

Test of the invasive pathogen hypothesis of bumble bee decline in North America

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Emergent fungal diseases are critical factors in global biodiversity declines. The fungal pathogen *Nosema bombi* was recently found to be widespread in declining species of North American bumble bees (*Bombus*), with circumstantial evidence suggesting an exotic introduction from Europe. This interpretation has been hampered by a lack of knowledge of global genetic variation, geographic origin, and changing prevalence patterns of *N. bombi* in declining North American populations. Thus, the temporal and spatial emergence of *N. bombi* and its potential role in bumble bee decline remain speculative. We analyze *Nosema* prevalence and genetic variation in the United States and Europe from 1980, before an alleged introduction in the early 1990s, to 2011, extracting *Nosema* DNA from *Bombus* natural history collection specimens from across this time period. *Nosema bombi* prevalence increased significantly from low detectable frequency in the 1980s to significantly higher frequency in the mid- to late-1990s, corresponding to a period of reported massive infectious outbreak of *N. bombi* in commercial bumble bee rearing stocks in North America. Despite the increased frequency, we find no conclusive evidence of an exotic *N. bombi* origin based on genetic analysis of global *Nosema* populations; the widespread *Nosema* strain found currently in declining United States bumble bees was present in the United States before commercial colony trade. Notably, the US *N. bombi* is not detectably different from that found predominantly throughout Western Europe, with both regions characterized by low genetic diversity compared with high levels of diversity found in Asia, where commercial bee breeding activities are low or nonexistent.

Bombus | Microsporidia | *Nosema bombi* | pollinator | conservation

There is growing concern that emerging infectious diseases in wild animals pose increasing risks to biodiversity and ecosystem services (1). Although quantitative data are accumulating on the deteriorating status of bumble bee (*Bombus*) pollinator populations throughout North America (2–7), the factors causing species decline remain uncertain and controversial. In the United States, shrinking or disappearing populations have been ascribed principally to an invasive virulent strain of fungal pathogen, *Nosema bombi* (Microsporidia), hypothesized to have been introduced from Europe in the early 1990s via commercial development of bumble bee colonies for pollination [“European *Nosema* invasion hypothesis” (ENIH)] (2, 4, 8, 9). However, this important hypothesis has remained largely untested, despite a highly publicized report documenting significant positive correlations between decline status of bumble bees in the United States and *N. bombi* prevalence (4). Because *Nosema* diminishes bumble bee colony fitness by reducing reproductive performance of sexuals (males and gynes) and lowering the survival rate of workers (e.g., ref. 10), there are compelling reasons to investigate exotic pathogen release as a causal factor in North American bumble bee decline.

The ENIH was based on circumstantial evidence from multiple associated observations. First, large-scale commercial bumble bee pollination of crops began in Europe in the late 1980s, using Europe’s native *Bombus terrestris*. From 1992 through 1994, with the goal of expanding US markets, queens of *Bombus occidentalis* and *Bombus impatiens* were exported to Europe for colony rearing, grown in facilities used for rearing *B. terrestris*.

Subsequently, these colonies were imported back into the United States for use in open-field and greenhouse pollination (11). Around this time, commercial colony production by other companies began in eastern Canada (1990), using wild queens of *B. impatiens*, and in California (1992) using *B. occidentalis*. However, *B. occidentalis* was abandoned by both major producers in North America shortly after 1997 because of infestation of the rearing stock with *N. bombi* (11), and soon afterward, wild *B. occidentalis* populations began to decline precipitously (2) along with this species’ close western US relative *Bombus franklini* (2). Both species belong to the subgenus *Bombus sensu stricto*, which includes *B. terrestris*. Parallel declines were soon detected in the two eastern species of *Bombus s. s.*, *Bombus affinis* and *Bombus terricola* (3, 12, 13). These declines were confirmed by more recent surveys, which also showed steep population declines among eastern *Bombus s. s.* species and members of a second subgenus (*Thoracobombus*), including *Bombus pennsylvanicus* and *Bombus fervidus* (4–6, 13, 14). Finally, these declines were occurring in the absence of a general decline among all bumble bee species, suggesting a targeted cause rather than more general environmental causes (8). Indeed, of the species examined to date, the declining North American bumble bees exhibit significantly elevated *N. bombi*

Significance

Wild bumble bees are experiencing population declines globally. Causes of declines in North American populations are unclear, although declining species are more frequently infected by the pathogen *Nosema bombi*. A widely accepted hypothesis suggests that contact with European species during domestication led to the introduction of exotic *N. bombi*. By screening museum specimens, we show that *N. bombi* prevalence increased significantly in declining species in the early to mid-1990s, coincident with *N. bombi* outbreaks in North American commercial stocks. There is no evidence that exotic *Nosema* strains were introduced from Europe. Regardless of geographic origins, the temporal connection between *N. bombi* epizootics in commercial *Bombus* stocks and increases in wild populations suggests a substantial risk of pathogen transmission with domestication.

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Data deposition: Nine Roche 454 libraries sequenced for locus discovery have been deposited in the Sequence Read Archive (SRA) database (BioProject, www.ncbi.nlm.nih.gov/bioproject, accession no. PRJNA289884). Sequences and alignments for rRNA and genomic loci and additional data information tables have been deposited in the Dryad Digital Repository (dx.doi.org/10.5061/dryad.83fb8).

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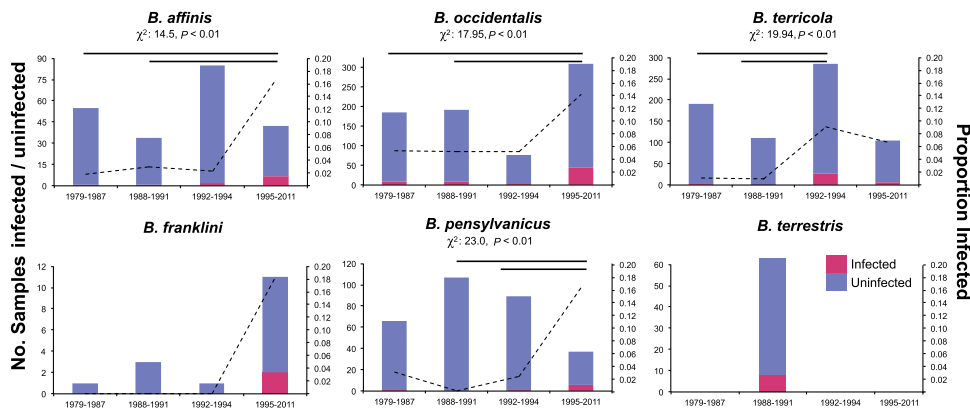


Fig. 1. Number of specimens screened and prevalence level per time period of *Nosema* for five target North American bumble bee species and the European *B. terrestris* (pink, infected bees; blue, uninfected bees; left y axis and bars, number of bees; right y axis and dashed lines, proportion out of the total screened). For species with sufficient sample sizes, results of χ^2 tests of independence of proportions are shown above each bar graph. Horizontal lines indicate pairs of time periods in which *Nosema* prevalence is significantly different at $P = 0.05$ based on arcsine-transformed prevalence data.

prevalence compared with more stable codistributed species (4). Collectively, these observations are consistent with the hypothesis that *N. bombi* jumped from the European commercial *B. terrestris*—during a 3-y period of export of North American queens to Europe and import of colonies reared from those queens—and was subsequently transmitted into wild bumble bee populations in North America. However, the observations to date do not provide quantitative data to test the *Nosema* invasion hypothesis.

An analysis of the ENIH would require genetic comparisons of *Nosema* strains from bumble bees collected before and subsequent to commercial introduction in 1992. Data on genetic variation in *Nosema* from both the native range and the invasive range can be used to test whether an invasion event actually occurred. Furthermore, evaluating *Nosema* frequencies in host populations over time, before and subsequent to the population declines, could reveal whether *N. bombi* prevalence has increased in declining populations in association with commercial production for pollination. To reveal the presence/absence of *Nosema* before and after the hypothesized early-1990s invasion, we use a technique to extract DNA nondestructively from museum specimens of *Bombus* species that currently exhibit moderate to high *N. bombi* prevalence in wild populations (*SI Methods*). We show that *N. bombi* was present in the United States historically but increased in incidence concomitantly with commercial *Bombus* trade. We also show that US and European *Nosema* are highly genetically similar relative to the genetically diverse *Nosema* found in Asian *Bombus*.

Results

Temporal Patterns of *Nosema* Prevalence. To examine changing patterns of *Nosema* prevalence over time in five declining North American bumble bee species (*B. affinis*, *B. franklini*, *B. occidentalis*, *B. terricola*, and *B. pensylvanicus*) (4), we used PCR to detect *Nosema* in pinned museum specimens collected from 1979 to 2011 (details are provided in *SI Methods*). We focus on these declining species because surveys of contemporary populations show moderate to high infection prevalence (e.g., ~15–37%), whereas populations of nondeclining North American species exhibit negligible infection rates [e.g., ~0.3–1% (4)] and are not of concern in our investigation. *Nosema* detection techniques were worked out initially by successful PCR screens of pinned historical European *B. terrestris* collected from populations with high known *N. bombi* prevalence (collected 1988 to 1991; $n = 63$; Table S1). Following successful retrieval and PCR detection of *N. bombi* DNA from dry European specimens, we screened historical collections of the five declining North American species (total samples: 2,048; Table S1), examining at least 216 individuals per species (mean: 492.3), except *B. franklini* ($N = 16$), which was difficult to obtain in large numbers because of its recent precipitous collapse (2), historically small geographic

range (2), and rarity in collections and is thus excluded from most statistical analyses (Fig. 1).

Comparisons of *Nosema* prevalence in bumble bees sampled over >30 y revealed nonrandom changes over time. In all examined North American *Bombus* species with substantial sample sizes, *Nosema* was detected before 1992 (Figs. 1 and 2 and Fig. S1), but significant increases in prevalence occurred after 1992 (Figs. 1 and 2, *SI Methods*, and Fig. S1). Change-point analysis indicates shifts in year-prevalence regression slopes between 1990 and 1995 (Fig. 2). All species show a positive trend in prevalence over time after 1992, except *B. terricola*, which shows a prevalence peak about 1992, followed by stability thereafter. In *B. franklini*, which was listed as severely threatened in 2003 (15), *Nosema* was detected only during the period of 1995 to 2011. The high *Nosema* levels amplified from the more recently collected *Bombus* samples are unlikely the result of greater sensitivity of PCR detection in younger samples based in part on the fact that all control *B. terrestris* samples exhibited constant prevalence over time—the 20- to 30-y-old historical samples exhibited similar detection frequencies (12.7%, $n = 63$) to those of freshly collected samples (this study, 18.4%; $n = 440$) (binomial test, $P = 0.15$). Furthermore, no temporally correlated amplification success was found for the nuclear long-wavelength opsin (*LWRh*) gene in *Bombus* samples collected between 1982 and 2004 ($n = 47$ bees; generalized linear model, binomial errors, null deviance vs. residual deviance: $\chi^2_1 = 0.0558$, $P = 0.8133$; Fig. S2). A less-stringent *N. bombi* PCR detection threshold (at least one-third of samples had positive PCR) produced qualitatively comparable results (Fig. S3 and Table S2).

Global Pattern of *Nosema* Diversity. To obtain a global perspective of *Nosema* diversity in bumble bees, we used pyrosequencing and Sanger sequencing to obtain a 298-bp portion of the small subunit (SSU) rRNA of *Nosema* from our North American samples ($n = 35$ modern and 7 historical samples; 16 species) and three European bumble bee species ($N = 10$ bees) (*SI Methods* and Table S3). We combined these data with those from 113 corresponding SSU rRNA GenBank sequences of Asian taxa (27 species sampled in China) and nonredundant European and North American taxa (Table S3). Filtered splits phylogenetic network analysis produced 11 *Nosema* SSU clades (Fig. 3 and Fig. S4), but the majority (97.1%, $n = 34$) of both the historical and modern North American *Nosema* isolates fell into a single clade (*N. bombi* s. s.), along with 91% of European isolates ($n = 22$) (Fig. 3). Only two North American samples, both from nondeclining *Bombus* (*Pyrobombus*) species (4), produced distinct haplotypes (Table S3 and Fig. S4; *B. impatiens*, clade 147; *Bombus mixtus*, clade 27), and only 2 of 22 European samples (*Bombus lapidarius* and *Bombus lucorum*) yielded *Nosema* sequences that fell outside the *N. bombi* clade (Fig. 3, Mixed hosts/*Nosema* “A” & “B”).

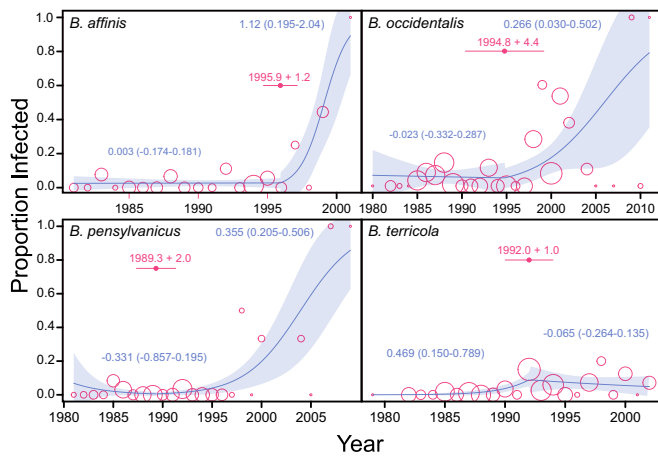


Fig. 2. Yearly proportion of four declining bumble bee species infected with *Nosema* (open circles). The size of each circle is proportional on a logarithmic scale to the number of bees screened in a given year. Yearly infection rate data are fitted with a piecewise quasibinomial regression curve and its 95% CI (shaded area). For each species, the change point in time (i.e., year) with SE is indicated by a filled circle, with an error bar on either side. Numbers beside curves are estimated slope parameters for each segment of the piecewise regression (95% slope CI in parentheses). For *B. affinis*, *B. pensylvanicus*, and *B. occidentalis*, *Nosema* prevalence did not vary with time before the estimated change points (i.e., 95% CI of slope parameters include 0), but the prevalence–time relationship became significantly positive (with 95% CI of slope parameters excluding 0) after the change points.

This “mixed-hosts” clade comprises *Nosema* from highly diverse hosts [western honey bee *Apis mellifera*, bumble bees, and several moth species (Table S3)] and appears closely related to *Nosema ceranae*, commonly found in honey bees. All *Nosema* from historical North American specimens fell into the *N. bombi* clade, except for sequences from a single *B. terricola*, which produced a highly distinct sequence (clade 1) more closely related to isolates from the mixed-hosts and *N. ceranae* clades (Fig. 3 and Fig. S4). In contrast to the extremely low *Nosema* diversity found in North American

and European samples, *Nosema* from Chinese *Bombus* is highly diverse, falling into six clades distributed across the network (16).

Genetic Differentiation Between *Nosema bombi* from Europe and North America. To probe more deeply into possible genetic differentiation between European and North American *N. bombi*, we conducted deep amplicon pyrosequencing of 31 *N. bombi* isolates (SI Methods and Table S4) using six previously unidentified genomic markers [246.3 ± 96.4 bp per locus (±SD); >1,000 reads per locus (SI Methods)]. The small number of discovered putative polymorphisms out of a preliminary screen of 287 alignments from reduced representation sequencing of nine *N. bombi* isolates (SI Methods and Table S4) suggests low variation across the genome. Despite high sequence coverage of polymorphic loci (>1,000 reads per locus), aimed at saturating detection of variation within individual bees, the overall level of *N. bombi* genetic variation was low for both North American and European isolates (average per locus heterozygosity, 0–0.456) and similar between North American and European samples (locus-specific allelic heterozygosity: Skillings–Mack statistic, 127.67; $P = 0.109$; 1,000 simulations). Hierarchical Bayesian analysis of molecular variance (17) revealed no significant differentiation between *N. bombi* from Europe and North American [$\Phi_{CT} = 0.00$; 95% confidence interval (CI), –0.01 to 0.01] but showed that most existing variation occurs among isolates within each region ($\Phi_{ST} = 0.26$; 95% CI, 0.19–0.46) (SI Methods and Fig. S5). The lack of differentiation among isolates between continents is supported by the clustering of European *N. bombi* with the majority of North American *N. bombi* on a neighbor-joining tree constructed from the genomic allele frequency data (Fig. 4, D_A distances).

Discussion

Significantly higher *N. bombi* prevalence in US *Bombus* species undergoing population declines relative to healthy species has been documented nationwide (4) and at local and regional levels (2, 3, 5–7, 12, 13). However, resolving the essential question of cause and effect has proven elusive: Did *N. bombi* infection actually cause the precipitous declines in *Bombus* populations, or is *N. bombi* naturally more prevalent in species experiencing decline from other causes? Our examination of *N. bombi* prevalence over a 30-y time span suggests that prevalence has not been continuously high in declining

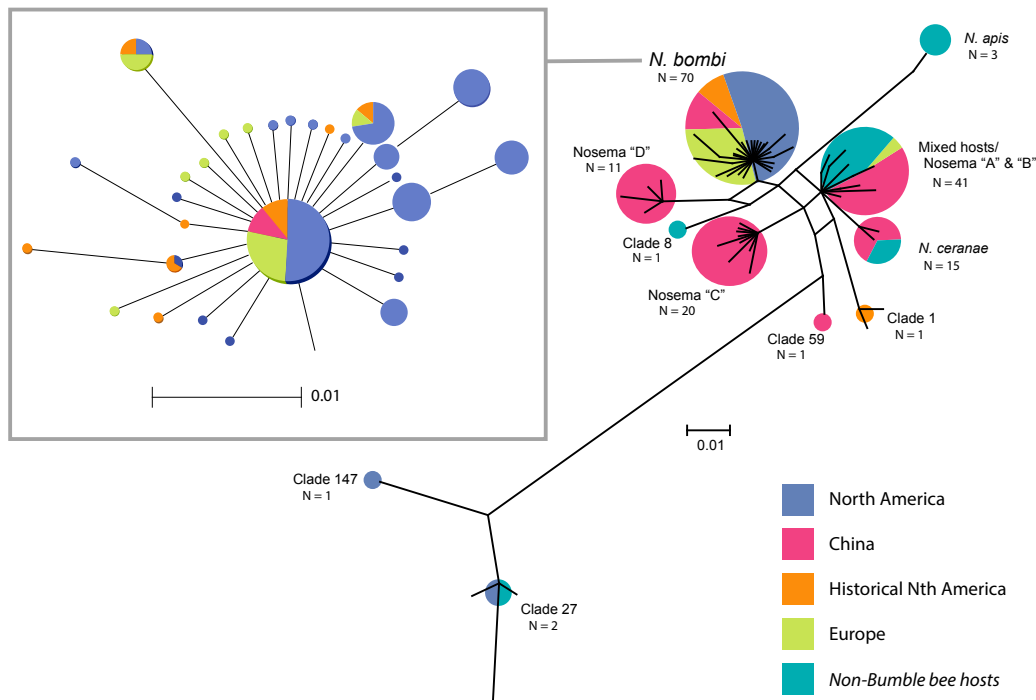


Fig. 3. Filtered splits phylogenetic network showing relationships among *Nosema* clades. Clades may contain members that are attributable to known species (e.g., *N. apis*) that have been previously named in the literature (e.g., *Nosema* “D”) or newly delineated (e.g., clade 1). For each clade, the number of host individuals (represented both numerically and by size of circle at the tips) and their geographic origins are also shown. Clade memberships can be found in Table S3.

degradation; however, our positive *B. terrestris* and *LW Rh* controls suggest the patterns are robust.

The last decade has seen rising acceptance of the view that bumble bee decline in North America is the result of exposure to an exotic European *N. bombi* strain introduced into native North American populations from European-bred colonies in the 1990s (2, 9). Despite the temporal increase we found in *Nosema* prevalence in declining species, there is no evidence that a distinct strain of *N. bombi* was introduced from Europe in the 1990s. The same SSU genotype currently found in host populations throughout Europe and North America was in the United States long before any known commercial trade began between Europe and North America. Moreover, the low, near identical *Nosema* genetic diversity found throughout North America and Europe precludes the introduction of a distinct *N. bombi* strain. Both historical and current North American isolates are essentially undifferentiated from those in Europe. The low SSU diversity is in stark contrast to the extensive *Nosema* diversity found in Chinese bumble bees (16). Although not all of the Chinese *Nosema* sequences necessarily represent actual infections, the greater diversity of *Nosema* SSU sequences detected in Asia nonetheless lies in stark contrast to Europe and North America. Our random genomic markers further support the low genetic differentiation of *Nosema* across Europe and North America. Such low diversity outside of Asia could have arisen through multiple mechanisms, including the natural spread of a Holarctic *Nosema* lineage before *Bombus* domestication, or more recently as a result of intensive *Bombus* domestication in Europe and North America, which has not occurred in China (27). Given the high parasite levels found today in European commercial bumble bees (28), together with the knowledge that commercial foraging bees come and go from “leaky” greenhouses (18, 21) and have been used in open-field pollination, a sweep of a common *N. bombi* strain is possible. In this scenario, the *N. bombi* strain resulting in increasing North American prevalence could conceivably reflect a secondary introduction from Europe. If North American and European *N. bombi* are similar because of recent shared evolutionary history, it will be challenging to identify the signatures of a recent secondary introduction even if one were to have occurred. Regardless of *N. bombi*'s geographic origin, the strong temporal connection between *N. bombi* epizootics in commercial *Bombus* stock in the mid-1990s and escalating prevalence of a genetically similar *Nosema* in declining wild host populations suggests a substantial risk of pathogen transmission from commercial stocks.

In contrast to an invasion hypothesis, increased *Nosema* prevalence in North America could simply reflect a natural increase in native *N. bombi*. A natural increase in the wild of native *N. bombi* during the period of increased prevalence could have contributed to the collapse of commercial *B. occidentalis* breeding following stock replenishment with infected wild *B. occidentalis* queens (27). Alternatively, if native queens had even low *N. bombi* prevalence, infections could spread rapidly among high-density colonies in breeding facilities and get transmitted back into wild populations as an epizootic because bumble bees are excellent dispersers (4). Moreover, the artificial conditions of intense breeding of a single narrowly collected species grown in dense facilities are ideal for selection for increased virulence of microbial parasites (29). Given the lack of genetic diversity in Microsporidia genomes in general (30), higher-resolution genomic data will be needed to fully tease apart all signatures of genetic variation in *N. bombi*. Indeed, genotype-by-genotype host–parasite interactions could be important in bumble bee *Nosema* infection (31). Identifying such highly localized functional variation in North American and European *N. bombi* isolates will require fine resolution whole-genome data.

The observations that declining bumble bee species exhibit higher *N. bombi* prevalence, and that increases in prevalence appear temporally correlated with the onset of *Bombus* declines in North America, are reminiscent of reports of other *Nosema* pathogens known to cause widespread threats to honey bees, including *N. ceranae*, which has been implicated as one factor in Colony Collapse Disorder worldwide (32). Other fungal pathogens are decimating populations of frogs (*Batrachochytrium dendrobatidis*)

(33) and bats (*Geomyces destructans*) (34) throughout their native ranges. However, confirming a direct causal link between *N. bombi* and North American bumble bee decline will require further research. Increased prevalence does not necessarily lead to increased pathogen impact on the host (35), and our detection methods do not distinguish individuals carrying infectious and noninfectious *Nosema*. Although stable and declining species clearly exhibit significantly different prevalences (4), patterns among declining species may differ. Temporal increases were observed across declining species but were more dramatic in *B. occidentalis*, *B. pensylvanicus*, and *B. affinis* than in *B. terrestris* (4). Comparative studies of susceptibility in declining and stable species are needed to reveal whether the increased prevalence in declining species is the result of higher susceptibility to the pathogen or greater *N. bombi* virulence in some species.

Understanding the transmission mode of *N. bombi* is also essential for understanding whether *Nosema* could have caused the observed population declines. Finally, additional factors may also have been involved. Recent reviews of pollinator declines are leaning toward the position that multiple stressors acting in concert are likely causing pollinator decline worldwide (36). These stressors include other pathogens reported from commercially produced bumble bee colonies (28, 37), loss of floral and nesting resources, agrochemicals, and changing climate (36, 38). Increasing physiological stress attributable to environmental degradation is likely to enhance the effects of pathogens. Enhanced procedures put into place by the commercial pollination industry to reduce pathogen infection and transmission, and to curtail intercontinental and interregional trade in commercial colonies, will lower the elevated health risks to wild pollinators witnessed globally over the last two decades.

Methods

Screening for *Nosema* in Bumble Bee Museum Specimens. We screened 2,048 bumble bee specimens from five declining North American species (*B. affinis*, *B. franklini*, *B. occidentalis*, *B. pensylvanicus*, and *B. terrestris*) collected from 1979 to 2011. Historical (1988 to 1991) European *B. terrestris* and freshly collected samples from regions known to harbor *N. bombi* were screened as positive controls (Tables S1 and S5). DNA was extracted nondestructively from pinned specimens (SI Methods). An ~120-bp fragment of the *Nosema* internal transcribed spacer (ITS) region of the rRNA gene was PCR-amplified with dedicated primers (ITS-f2 and ITS-r2) (25) to detect the presence of *Nosema*. Both extraction and PCR preparation were conducted in a clean environment, free from PCR products and other contaminants. All amplifications were replicated at least three times. A sample was considered *Nosema*-positive when at least 50% of PCR replicates successfully amplified the expected product (see Table S2 and Fig. S3 for results with a lower-stringency threshold). To examine whether specimen age adversely affected PCR success, besides using historical *B. terrestris* with high *Nosema* prevalence as a control, we amplified a 117-bp fragment of the *Bombus* *opsin* (*LW Rh*) gene from 47 bumble bee specimens of varying ages (1982 to 2004) (Table S1 and Fig. S2).

To detect temporal changes in *Nosema* prevalence in the four North American bumble bee species with large sample sizes, we conducted χ^2 tests of independence of the proportion of infected bees across four time periods (SI Methods), as well as multiple comparison tests. We also conducted piecewise general linear modeling (GLM) (family, quasibinomial), a regression model with change points, to determine whether the year–*Nosema* prevalence relationship changed significantly for each species around 1992 (39). In this analysis, we used the R-package “segmented” to determine the time point at which the regression slope parameters changed (40). We also conducted analysis of deviance to determine whether the piecewise models provided significantly better fit to the data compared with models without change points (SI Methods).

Small Subunit rRNA Sequencing. We sequenced a 298-bp fragment of the *Nosema* SSU rRNA gene from infected North American ($n = 35$) and European ($n = 10$) bumble bees collected recently (2009 to 2011), using custom primers targeting conserved sites (SI Methods). In addition, we included seven infected North American museum specimens (Table S3). PCR products were cloned and Sanger-sequenced and/or pyrosequenced. Cloning and sequencing allowed detection of potential coinfections, as commonly seen in the honey bees (26, 41). Demultiplexing and quality control of pyrosequencing reads were performed with Mothur version 1.28 (42). To provide additional geographic context of global *Nosema* diversity, we supplemented the dataset with 113 nonredundant GenBank sequences derived from a variety of *Nosema* and related *Nosema* taxa [species]. SplitsTree version 4.12.6 (43) was used to construct a network using the neighbor-net method and uncorrected p-distances (44) (SI Methods).

Multilocus Amplicon Sequencing. We used reduced representation genome pyrosequencing of nine *N. bombi* isolates [each from a single infected *Bombus* individual (Table S4)] to identify potentially informative genetic loci beyond the rRNA gene (SI Methods). The amount of genetic variation uncovered was low, although we identified six loci for application of deep amplicon pyrosequencing on *N. bombi* isolates from seven European *B. terrestris* specimens and 24 North American specimens (15 species) (Table S4). Using POPTREEW, we constructed a neighbor-joining tree of among-isolate relationships based on combined allele frequency data and pairwise D_A distance matrix (45). We also tested whether heterozygosity of North American *N. bombi* isolates was significantly lower than that of isolates from European species using the Skillings-Mack test (46). To estimate differentiation among European or North American

N. bombi isolates at multiple levels, we applied a hierarchical Bayesian analysis of molecular variance at three levels: within isolates (Φ_{ST}), among isolates-within regions (Φ_{SC}), and between regions (Φ_{CT}) (SI Methods).

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