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Distribution of *Nosema ceranae* in the European honeybee, *Apis mellifera* in Japan

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ABSTRACT

The microsporidian species, *Nosema apis* and *Nosema ceranae* are both known to infect the European honeybee, *Apis mellifera*. Nosema disease has a global distribution and is responsible for considerable economic losses among apiculturists. In this study, 336 honeybee samples from 18 different prefectures in Japan were examined for the presence of *N. apis* and *N. ceranae* using a PCR technique. Although *N. ceranae* was not detected in most of the apiaries surveyed, the parasite was detected at three of the sites examined. Further, *N. ceranae* appears to be patchily distributed across Japan and no apparent geographic difference was observed among the areas surveyed. In addition, the apparent absence of *N. apis* suggests that *N. ceranae* may be displacing *N. apis* in *A. mellifera* in Japan. Partial SSU rRNA gene sequence analysis revealed the possible existence of two *N. ceranae* groups from different geographic regions in Japan. It seems likely that these microsporidian parasites were introduced into Japan through the importation of either contaminated honeybee-related products or infected queens. This study confirmed that PCR detection is effective for indicating the presence of this pathogen in seemingly healthy colonies. It is therefore hoped that the results presented here will improve our understanding of the epidemiology of Nosema disease so that effective controls can be implemented.

1. Introduction

Microsporidia are obligatory intracellular parasites that are commonly found in insects and other invertebrates (Larsson, 1986). Of these parasites, two species belonging to the genus *Nosema* have been confirmed as pathogens in honeybees; *Nosema apis* and *Nosema ceranae* are parasites of the European honeybee, *Apis mellifera* and Asian honeybee, *Apis cerana*, respectively (Zander, 1909; Fries et al., 1996; Fries, 2010). The Nosema disease caused by these parasites has spread worldwide and is currently responsible for considerable economic losses in apiculture (Bailey and Ball, 1991; Fries, 1997, 2010). Although *A. mellifera* was previously considered to be the exclusive host of *N. apis* (Matheson, 1993), which causes a decrease in both honeybee lifespan and bee populations during winter (Ritter, 2001), the congeneric *N. ceranae* was recently observed in *A. mellifera* colonies (Huang et al., 2007; Higes et al., 2006). While *N. ceranae* was first reported in its original host, the Asian honeybee *A. cerana*, by Fries et al. (1996), the recent spread of this microsporidian species in both Europe (Klee et al., 2007) and the USA (Chen et al., 2008) suggests that the host specificity of this parasite has broadened to include both *A. cerana* and *A. mellifera* within the last decade (Fries et al., 1996; Chen et al., 2008). Using experimental infection trials of caged worker bees, Higes et al. (2007) demonstrated that Nosema disease caused by *N. ceranae* in European honeybees is considerably more prevalent than that caused by *N. apis*.

In Japan, commercially reared *A. mellifera* have widely been used as pollinators of greenhouse crops such as strawberries during the winter season. Given their high pollination efficiency, there has been a marked increase in the demand for honeybees for use as pollinators. However, in an attempt to satisfy this demand, queens have been imported into Japan and it is possible that honeybee diseases, such as *Nosema* disease, may be introduced into the country via this route. Despite the global prevalence of *Nosema*, little is known about the current distribution and presence of *Nosema* species in Japan. The aim of this study was to investigate the occurrence and incidence of the *Nosema* parasite in *A. mellifera* in Japan using PCR techniques.

2. Materials and methods

2.1. Samples

In summer of 2009, *A. mellifera* worker bees from 25 apiaries belonging to volunteer beekeepers were collected from several prefectures in Japan (Table 1). Five colonies were randomly selected from each apiary and three individual worker bees from each colony were subjected to DNA analysis. Reference DNA samples of *N. apis* and *N. ceranae* were provided by Dr. R. Martin-Hernandez (Centro Apícola Regional, Junta de Castilla-La Mancha, Spain).

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Table 1
Location and sample number of *N. ceranae* observed in apiaries in the study area.

Colonies	1			2			3			4			5		
Individuals	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1. Hokkaido1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2. Hokkaido2	–	–	–												
3. Hokkaido3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4. Aomori	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5. Miyagi	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6. Yamagata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	-
7. Nagano	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8. Shizuoka	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9. Aichi	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10. Gifu	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11. Mie1	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+
12. Mie2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13. Mie3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14. Wakayama1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15. Wakayama2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16. Wakayama3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17. Nara	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18. Tottori	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19. Hiroshima1	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
20. Hiroshima2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
21. Kagawa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22. Saga	–	–	+	+	–	–	–	–	–	–	–	–			
23. Nagasaki	–	–	–												
24. Ohita	–	–	–												
25. Miyazaki	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+: PCR positive.
–: PCR negative.
□: Failed DNA extraction

2.2. DNA extraction

Worker bees were washed with 10% NaOH solution and rinsed twice with ddH_2O (deionized distilled water). DNA was then extracted from a single worker bee using a FastDNA Kit (MP Biomedicals, OH) according to the manufacturer's instructions.

2.3. Polymerase chain reaction

PCRs were carried out in 50 μl reaction volumes containing 1 μl of DNA template (100 ng in 1 μl), 5 μl of 10X PCR buffer, 2.5 mM $MgCl_2$, 250 μM of dNTPs, 0.5 U of KOD (named after *Thermococcus kodakaraensis*) *Taq* DNA polymerase (Toyobo Co. Ltd., Japan) and 0.2 μM of each primer on a thermal cycler (PTC-200, MJ Research Inc., MA). The primers used were as follows: SSU-res-f1: (5′-GCC TGA CGT AGA CGC TAT TC-3′) and SSU-res-r1: (5′-GTA TTA CCG CGG CTG CTG G-3′) (Klee et al., 2007). This primer set was designed to amplify the rRNA gene in *N. ceranae*. Each PCR reaction run independently. *N. ceranae* F: (5′-CGG ATA AAA GAG TCC GTT ACC-3′) and *N. ceranae* R: (5′-TGA GCA GGG TTC TAG GGA T-3′); *N. apis* F: (5′-CCA TTG CCG GAT AAG AGA GT-3′) and *N. apis* R: (5′-CAC GCA TTG CTG CAT CAT TGA C-3′) (Chen et al., 2008). The PCR amplification conditions consisted of an initial denaturation step at 95 °C for 60 s, followed by 35 cycles of 95 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s. PCR products were electrophoresed on 1.5% agarose, stained with ethidium bromide, and visualized under UV light. The PCR fragment was subcloned into a pGEM®-T Easy Vector (Promega Corp., WI) and sequencing was performed using a DNA sequencer (ABI 3730, Applied Biosystems Inc., CA) and a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Three clones of each fragment were sequenced. To identify individual polymorphic nucleotide positions, partial gene sequences from different regions of the small subunit (SSU) rRNA in *N. ceranae* were obtained from the GenBank database (Accession Nos. FJ789799, U26533, DQ078785 and DQ329034; http://www.ncbi.nlm.nih.gov/) and aligned using GENETYX-MAC (ver. 9.0, Software Development Co. Ltd., Japan).

3. Results

European honeybees (336 from 112 colonies) from 18 different prefectures in Japan were examined for the presence of *N apis* and *N. ceranae* using a PCR-based assay. The *N. ceranae*-specific primers successfully amplified an amplicon measuring approximately 250 bp (Chen et al., 2008) (Fig. 1). Conversely, no amplification was observed using the *N. apis*-specific primers indicating *N. apis* was not detected in any of the 336 samples. PCR products were sequenced and subjected to a BLAST search. All of the cloned PCR fragments showed high similarity to *N. ceranae* (Fries et al., 1996, Genbank Acc. No. U26533). The results of the PCR detection assays for all 336 *N. ceranae* samples are shown in Table 1, and the locations of these samples were plotted on a map of Japan map to visualize the distribution of *Nosema* species (Fig. 2). Of the 112 colonies surveyed, *N. ceranae* was detected in five colonies from three apiaries in the prefectures of Mie, Hiroshima and Saga (4.5%). All three of the honeybee specimens from colony 5 in the Mie1 apiary tested positive for *N. ceranae*. In the other colonies

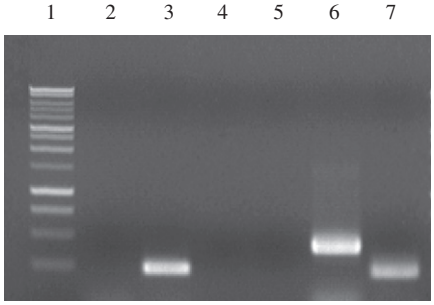

Fig. 1. Typical PCR products amplified using *N. ceranae*-specific primers electrophoresed on agarose and stained with ethidium bromide. Lanes 2, 4, 6; *N. apis*-specific products. Lanes 3, 5, 7: *N. ceranae*-specific primers. Lane 1: 1 kb DNA marker; Lanes 2–3: samples from Mie; Lanes 4–5: ddH_2O as a negative control; Lanes 6–7: *N. apis* and *N. ceranae* reference DNA, respectively.