, sowie *N. apis* sense und antisense. Damit konnten wir *Nosema ceranae* in drei Proben aus den Provinzen Artvin, Hatay und Muğla, und *N. apis* in Proben aus den Provinzen Sivas, Izmir, Bitlis and Gaziantep nachweisen (Tab. I). Alle positiven Honigbienenproben konnten der ‘C’ Linie von *A. mellifera* zugeordnet werden. DNA-Sequenzierungsanalysen von *N. ceranae* Proben zeigten keine genetische Variabilität in dem 208 bp Fragment der kleinen Untereinheit (SSU) der 16S RNA von *N. ceranae* aus der Türkei. Und in einer TCS und BLAST-Analyse erwies sich der 16S SSU Genotyp aus der Türkei als identisch mit *N. ceranae* DNA-Sequenzen aus Europa, Australien und den USA (Abb. 3). Die TCS-Analyse zeigte auch, dass es sich hierbei um den basalen, ursprünglichen Genotyp innerhalb der sechs *N. ceranae* Genotypen handelt.
In dieser Untersuchung konnten wir zeigen, dass sowohl *N. apis* als auch *N. ceranae* in Honigbienen aus der Türkei vorkommen. *N. ceranae* war bisher in geographisch entlegenen Gebieten der Türkei, sowie in Regionen der Ägäis, des Mittelmeers und des Schwarzen Meeres nachgewiesen worden. Aydin et al. (2005) fanden *Nosema* sp. in sechs Regionen der Türkei, und hier überwiegend in Marmara- und Schwarzmeergebieten. Wir konnten zwar keine *N. apis*- oder *N. ceranae*-Vorkommen in Marmara, Zentral- oder Ostanatolien nachweisen, jedoch aber in Südostanatolien, einer Region in der Aydin et al. (2005) keine *Nosema* sp. gefunden hatten. Aufgrund der geringen genetischen Variabilität zwischen *N. ceranae*-Linien schlagen wir vor, dass künftige Forschungsprojekte sich weniger auf die Untersuchung genetischer Unterschiede in der Virulenz, sondern vielmehr auf die Untersuchung spezifischer *N. ceranae* Virulenzgene konzentrieren sollten, sowie auf Gene, die bei Honigbienen eine Rolle in der *Nosema*-Suszeptibilität spielen könnten. Hierzu sollten auch klimatische Variablen und andere Umgebungsbedinungen in Betracht gezogen werden, die für die Prävalenz und Übertragung von *N. ceranae* von Bedeutung sein können. Daneben kann es durchaus sein, dass bestimmte Bienenlinien mit *N. ceranae*-Infektionen besser zurechtkommen als andere, oder dass einfach eine erhöhte genetische Variabilität durch Zuchtprogramme und Einkreuzung wilder Populationen, die die Krankeit überlebt hatten, es erlauben könnte, dass Bienen adaptive Mechanismen im Umgang mit der Krankheit entwickeln.

***Apis mellifera* / *Nosema ceranae* / *Nosema apis* / molekulare Diagnostik / Türkei**

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