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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

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homogenized midgut sections, we defined the levels of infection intensity as follows for 20 visual fields per gut tissue smear: *low infection level* (<2 spores per visual field = 1–1000 spores/μl); *moderate infection level* (2–20 spores/visual field = 1000–100,000 spores/μl); *high infection level* (>20 spores/visual field ⩾100,000 spores/per microliter). Because detectable *C. bombi* infections were generally of very high intensity, we did not evaluate host individuals for infection intensity.

Diagnosis of microsporidia by light microscopy is generally sufficiently accurate, but low level infections can be missed. We confirmed our prevalence data by conducting PCR detection of random populations. An average false negative diagnosis rate of 5% for low level or early stage infections suggests that calculated prevalence estimates should be considered slightly conservative.

2.3. Molecular identification

DNA was extracted from infected midgut tissues using a modified Chelex® 100 method (Walsh et al., 1991). Tissue pieces of approximately 2 mm in diameter were added to 150 μl of 5% Chelex® 100 resin and 5 μl proteinase K (20 mg/ml). The sample was incubated at 55 °C for 1 h, followed by 95 °C for 15 min to denature enzymes. Samples were centrifuged (6000 rpm) and the supernatant was stored at −20 °C.

A 573 bp region, including the ITS and flanking SSU and LSU, was amplified in *N. bombi* using the oligonucleotide primer pair 1061f (Weiss and Vossbrinck, 1998) and 228r (Vossbrinck et al., 1993). Primers used to amplify and sequence a 417 bp region of the *Crithidia* spp. 18s rRNA gene were SEF and SER (Meeus et al., 2010). PCR reactions consisted of 5 μl 5× Promega GoTaq flexi buffer, 2 μl 25 mM $MgCl_2$, 0.5 μl dNTPs (10 mM each), 0.125 μl 5U Taq polymerase (Promega), and 2.5 μl each forward and reverse primers (2.5 μM), 4 μl of sample DNA per reaction, and nucelotide-free water was used for negative controls. PCR initial denaturation was 95 °C for 45 s, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C (*N. bombi*) or 60 °C (*Crithidia* spp.) for 30 s and extension at 72 °C for 90 s. Successfully amplified PCR products were purified using the ExoSAP-IT clean-up method (Affymetrix, Inc.) and sequenced using PCR primers on an ABI 3730XL capillary sequencer at the University of Illinois Core DNA Sequencing Facility of the W.M. Keck Center for Comparative and Functional Genomics. Sequences were analyzed and aligned using the BioEdit sequence alignment editor (Hall, 1999).

The published 18s rRNA gene sequence for *C. expoeki* differs from *C. bombi*; however, the particular sequence we used is not yet known for this new species and, therefore, *Crithidia* species cannot be distinguished with our data. Hereafter, we refer to isolates of this genus as "*Crithidia*".

2.4. Polymorphism in N. bombi

Variation in *N. bombi* was initially detected by the presence of multiple peaks and clear frame shifts starting in the ITS region in DNA sequence chromatograms. We used TA cloning and sequencing to detect the presence of the multiple sequence variants within a single isolate that might explain this pattern. We selected a *B. pensylvanicus* isolate for which direct sequencing (see above) resulted in noticeable chromatogram deterioration immediately following the second GTTT repeat of the ITS region. The ITS fragment was amplified using the PCR conditions described above, using primer pair 1061f and 580r (Baker et al., 1994). The PCR product was visualized and extracted from a 1.5% agarose gel and purified using a QIAquick gel extraction kit (Qiagen). The purified product was cloned into pGem-T easy vector (Promega) following the manufacturer's protocol. The cloned vector was transferred into *E. coli* DH5α using the chemical transformation method of Chung et al. (1989). Colonies containing the recombinant vector were selected using primers, L-ITS for a larger variant and SNP-ITS for a variant with a single nucleotide polymorphism (SNP) (Table 1), designed specifically to detect the different alleles. The PCR screen was confirmed by randomly choosing one colony of each variant and sequencing the plasmid insertion. The ratio of the variants was determined by screening 70 colonies from this isolate.

To reveal the presence and the ratio of the allelic variant containing the SNP (here termed "L8") in other *N. bombi* isolates, quantitative PCR (qPCR) using the SYBR green method was conducted. We employed an approach that involved two independent reactions for each isolate. One qPCR specifically targeted the allelic variant, the other quantified total *N. bombi* DNA including all variants. We divided copy numbers of L8 by total copy numbers from the same DNA sample to obtain a ratio of alleles. Preliminary cloning results suggested that the ratio of L8 was likely to be low, making detection of the variant problematic in low spore density samples. We therefore used a nested PCR, whereby PCR products from initial amplification of the ITS region were then used as the template for qPCR. For the initial PCR, we used *pfx* DNA polymerase (Invitrogen), which has proofreading activity and reduced the possibility of enzyme errors. We used primer pair 1061f/580r for two replicates per isolate and another, 860f/228r (Table 1), for a third replicate to minimize the chance of preferably amplifying certain alleles. The second, nested PCR (qPCR) was performed using 100× diluted PCR product from the first reaction and 150 pmol of each primer. SYBR Green Master premix (ABI) was used for the qPCR. Absolute quantification was performed referencing the standard curve based on serial dilution of the plasmid with the insertion, which was constructed in the previous cloning and sequencing. The qPCR runs were conducted using standard two-step PCR reaction (40 cycles in 95 °C 30 s for denature and 64 °C 1 min for annealing and extension) on an ABI HT7900, and results were analyzed using the ABI SDS 2.2.2 Program. Mean ratios were then calculated from all replicates.

Two additional isolates received from R. Paxton were added to the qPCR analyses; one isolate was collected from *Bombus pascuorum* in Northern Ireland and another from *B. terrestris* in the Netherlands. Both 1061f/580r and 860f/228r PCR products were tested.

2.5. Statistics

We analyzed survey data for both *N. bombi* and *Crithidia* using a Generalized Linear Model analysis (GLM) for weighted binomial proportion data to assess significant differences in relative prevalence among species; to assess differences among subgenera we used a Linear Mixed Effects Model (LME) with subgenus as a fixed effect and species as a random factor, compensating for multiple comparisons by Holm–Bonferroni correction to $\alpha = 0.05$ in both analyses. To compare differences in site prevalence and intensity of infection, we used Fisher's Exact Test, correcting for multiple comparisons with the Holm–Bonferroni method. All analyses were performed using R v2.10.1 (R Development Core Team, 2009).

3. Results

3.1. Pathogen diversity

A total of 9909 bumble bees representing 36 species (518 in 2007; 5156 in 2008, and 4235 in 2009) were examined from 38 states across the contiguous United States. The most common pathogens found were the microsporidium *N. bombi* and the trypanosome *Crithidia*. Other pathogens, including nematodes, conopid fly larvae, and several morphotypes of protozoa were recovered but were not identified to species, and are not discussed.

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Table 1
Primers, oligonucleotide sequence, annealing temperatures (Ta), and expected fragment length in basepairs used for PCR and qPCR amplification of *Nosema bombi* ribosomal RNA gene.

Primer	Sequence (5′–3′)	Ta (°C)	Exp. fragment size (bp)	Comments
L-ITS-f	GTA TAA GTT TRT TTG TTT GTA TGT CAT	58	460	Primer to detect long ITS allele (L1)
SNP-ITS-f	GAT CAT AAT CAG GAA GTA TAA GTT TA	56	475	Primer to detect ITS allele with SNP (L8)
qSNP-ITSf	CAG GAT CAT AAT CAG GAA GTA TAA GTT TAT	65	85	qPCR primers to detect allele with SNP (L8)
qSNP-ITSr	CGA CCT TCA TCG TTA TGG TAT CC	66	85	
qSSUf	CGC CCG TCG CTA TCT AAG	65	122	qPCR primers for quantification of total PCR product
qSSUr	TAT GAT CCT GCT AAT GGT CTC C	65	122	
860f	GGA GTG GAT TGT GCG GCT	58	656	PCR primer complimenting primer 228r

3.2. Crithidia spp.

Crithidia isolates were recovered from 62 of 284 sites (21.8%) in 24 states. Across all sites and years, 2.7% of the bumble bees (270 specimens) were infected with the trypanosomatid. Infection rates were similar in eastern and western bees (2.4% and 3.0%, respectively), and the pathogen infected 15 of 36 collected bumble bee species (41.7%) (Table 2).

Crithidia most commonly occurred in *B. impatiens* and *B. bimaculatus* in the East, and *B. bifarius* and *B. mixtus* in the West, but also infected ten other species, including *B. pensylvanicus* and *B. occidentalis* (Table 2). The total prevalence of *Crithidia* was significantly higher in *B. mixtus* than in other western species (9.6%, $P < 0.05$, GLM), and was higher in the eastern *B. impatiens* (3.3%) than in *B. pensylvanicus* and *B. griseocollis* ($P < 0.05$, GLM). Prevalence of *Crithidia* was relatively low in most bumble bee populations, rarely more than 10% (Fig. 1). The highest prevalences were recorded in a California population of *B. mixtus* (32.0%; $N = 68$) and an Illinois population of *B. impatiens* (26.7%; $N = 45$). Some populations of *B. mixtus* from California and of *B. impatiens* from Illinois were infected with both *N. bombi* and *Crithidia*, but *B. mixtus* was the only host species in which mixed infections were

Table 2
Prevalence of *Crithidia* species, and prevalence and allelic variation of *Nosema bombi* in 36 US and two European bumble bee species.

Species	Subgenus	Distribution[1]	Total # of bees collected	*Crithidia* species	*Nosema bombi*				
				Prevalence (%)	Prevalence (%)	Alleles[2]	*N*[3]	Ratio (%)[4]	SD
B. affinis	*Bombus* s. str.	E	14	0.00	50.00	S1, L1, **L8**	4(4)	**9.66**	**8.24**
B. appositus	*Subterraneobombus*	W	70	0.00	0.00	n.a.			
B. auricomus	*Bombias*	E	63	0.00	1.59	n.a.			
B. balteatus	*Alpinobombus*	W	18	0.00	0.00	n.a.			
B. bifarius	*Pyrobombus*	W	2096	2.72	0.57	S1, L1	1(1)	**0.44**	–
B. bimaculatus	*Pyrobombus*	E	1070	2.90	0.28	n.a.			
B. borealis	*Subterraneobombus*	E	10	0.00	0.00	n.a.			
B. californicus	*Thoracobombus*	W	49	0.00	4.08	n.a.			
B. caliginosus	*Pyrobombus*	W	5	0.00	0.00	n.a.			
B. centralis	*Pyrobombus*	E, W	183	0.55	0.55	n.a.			
B. citrinus	*Psithyrus*	E	1	0.00	0.00	n.a.			
B. fernaldae	*Psithyrus*	W	27	0.00	3.70	n.a.			
B. fervidus	*Thoracobombus*	E, W	55	1.82	16.36	S1, **L8**	1(1)	**6.53**	–
B. flavifrons	*Pyrobombus*	E, W	119	1.68	0.84	n.a.			
B. fraternus	*Cullumanobombus*	E	4	0.00	0.00	n.a.			
B. frigidus	*Pyrobombus*	W	12	25.00	8.33	n.a.			
B. griseocollis	*Cullumanobombus*	E, W	476	0.00	0.42	S1, **L8**	1(1)	**4.26**	–
B. huntii	*Pyrobombus*	W	142	0.70	0.70	n.a.			
B. impatiens	*Pyrobombus*	E	2864	3.32	0.73	S1,**L8**	3(3)	**2.35**	**3.75**
B. insularis	*Psithyrus*	E, W	83	0.00	3.61	n.a.			
B. melanopygus	*Pyrobombus*	W	70	7.14	4.29	n.a.			
B. mixtus	*Pyrobombus*	W	468	9.62	11.75	S1, L1, **L8**	6(3)	**2.04**	**2.48**
B. morrisoni	*Cullumanobombus*	W	1	0.00	0.00	n.a.			
B. nevadensis	*Bombias*	E, W	48	0.00	0.00	n.a.			
B. occidentalis	*Bombus* s. str.	W	172	2.33	37.21	S1, L1	8(5)	**0.56**	**0.64**
B. pensylvanicus	*Thoracobombus*	E	545	0.18	15.23	S1, **L8**	15(10)	**17.7**	**11.18**
B. perplexus	*Pyrobombus*	E	2	0.00	50.00	n.a.			
B. rufocinctus	*Cullumanobombus*	E, W	130	0.00	0.77	n.a.			
B. sitkensis	*Pyrobombus*	W	51	0.00	1.96	n.a.			
B. suckleyi	*Psithyrus*	W	4	0.00	25.00	n.a.			
B. sylvicola	*Pyrobombus*	W	43	2.33	0.00	n.a.			
B. ternarius	*Pyrobombus*	E	15	20.00	0.00	n.a.			
B. terricola	*Bombus* s. str.	E	32	0.00	9.38	S1, L1	1(1)	**0.7**	–
B. vagans	*Pyrobombus*	E	36	0.00	0.00	n.a.			
B. vandykei	*Pyrobombus*	W	28	0.00	0.00	n.a.			
B. vosnesenskii	*Pyrobombus*	W	903	2.21	1.33	S1, **L8**	2(2)	**0.39**	**0.48**
B. pascuorum	*Thoracobombus*	Europe	n.a.	n.a.	n.a.	S1, L1	1(1)	**0.00**	–
B. terrestris	*Bombus* s. str.	Europe	n.a.	n.a.	n.a.	S1, L1	1(1)	**0.00**	–

[1] Species collected to the east (E) and/or west (W) of 104th longitude W.
[2] Types of alleles found through direct sequencing analysis.
[3] Total number of isolates evaluated (in parentheses: number of isolates evaluated through qPCR).
[4] Mean values from all isolates for the ratio of L8 to S1, calculated from qPCR analysis.

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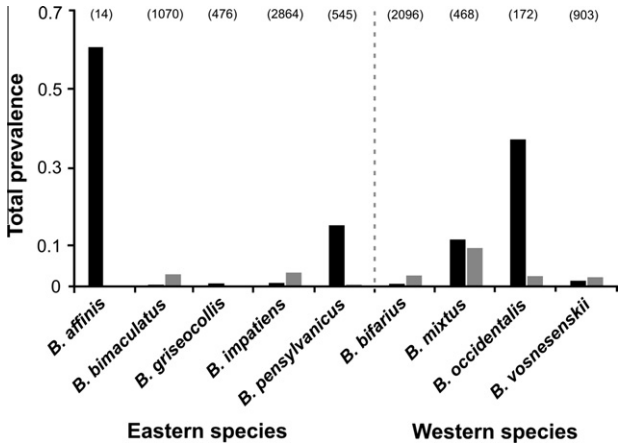

Fig. 1. Prevalence of *Nosema bombi* (black bars) and *Crithidia* (gray bars) in US bumble bee (*Bombus*) species. Numbers in parentheses above columns indicate total sample size.

observed in individual bees. All infected specimens belonged to the subgenera *Bombus* sensu stricto, *Pyrobombus*, and *Thoracobombus*. *Crithidia* were not recovered from species in subgenera *Bombias*, *Cullumanobombus*, *Psithyrus* and *Subterraneobombus*. Differences in infection among subgenera were not significant ($P > 0.05$, LME). Sequencing of random infected samples from each population confirmed identity with a published partial SSU region of *C. bombi* (Meeus et al., 2010).

3.3. N. bombi

N. bombi was geographically widespread and occurred in 22 of 36 collected bumble bee species (61.1%, Table 2) at 86 sites (30.3%) in 26 states across the US (Fig. 2).

Across all sites and years, *N. bombi* was recovered from 2.9% of surveyed bumble bees (2.1% in eastern species; 3.4% in western species), with 289 infections observed. The highest overall prevalence was recorded from *B. pensylvanicus* in the East (83 of 545, 15.2%), and *B. occidentalis* in the West (64 of 172, 37.2%) (Cameron et al., 2011). Significantly fewer infections were recorded for the other target species ($P < 0.001$, GLM). Although significantly fewer infections were recorded for *B. mixtus* than for *B. occidentalis*, significantly more infections were observed in *B. mixtus* than in other abundant western species, *B. bifarius* and *B. vosnesenskii* ($P < 0.001$, GLM) (Fig. 1).

More specimens of the subgenus *Bombus* sensu stricto were infected than those of the subgenera *Cullumanobombus* or *Pyrobombus* (33.9%, $P < 0.05$, LME); none of the other subgenera differed significantly from each other.

Of the bumble bee species that were collected at $\geqslant$20 sites, *N. bombi* was present in *B. pensylvanicus*, *B. occidentalis* and *B. vosnesenskii* individuals at 45.3%, 46.1% and 35.7% of sites, respectively (Fig. 3). *Bombus mixtus* was infected at seven of 37 sites; *B. affinis* was infected at four of five sites. *N. bombi* was not frequently detected in the two most common species, *B. impatiens* (10 of 131 sites) and *B. bifarius* (7 of 88 sites), nor did it commonly occur in *B. bimaculatus* (3 of 95 sites) or *B. griseocollis* populations (2 of 43 sites). *N. bombi* occurred in *B. pensylvanicus* at significantly more sites than for *B. impatiens* and *B. bimaculatus* ($P < 0.001$, Fisher's Exact Test); the number of sites with *N. bombi*-infected *B. occidentalis* in the West was not significantly higher than for *B. vosnesenskii* ($P = 0.45$, Fisher's Exact Test). *N. bombi* occurred significantly less often in *B. bifarius* populations than in *B. occidentalis* or *B. vosnesenskii* ($P < 0.01$, Fisher's Exact Test) (Fig. 3).

The intensity of *N. bombi* infections ranged from less than 1000 spores/μl of tissue suspension to 867,500 spores/μl. High intensity infections occurred more often in *B. pensylvanicus* (38.6%) than in *B. impatiens* (4.8%, $P < 0.01$, Fisher's Exact Test), and more often in *B. occidentalis* (64.1%) than in *B. mixtus* (18.2%, $P < 0.001$, Fisher's Exact Test). With the exception of *B. vosnesenskii*, in which a third of all infections (4 of 12) were of high intensity, all other target species had less than 20% high intensity infections, with none detected in *B. bimaculatus* and *B. griseocollis* (Fig. 3).

3.4. Morphological and genetic variation in N. bombi

The mature infective spores of *N. bombi* are slightly elongate oval. Frequent observation of empty spores in the tissue smears indicated intracellular germination (Solter and Maddox, 1998; Cali and Takvorian, 1999). Under light microscopy (400×), mature spores are identical in shape to those found in European bees (Fries

Fig. 2. Distribution of *Nosema bombi* in *Bombus* species of the continental United States. Total number of infections recorded for populations of (a) *B. occidentalis*, (b) *B. pensylvanicus*, (c) *B. bifarius*, (d) *B. bimaculatus*, (e) *B. vosnesenskii*, (f) *B. impatiens*, (g) *B. mixtus*, and (h) *B. griseocollis*.

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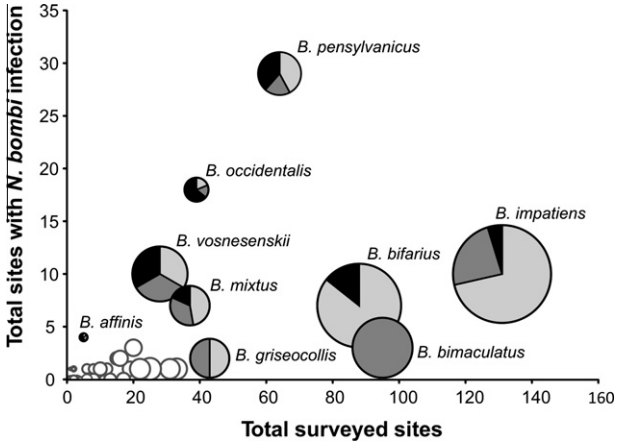

Fig. 3. *Nosema bombi* prevalence at surveyed sites for all collected host species. Circle size indicates relative total number of individuals collected per species. Pie chart elements indicate the proportion of infection intensity for all target species and *B. affinis*; light grey = low level infection, medium grey = medium level infection, dark grey = high level infection. White circles in bottom left hand corner represent bumble bee species with <150 individuals collected.

et al., 2001). In *B. mixtus*, however, spores appeared to be more elongate, and in *B. vosnesenskii* the spores were slightly reniform. These variations were observed in several but not all infected individuals of these hosts.

Ribosomal RNA sequences were identical to those of *N. bombi* isolates from Europe (Tay et al., 2005); however, closer inspection of deteriorations in sequence quality for some host species, beginning immediately after the GTTT tetranucleotide repeats, suggested the possibility of some within-host sequence variants. Cloning and sequencing revealed a second allele (L1), identical to the first but with an additional GTTT repeat, in *B. affinis*, *B. occidentalis*, *B. terricola* and *B. mixtus*. This allele has previously been reported in Europe (Tay et al., 2005). A third, novel allele containing a single nucleotide polymorphism (a G-to-A transition in the second tetranucleotide) and named "L8" to conform to the nomenclature used for European isolates (Tay et al., 2005), also appeared in a number of *Bombus* species (Table 2).

The ratio of long alleles, $(GTTT)_3$, to short, $(GTTT)_2$, was low. We used qPCR to calculate ratios of >0.03% for the L8 allele (GTTTATTTGTTT) for nearly all specimens that were inspected in this analysis (Table 2). Ratios of the L8 allele varied from 0.03% in one *B. impatiens* and 0.09% in one *B. occidentalis* to 33.9% in one *B. pensylvanicus* individual. The L8 allele was not found in the two European samples. Sequence analysis and qPCR produced similar results, indicating that the variant was present at ratios of at least 1% in *B. affinis*, *B. fervidus*, *B. griseocollis*, *B. impatiens*, *B. mixtus* and *B. pensylvanicus* (Table 2). The variant was present in *B. bifarius*, *B. occidentalis*, *B. terricola*, and *B. vosnesenskii* at levels between 0.03% and 1.0%, however, only one *B. terricola* individual and one *B. bifarius* individual was sequenced. The sequence of the L8 allele is available in GenBank, accession number JN624385.

We found more than two variants for some host species using direct sequencing. While some *B. affinis* isolates appeared to have S1 and L1 variants, others had S1 and L8. Some specimens of *B. mixtus* appeared to contain only S1 variants, while in others we also found L1.

4. Discussion

In this broad nationwide survey of pathogens from 36 species of US bumble bees, we have shown that the microsporidium *N. bombi* and one or more trypanosome species in the genus *Crithidia* commonly occur in US bumble bee populations and were present in approximately half of the species we evaluated. Because half the species were collected in low numbers (<60 individuals) it is possible that both pathogens occur in additional, less-well sampled species but were not observed in this study. For the eight target species (*B. bimaculatus*, *B. griseocollis*, *B. impatiens*, and *B. pensylvanicus* in the East, and *B. bifarius*, *B. mixtus*, *B. occidentalis*, and *B. vosnesenskii* in the West), however, we sampled over 8000 specimens throughout their respective ranges in the contiguous United States providing a detailed look at the prevalence and distribution of pathogens in these pollinators.

Crithidia was most commonly present in bumble bee species that have apparently stable populations (*B. impatiens* and *B. bimaculatus* in the East, *B. bifarius* and *B. mixtus* in the West) as recently reported in a less extensive survey (Kissinger et al., 2011). Unlike in Switzerland, where 10–35% of bumble bees were reported to be infected with *C. bombi* (Shykoff and Schmid-Hempel, 1991; Korner and Schmid-Hempel, 2005), a prevalence of less than 3% was observed in the declining *B. occidentalis* and *B. pensylvanicus* and in less than 3% of all *Bombus* species overall. Similar to the Switzerland studies, *Crithidia* was most commonly observed in the subgenera *Bombus* sensu stricto and *Pyrobombus* (Shykoff and Schmid-Hempel, 1991; Korner and Schmid-Hempel, 2005).

C. bombi was recently implicated as a causal factor in bumble bee decline and was the focal species for the hypothesis of pathogen spillover from greenhouses into native populations in eastern Canada (Colla et al., 2006; Otterstatter and Thomson, 2008). We found no evidence to suggest that *Crithidia* infections are involved in species decline in the US *Crithidia* was widespread in stable bumble bee populations throughout the US and at low prevalence in declining species, but some of the sites with highest prevalence, two with 18% and 24%, for example, were at least 20 km from the nearest greenhouses, a much greater distance than the typical foraging range of most bumble bees (Osborne et al., 2008). Whether spillover is occurring or has occurred cannot be ascertained from this survey, but it does not appear that *Crithidia* presence in US bumble bees is the result of invasion of exotic species.

We isolated *N. bombi* from nearly two thirds of the *Bombus* species we evaluated. *N. bombi* is geographically widespread in US bumble bee populations, even though the majority of the species with no record of microsporidian infection were sampled at a low rate (<100 individuals). Relatively high *N. bombi* prevalence in two declining species was previously reported for US bumble bees (Cameron et al., 2011). We show that *N. bombi* infections in these species are found at significantly more sites than for other species, and that higher intensity infections are produced in *B. occidentalis* and *B. pensylvanicus* than in other species. The overall distribution and prevalence of *N. bombi* in all surveyed US bumble bee species corroborates those of previous, more localized, surveys where *N. bombi* was found at low levels in some common species (Sokolova et al., 2010; Kissinger et al., 2011), although our values are somewhat lower than those reported (~15%) in a recent study of several species in Massachusetts (Gillespie, 2010).

This larger study allows us to make some general comparisons about infection of *N. bombi* across *Bombus* subgenera. Three *Bombus* sensu stricto species show evidence of recent declines, and all have relatively high infection prevalence, although two of the species, *B. affinis* and *B. terricola*, were poorly sampled compared to the third member *B. occidentalis*. Likewise, *Thoracobombus* species have shown evidence of recent population declines, most notably *B. pensylvanicus* (Cameron et al., 2011). *B. pensylvanicus* had the second highest *N. bombi* prevalence in this study of species, with >50 samples. Prevalence in two of its less well-studied relatives, *B. fervidus* and *B. californicus*, was also high, suggesting that *Thoracobombus* other than *B. pensylvanicus* deserve closer consideration by researchers. *Pyrobombus*, the dominant subgenus

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surveyed in this study (17 species, including *B. impatiens*, *B. bimaculatus*, *B. bifarius*, and *B. vosnesenskii*) exhibited low infection levels as a group. Although the overall prevalence of *N. bombi* in *B. mixtus* (*Pyrobombus*), a species that appears to be stable, was also relatively high, >10%, the prevalence in two collections (34% in 2007 and 43% in 2008) of one population in California constituted most of the infections recorded. The overall prevalence in *B. mixtus* in all other sites combined was 2.6%, illustrating the variation in pathogen density in a single host species at different sites and time periods. This site-specific variation in prevalence is similar to that reported for European bumble bees (Paxton, 2008, unpublished report), and we also note that one European study reported that species of the subgenera *Bombus* sensu stricto, *Megabombus* and *Thoracobombus* appeared to have a slightly elevated prevalence of *N. bombi* (Shykoff and Schmid-Hempel, 1991).

Variation in *N. bombi* infection was also found at the genetic level with specific allelic differences in the ITS region between isolates from Europe and the US, and at differing ratios among US bumble bee species. The L8 allele, a variant of the common L1 allele that contains a SNP at the second GTTT tetranucleotide, was found at ratios above 8.7% in all inspected *B. pensylvanicus* isolates, but its presence in *B. occidentalis* was always below 1.7%. It is possible that the very low ratios in *B. occidentalis* and three other species were the result of an enzyme error, but multiple repeats confirmed the presence and levels of the allele. High standard errors may be related to the occurrence of alleles at the limit of detection. Sample sizes were not sufficient to determine whether differences in allelic ratios among the isolates indicate the presence of distinct *N. bombi* strains. In addition, the rRNA gene is not a reliable population genetic marker. There are multiple, possibly different copies of the rRNA gene in the microsporidian genome (Brugere et al., 2000) and different variants appear even within a single spore (O'Mahony et al., 2007). While the shorter S1 allele that is commonly found in European *N. bombi* isolates was also isolated from all host species in the US, the L8 allele appears to be unique to North American isolates. In Europe, different alleles did not appear to be associated with particular host species or subgenera (Tay et al., 2005), nor are they in the US with the possible exception of undetected L8 alleles in *B. occidentalis*. Should the L8 allele not exist in European isolates, its presence in US bumble bees could suggest that a distinct strain of *N. bombi* occurs in the US and that allelic variations among the different isolates may be the result of host-pathogen coevolution on the continent. A far more comprehensive study would be required to determine the range of variation of *N. bombi* across Europe and the US and whether differences between the isolates represent a phylogenetic association.

N. bombi was reported infecting bumble bees in Eastern Canada as early as the 1940s (Fantham et al., 1941; Macfarlane, 1974), so any recent introductions most likely would be in addition to natural occurrence of the pathogen. We found no differences, with the exception of the L8 allele, in the SSU-rRNA and ITS gene sequences between the *N. bombi* isolates and samples isolated from the European *B. terrestris*. Because *B. occidentalis* and *B. impatiens* were reared in laboratories with *B. terrestris* in Europe (Flanders et al., 2003) and then transported to California where *B. occidentalis* was reared for commercial use (Thorp, 2008), the potential exists that a European strain of *N. bombi* was released in the US and may have impacted *B. occidentalis* populations. This study, however, did not provide the necessary data to explore this hypothesis.

We know little about the role of pathogens in North American bumble bee populations, including that of viruses, which we did not survey. The data we present are derived from collections made over a period of 3 years and, although this was an extensive undertaking, they are inadequate to fully understand the role of pathogens in *Bombus* spp. population dynamics. We have not yet determined the differential infectivity or virulence of the pathogens to individual *Bombus* species, and a higher prevalence level of a pathogen in a particular host does not imply that a host is more affected by the pathogen or that an epizootic is occurring. Rather it could indicate that a species has higher tolerance for a particular pathogen. In addition, it is possible that high prevalence could be the consequence rather than a cause of a population decline (Whitehorn et al., 2011).

The widespread distribution of *Crithidia* in United States *Bombus* populations and low enzootic prevalence in several *Bombus* species suggest a stable interaction between the pathogen and its hosts. *N. bombi* is also a widespread pathogen in US bumble bee populations and the prevalence varies extensively between host populations and among host species. The correlation between *N. bombi* and declining host species may imply that the pathogen is involved in recent population changes of particularly susceptible species, but until differences in host susceptibility and pathogen strains, as well as other host-pathogen interactions are investigated for North American hosts, the role of *N. bombi* in bumble bee population dynamics cannot be determined. The discovery of a possible new allele in *N. bombi*, however, suggests that there may be unique naturally occurring isolates in North America and that the role of this pathogen in bumble bee population dynamics may differ between species and subgenera.

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