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Interspecific geographic distribution and variation of the pathogens *Nosema bombi* and *Crithidia* species in United States bumble bee populations

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ABSTRACT

Several bumble bee (*Bombus*) species in North America have undergone range reductions and rapid declines in relative abundance. Pathogens have been suggested as causal factors, however, baseline data on pathogen distributions in a large number of bumble bee species have not been available to test this hypothesis. In a nationwide survey of the US, nearly 10,000 specimens of 36 bumble bee species collected at 284 sites were evaluated for the presence and prevalence of two known *Bombus* pathogens, the microsporidium *Nosema bombi* and trypanosomes in the genus *Crithidia*. Prevalence of *Crithidia* was ⩽10% for all host species examined but was recorded from 21% of surveyed sites. *Crithidia* was isolated from 15 of the 36 *Bombus* species screened, and were most commonly recovered from *Bombus bifarius*, *Bombus bimaculatus*, *Bombus impatiens* and *Bombus mixtus*. *Nosema bombi* was isolated from 22 of the 36 US *Bombus* species collected. Only one species with more than 50 sampled bees, *Bombus appositus*, was free of the pathogen; whereas, prevalence was highest in *Bombus occidentalis* and *Bombus pensylvanicus*, two species that are reportedly undergoing population declines in North America. A variant of a tetranucleotide repeat in the internal transcribed spacer (ITS) of the *N. bombi* rRNA gene, thus far not reported from European isolates, was isolated from ten US *Bombus* hosts, appearing in varying ratios in different host species. Given the genetic similarity of the rRNA gene of *N. bombi* sampled in Europe and North America to date, the presence of a unique isolate in US bumble could reveal one or more native North American strains and indicate that *N. bombi* is enzootic across the Holarctic Region, exhibiting some genetic isolation.

1. Introduction

Naturally occurring pathogens typically cycle at fluctuating prevalence levels in populations of their host species. At enzootic levels, they may interact with other natural enemies to suppress population numbers; when epizootics occur, one pathogen species may cause significant impacts on the viability of a host population (Anderson and May, 1981). Several pathogens are known to occur in bumble bees (*Bombus* spp.) and may play key ecological roles in the population dynamics of their hosts, which are among the most important pollinators globally in both agriculturally intense settings (Velthuis and Van Doorn, 2006) and in natural settings where they are cornerstone species in pollination networks (Memmott et al., 2004). Most of these studies have been conducted in Western Europe and have focused on the most common host species, *Bombus terrestris* L. and *Bombus lucorum* L. (Shykoff and Schmid-Hempel, 1991; Korner and Schmid-Hempel, 2005; Rutrecht and Brown, 2009). Little is known about the pathogen complex of the approximately 52 North American *Bombus* species.

Reports of locally declining bumble bee species, local extinctions and range reductions have been published in Western Europe and in North America over the past few decades (e.g. Williams, 1982; Biesmeijer et al., 2006; Fitzpatrick et al., 2007; Inoue et al., 2008; Colla and Packer, 2008). Four species have become locally extinct in 11 European countries over the last 60 years (Kosior et al., 2007). In the US, one species, *Bombus franklini* (Frison), has apparently disappeared from its native range and is recognized as endangered by the International Union for Conservation of Nature (IUCN) (Kevan, 2008). While the status of bumble bees in North America has become a major focus for scientists and conservationists, research on long-term population dynamics are lacking.

Cameron et al. (2011) recently investigated distribution changes, population genetic structure and pathogen prevalence

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in eight bumble bee species over 3 years throughout the contiguous Unites States. Comparisons of current relative abundance data with historical data from museum collections and surveys of historical distributions indicated significant reductions in the geographic ranges of multiple species over the last 20–30 years. Significant range contractions were documented in the US for *Bombus affinis* Cresson (87%), *Bombus pensylvanicus* (DeGeer) (23%), *Bombus terricola* Kirby (31%) and *Bombus occidentalis* (Greene) (28%). Reductions in genetic diversity were also recorded for those US populations reported to be declining. Additionally, Cameron et al. (2011) showed that prevalence of the microsporidium *Nosema bombi* was significantly higher in two declining species, *B. pensylvanicus* and *B. occidentalis*, than in other targeted species.

The possibility that exotic pathogens or strains, introduced through commercial transport of bumble bees for pollination of greenhouse crops, could have invaded native US bumble bee populations and played a critical role in subsequent declines has recently been suggested (Thorp and Shepherd, 2005; Colla et al., 2006; Otterstatter and Thomson, 2008). *Crithidia bombi* and the newly described *Crithidia expoeki* (Schmid-Hempel and Tognazzo, 2010) are extracellular trypanosomatid parasites that occur in the midgut lumen and rectum of bumble bees. Replication of *C. bombi* in the host is rapid but the effects on individuals are usually subtle, including reduced pollen loads carried during foraging trips (Shykoff and Schmid-Hempel, 1991), variation in foraging behavior (Otterstatter and Thomson, 2006), and increased development of ovaries in workers (Shykoff and Schmid-Hempel, 1991). Colony-level effects, however, include slower colony growth rate and reduction in colony fitness (Brown et al., 2003). The prevalence and intensity of *C. bombi* infections are lower in *B. terrestris* colonies with high genetic variation (Liersch and Schmid-Hempel, 1998), and more than one *C. bombi* genotype can occur within individual bumble bee nests, indicating strong genotypic interactions between *C. bombi* and its host (Schmid-Hempel and Reber Funk, 2004; Popp and Lattorff, 2010).

The bumble bee microsporidium *N. bombi* has also elicited recent attention. *N. bombi* is an obligate intracellular pathogen that produces systemic disease in its host (Fries et al., 2001; Larsson, 2007); the effects are generally chronic, including reduction in individual reproduction rate (Otti and Schmid-Hempel, 2007, 2008), life span (Fantham and Porter, 1914; Schmid-Hempel and Loosli, 1998; Rutrecht and Brown, 2009) and colony growth (Rutrecht and Brown, 2009). There has been a limited amount of research on susceptibility of European bumble bee species to *N. bombi* (Schmid-Hempel and Loosli, 1998; Otti and Schmid-Hempel, 2007, 2008; Rutrecht and Brown, 2009), and none for American bumble bees. One European study found significant differences in susceptibility of different host species to *N. bombi* spores harvested from *B. terrestris* (Schmid-Hempel and Loosli, 1998), but Rutrecht et al. (2007) reanalyzed these data and did not find differences.

N. bombi, *Crithidia* spp. or a combination of species might play a role in recent population fluctuations in North America, but there is little information about the distribution of these pathogens in the Nearctic region. Analysis of molecular markers provides a tool for investigating the origins of potentially invasive species, but no population genetic studies of *Crithidia* spp. or *N. bombi* have been conducted. Genetic investigations of *N. bombi* have relied on comparative sequence data for the small subunit (SSU) and the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene. In Europe, the nucleotide sequences of the *N. bombi* SSU-rRNA gene were found to be identical for isolates from three bumble bee species (Fries et al., 2001); these results were corroborated in another study of eight European bumble bee host species (Tay et al., 2005). Analyses of a small number of North American *N. bombi* samples identified no unique genetic variation in the rRNA region (Cameron et al., 2011; Kissinger et al., 2011). In Europe, however, variability in the SSU and large (LSU) rDNA subunits was reported, including a 4 bp insertion in the ITS region. All European isolates possess a short 29-bp ITS region with a tetranucleotide repeat $(GTTT)_2$, as well as a long 33-bp ITS region with an insertion of a second repeat $(GTTT)_3$ (O'Mahony et al., 2007). Six distinct short alleles and seven long alleles, characterized by different combinations of ITS repeat units and base pair substitutions, were found in the surveyed species (Tay et al., 2005; O'Mahony, 2008, unpublished report). The variants were distributed across populations and showed no sign of species specificity (Tay et al., 2005). This variation suggested that more detailed investigation of this gene region could provide information about potential genotypic variation of North American *N. bombi*.

Here we expand on data and report new data from a multi-year survey of bumble bee species in the USA (Cameron et al., 2011) to further investigate the hypothesis that *Crithidia* spp. or *N. bombi* may play a role in *Bombus* population declines. We explore variation in prevalence of *N. bombi* and *Crithidia* spp. among 36 bumble bee species, as well as molecular variation of the *N. bombi* rRNA ITS region for comparison with European isolates.

2. Methods

2.1. Bumble bee sampling

We analyzed pathogens of 36 *Bombus* species sampled from 284 sites throughout the contiguous US between 2007 and 2009 (see Cameron et al., 2011, for details of sampling and field survey protocols). For the present study, populations were designated as a group of conspecific bumble bees found foraging at a single site, each site a minimum of 2 km distant from others.

Sampling was focused to compare pathogen presence, prevalence and intensity levels among eight bumble bee species that were hypothesized to be in decline or to be stable, but also included other *Bombus* species at some sites. We attempted to collect at least 60 specimens of the target species in each site, affording a >95% chance of detecting infections at a prevalence of 5%. The target western US bumble bee species were *B. occidentalis*, *B. bifarius*, *B. mixtus* and *B. vosnesenskii*. The species targeted for study in the eastern US were *B. pensylvanicus*, *B. impatiens*, *B. bimaculatus* and *B. griseocollis*. *B. occidentalis* in the western US and *B. pensylvanicus* in the eastern US have experienced range contractions (Cameron et al., 2011); the other *Bombus* species are considered to be stable in numbers and distribution.

2.2. Pathogen screening

Screening protocols are described in Cameron et al. (2011). Briefly, western *Bombus* spp. specimens were dissected in the field or in the laboratory in Logan, UT, and the digestive tracts were shipped on dry ice to the insect pathogen laboratory at the University of Illinois. Eastern specimens were frozen whole at −80 °C and were thawed for screening in the laboratory. Midgut tissues were smeared on glass slides and examined under phase-contrast microscopy (400× magnification). Tissues that were infected with either *N. bombi* or *C. bombi* were placed in 1.5 ml cryovials containing 30% glycerol and stored in liquid nitrogen.

Tissue samples with observable microsporidia infections were evaluated to determine identity of the pathogen (morphology of mature spores and rDNA gene sequence of selected samples in each population) and intensity of infection. To determine infection intensity, we evaluated total production of mature *N. bombi* spores in a host. The entire midgut was homogenized in a tissue grinder with 100 μl of water and spores were counted using a Petroff-Hauser hemocytometer. Based on repeated spore counts of