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Diversity of *Nosema* associated with bumblebees (*Bombus* spp.) from China ☆

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ABSTRACT

Bumblebees (*Bombus* spp.) are important pollinators of many economically important crops and microsporidia are among the most important infections of these hosts. Using molecular markers, we screened a large sample (n = 1,009 bees) of workers of 27 different *Bombus* spp. from China (Sichuan, Qinghai, Inner Mongolia, and Gansu provinces). The results showed that 62 individuals representing 12 *Bombus* spp. were infected by microsporidia with an overall prevalence of 6.1%. Based on the haplotypes (ssrRNA sequences), we confirmed the presence of *Nosema bombi*, *Nosema ceranae* and (likely) *Nosema thomsoni*. In addition, four new putatively novel taxa were identified by phylogenetic reconstruction: *Nosema A*, *Nosema B*-complex, *Nosema C*-complex and *Nosema D*-complex. In many cases, hosts were infected by more than one *Nosema* taxon. Possible caveats of sequence analyses are discussed.

1. Introduction

Bumblebees (*Bombus* spp.) are among the most important natural pollinators in temperate and sub-arctic ecosystems throughout the Northern Hemisphere (Bingham and Orthner, 1998), and may provide superior pollination services than the honeybees for many plant species (Winter et al., 2006). With these advantages, some bumblebees have become commercially bred species, notably for greenhouse pollination of a large variety of valuable crops (Velthuis and van Doorn, 2006). In the most noteworthy cases, the following species have been used around the world: *Bombus terrestris*, *Bombus lucorum*, *Bombus occidentalis*, *Bombus ignitus* and *Bombus impatiens* (Ruz, 2002; Velthuis and van Doorn, 2006; Li et al., 2008). Several species had been released in New Zealand for the purpose of pollination in the 19th century (*B. terrestris*, *Bombus ruderatus*, *Bombus hortorum*, and *Bombus subterraneus*) (Macfarlane and Gurr, 1995). However, such introductions not only provide benefits but also generates problems such as the risk of parasitic infections that might reduce the quality of pollination, or that can lead to the transfer of novel pathogens into the native fauna in a given region (Goka et al., 2000, 2001, 2006; Niwa et al., 2004; Colla et al., 2006; Otterstatter and Thomson, 2008).

Pathogens have been suspected to play a role in the decline of bee numbers, notably the honeybee colony collapse syndrome (Kevan and Phillips, 2001; Cox-Foster et al., 2007), and can also affect the ecology and distribution of bumblebees (Schmid-Hempel, 2001) as well as perhaps contribute to their decline (Cameron et al., 2011). Furthermore, workers infected by parasites often show subtly different foraging behaviours, such as altering the steadiness in flower visits (Otterstatter et al., 2005), having more difficulty in flower handling (Gegear et al., 2005) or processing floral information (Gegear et al., 2006), and by shifting their floral preferences (Schmid-Hempel and Schmid-Hempel, 1990; Schmid-Hempel and Stauffer, 1998). All of these changes must ultimately reflect on the costs and benefits of the foraging process and might therefore represent a substantial cost of parasitism.

The microsporidia are a large and cosmopolitan class of intracellular parasites, now recognised as a basic branch of the fungi (Wittner, 1999; Didier and Weiss, 2008; Corradi and Slamovits, 2011). As a result of obligate parasitism, microsporidia have unusually compressed and modified genomes, which is a subject of great interest for evolutionary biology (Corradi and Slamovits, 2011). More specifically, *Nosema* spp. that infect bees have more recently prompted considerable research efforts. For example, European populations of the honeybees (*Apis mellifera*) used to be infected primarily by

☆ *Note:* Nucleotide sequence data reported in this paper are available in GenBank under Accession Nos. JN872219 to J872293.

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Nosema apis. More than 10 years ago, *Nosema ceranae*, originating from the Asian honeybee, *Apis cerana* (Fries et al., 1996), transferred into *A. mellifera*. This invading pathogen is now common and seems to rapidly replace *N. apis* as the dominant microsporidian infection in many geographic locations (Fries et al., 2006; Higes et al., 2006; Huang et al., 2007; Paxton et al., 2007; Chen et al., 2008, 2009; Fries, 2010). *Nosema apis* has many effects on its honeybee hosts (Malone et al., 1995) and *N. ceranae* seems to be no less virulent (Higes et al., 2007; Mayack and Naug, 2009; Alaux et al., 2011).

Despite the reported presence of microsporidian infections for at least 21 different bumblebee species, only one microsporidian, *Nosema bombi*, has yet been investigated with respect to its general biology and ecology (Fantham and Porter, 1914; Skou et al., 1963; Showers et al., 1967; Schmid-Hempel and Loosli, 1998; Sokolova et al., 2010), virulence (Fisher, 1989; Otti and Schmid-Hempel, 2007, 2008; van der Steen, 2008), biological and morphological characteristics (Li et al., 2005; Larsson, 2007) and molecular genetics (Fries et al., 2001; Tay et al., 2005; Klee et al., 2006; O'Mahony et al., 2007). In the latter studies, intraspecific genetic variants of *N. bombi* infections, based on the ssrRNA and lsrRNA sequences, as well as polymorphism in the internal transcribed spacer (ITS), have been described (Tay et al., 2005; O'Mahony et al., 2007). Generally, *N. bombi* infects a wide range of *Bombus* spp. (Tay et al., 2005). A recent study has also reported the presence of *N. ceranae* in native bumblebees of Argentina (*Bombus atratus*, *Bombus bellicosus*, and *Bombus morio*) (Plischuk et al., 2009). Together with the reported genotypic variability of *N. bombi* (Tay et al., 2005; O'Mahony et al., 2007), there might be more extant variation in *Nosema* infections of bumblebees than hitherto considered.

Here, we report on the investigation of natural infections by microsporidia and the genetic diversity of *Nosema* in Chinese *Bombus* spp. using molecular genetic analyses through DNA sequencing (Fries et al., 2001; Tsai et al., 2009). China is a particularly interesting study location, since the present-day region of Sichuan, Western China, is considered to be the centre of evolution for the *Bombus* clade and contains the highest known diversity with at least 115 species, which is almost half of all known taxa worldwide (Williams et al., 2009). Our data allow not only the evaluation of the diversity of *Nosema* infections in the centre of origin of *Bombus*, but also the potential for the spread of microsporidia to new locations and other hosts in China and elsewhere.

2. Materials and methods

2.1. Collection and sample preparation

The bumblebees were collected with nets and as a random sample in any given locality in Sichuan, Inner Mongolia and Qinghai provinces, China, during July and August 2008, and after capture were transferred directly into pure ethanol. The samples were analysed a few months later in the laboratory. The exact locations are listed in Table 1 and shown in Fig. 1.

2.2. DNA preparation and amplification (PCR)

For the preparation of nucleic acid, the abdomen of the individual bee was cleanly cut off with scissors, immediately put into a vial and homogenised in 100 μl Krebs Ringer solution with a pestle. DNA was extracted from 50 μl of homogenate of a single bee abdomen using a DNA purification kit, Wizard® SV 96 Genomic DNA Purification System (Promega, Madison Wisconsin, USA). DNA extracts were kept at −20 °C until needed as a DNA template for the PCR.

Based on conserved ssrRNA regions, we selected the ssrRNA-f1: (5′-CAC CAG GTT GAT TCT GC CT-3′) and ssrRNA-r1b: (5′-TGT TCG TCC AGT CAG GGT CGT CA-3′) primer set (Tay et al., 2005) to amplify the coding region of microsporidian ssrRNA. PCR reactions were performed using a Mastercycler 5333 (Eppendorf) in 25 μl volume containing 5.0 μl of template DNA, 2.5 μl of 10× PCR buffer, 2.0 μl of dNTPs (200 μM), 0.25 μl of Taq polymerase (Takara, Dalian, China), and 1 μl of each forward and reverse primer (10 μM), plus 13.65 μl of water.

PCR parameters for amplification were as follows: initial DNA denaturation step of 5 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 30 s at 64 °C, and 30 s at 72 °C, and terminated with a final extension step at 72 °C for 10 min. For each run of the PCR, negative (water) and positive (previously identified positive sample) controls were run together with DNA extracts of isolates as template. PCR products were electrophoresed in 1.2% agarose gel containing 0.5 μg/ml GoldView (GV) and visualised under UV light. PCR products were purified using the Agarose Gel DNA Purification kit Ver.2.0 (Takara, Japan) and sub-cloned into the pMD19-T vector (Takara). One to three of these transformed bacterial clones of each PCR product (representing a single host individual) were suitable to be sequenced using M13-reverse and M13-forward universal primers in an automated fluorescence sequencing system ABI (Perkin–Elmer) with a Big-dye terminator v3.0 Cycle Sequencing Ready Reaction for an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

2.3. Sequence analysis

After manual editing and error checking, the sequences were further processed and aligned with MacVector 11.1 for Macintosh. We then used a BLAST database search in GenBank to identify and include the closest matches of the same sequence for other microsporidian taxa. Note that we used BLAST to compare all of our sequences against GenBank, which resulted in a set of closest matches that was used to place our sequences into context. This set was *N. bombi* (Accession Nos. AY008373, AY741110, GQ254295), *N. apis* (FJ789796, X73894), *N. ceranae* (GUI131043, NCU26533), *Nosema thomsoni* (EU219086), *Nosema portugal* (AF033316) and *Vairimorpha lymantriae* (AF033315), which were also aligned and included in the analyses.

Each of our sequences (i.e. a haplotype) was considered to represent a taxonomic unit. We then ran different analyses with the aligned sequences (taxonomic units), treating gaps as valid characters:

(i) A haplotype network was generated by first calculating the number of pairwise substitutions between the aligned sequences in MacVector. This distance matrix was then visualised with Hapstar 0.6 (Teacher and Griffiths, 2010).

(ii) *Substitution model:* For the phylogenetic analyses we first determined the best model for the base frequencies and substitution rates using jModelTest 0.1 with model parameters set to default values (Posada and Crandall, 1998; Posada, 2008). These values were entered in all analyses referring to the best model.

(iii) Due to the large phylogeny, we used Bayesian inference with MrBayes 3.1.2 for Macintosh as our method of choice to construct a tree from the aligned sequences. The best model for the substitution rates was used. A total of 2,500,000 generations were run so that the convergence (split frequencies) dropped below a criterion of <0.01 (final value was: 0.009382 and potential scale reduction factor (PSRF) = 1.004) (Hall, 2008). Trees were sampled every 100 generations and with a burn-in of 25%; a total of 18,750 trees were used for the consensus tree shown in Fig. 4.

(iv) To compare the results, we additionally constructed phylogenetic trees using PAUP 4.0b10 (Swofford, D.L., 2003. PAUP. Phylogenetic Analysis using Parsimony. Version 4. Sinauer