A molecular assay for the presence of the *Nosema* parasite in Alabama honeybees

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Abstract

Colony-Collapse Disorder (CCD) in honeybees has been postulated to be due to infection with the microsporidian *Nosema* (Higes *et al.* 2009) or infection with the Israeli acute paralysis virus (IAPV)(Cox-Foster *et al.* 2007). We have developed a simple molecular assay for the presence and identification of the species of *Nosema* in honeybees beginning with a single bee. Our protocol PCR-amplifies *Nosema* small-subunit ribosomal DNA (SSU rDNA) using the primers designed by Chen *et al.* (2008). Intestines (including the ventriculus) were dissected from honeybees and DNA was isolated using the Wizard Genomic DNA Extraction Kit (Promega). Non-specific *Nosema* SSU rDNA primers, or primers specific for *N. apis* or *N. ceranae* were used to PCR-amplify a short segment. Nosema-infected Alabama bees were confirmed by electrophoresis and sequencing to have *N. ceranae* SSU rDNA.

The microsporidian parasite *Nosema* is a common parasite of honeybees and may be involved in colony-collapse disorder (CCD), although some evidence indicates that it may be caused by the Israeli acute paralysis virus (IAPV)(Higes *et al.*, 2009; Cox-Foster *et al.*, 2007). We have developed a molecular assay for the presence of *Nosema* in Alabama honeybees following the protocol of Chen *et al.* (2008).

Materials and Methods

DNA was extracted either from whole abdomens or from intestines dissected out of honeybees. For dissections, bees were placed in insect Ringer's solution and the abdominal intestine, including the ventriculus, was removed. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) or the MoBio PowerSoil DNA Isolation Kit. Two species of *Nosema* are present in the U.S.: *N. apis* and *N. ceranae*. PCR-amplification of *Nosema* DNA used three small subunit ribosomal DNA (SSU rDNA) primer pairs (Higes *et al.*, 2009). One pair, Nosema-F (5' GGC AGT TAT GGG AAG TAA CA 3')/Nosema-R (5' GGT CGT CAC ATT TCA TCT CT 3'), recognizes either species. The other two pairs are species-specific: Napis-F (5' CCA TTG CCG GAT AAG AGA GT 3')/Napis-R (5' CAC GCA TTG CTG CAT CAT TGA C 3') and Nceranae-F (5' CGG ATA AAA GAG TCC GTT ACC 3')/(Nceranae-R (5' TGA GCA GGG TTC TAG GGA T 3'). DNA was isolated from bees from local colonies and from bees supplied by Dennis Barclift, Alabama State Apiarist, that had been cytologically confirmed to have *Nosema* spores present in their intestines. Amplification was confirmed by electrophoresis using either standard 2-3% agarose gels or 2% agarose in the E-Gel system (Life Technologies). For sequencing, amplicons were purified on a CloneWell 0.8% agarose gel (Life Technologies) and cloned using the TOPO-TA cloning kit (Invitrogen) followed by colony sequencing (GeneWiz). Alternatively, purified PCR product was sequenced by GeneWiz using the *Nosema* primers. Geneious software (Drummond *et al.*, 2011) was used for sequence analysis.

Results

PCR-amplified DNA from bees known to harbor *Nosema* using the three *Nosema* primers showed the presence of *N. ceranae* DNA upon electrophoresis (Figure 1). Amplification of several bees from the colonies of Malia Fincher, Samford University, failed to show the presence of *Nosema* (data not shown). Sequencing revealed that the *Nosema* DNA present in these samples was indeed *N. ceranae* DNA (Figure 2).

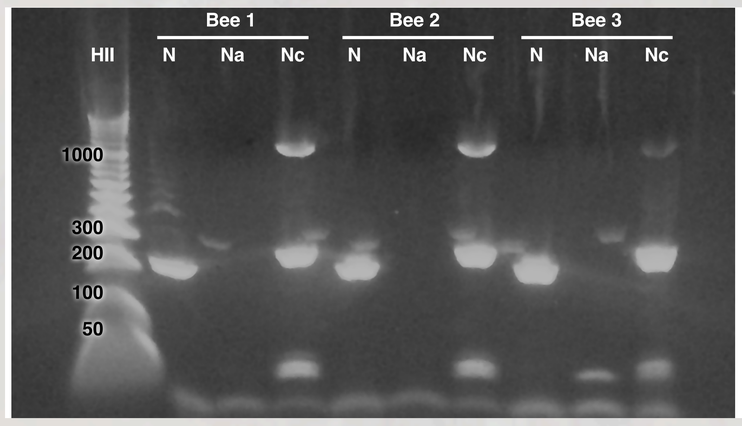

Figure 1. PCR-amplification of N. ceranae DNA. DNA was isolated from three honeybees known to have *Nosema* present. DNA was PCR-amplified from these bees using a pair of primers specific for *N. apis* (Na), a pair of primers specific for *N. ceranae* (Nc), and a pair of primers that recognize both species (N). This 2% agarose gel indicates that all three bees had *N. ceranae*, since the appropriate fragment (less than 300 bp) was amplified by the N and Nc primer pairs, but not by the Na primer pair. (The identity of the larger, approximately 1000 bp fragment in the Nc lanes remains unknown.) HII is the marker Hyperladder II (Bioline USA).

Discussion

We have demonstrated that this molecular assay can not only identify the presence of the *Nosema* parasite but can also identify the species present. This methodology should prove much quicker than cytological assessment, which does not easily identify species. Our future goals are to develop a protocol for determining the relative proportion of these two species present in a bee or colony. This would involve either denaturing gradient gel electrophoresis (DGGE) after the methodology of Bourgeois (2010) or next-generation sequencing. We also plan to develop similar protocols for detecting IAPV in honeybee colonies.

Acknowledgements

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NCBI: http://www.ncbi.nlm.nih.gov

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2Nc          1 CTTCGGGGAATCTTAAGTCTTTCTTCCAAGAAAGTAGGGCAAGCTATGCTCTTGGGATGT   60
               CTTCGGGGAATCTTAAGTCTTTCTTC AAGAAAGTAGGGCAAGCTATGCTCTTGGGATGT
DQ486027  4207 CTTCGGGGAATCTTAAGTCTTTCTTCTAAGAAAGTAGGGCAAGCTATGCTCTTGGGATGT 4266

2Nc         61 GAGATCCAGTGCCGGTTGGGAGAAGCCGTTACCCTTCGGGGAATCTTCAAAAAACACACA  120
               GAGATCCAGTGCCGGTTGGGAGAAGCCGTTACCCTTCGGGGAATCTTCAAAAAACACACA
DQ486027  4267 GAGATCCAGTGCCGGTTGGGAGAAGCCGTTACCCTTCGGGGAATCTTCAAAAAACACACA 4326

2Nc        121 ACCT-GGGGAGGGTTTGGCAAGCTGCTGACGCCGTAGCATTTGCGTTGGATCAGGTCAGA  179
               ACCT GGGGAGGGTTTGGCAAGCTGCTGACGCCGTAGCATTTGCGTTGGATCAGGTCAGA
DQ486027  4327 ACCTGGGGGAGGGTTTGGCAAGCTGCTGACGCCGTAGCATTTGCGTTGGATCAGGTCAGA 4386

2Nc        180 TCCTTAAACTGACGATTGAGTCAGGCGTGTT  210
               TCCTTAAACTGACGATTGAGTCAG CGTGTT
DQ486027  4387 TCCTTAAACTGACGATTGAGTCAG-CGTGTT 4416
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Figure 2. PCR-amplified fragment is N. ceranae DNA. DNA from Bees 2 and 3 (Figure 1) was purified on a CloneWel 0.8% agarose gel (Life Technologies) and sequenced (GeneWiz) using the Nceranae primers. The two fragments had the identical 210 nt sequence shown here as 2Nc. A BLAST search of the NCBI database using Geneious software (Drummond *et al.* 2011) showed this match with a published *N. ceranae* sequence (shown here as accession number DQ486027). The two sequences show identity at 208 of 211 sites, with two single base insertion/deletions and one G/C transition.