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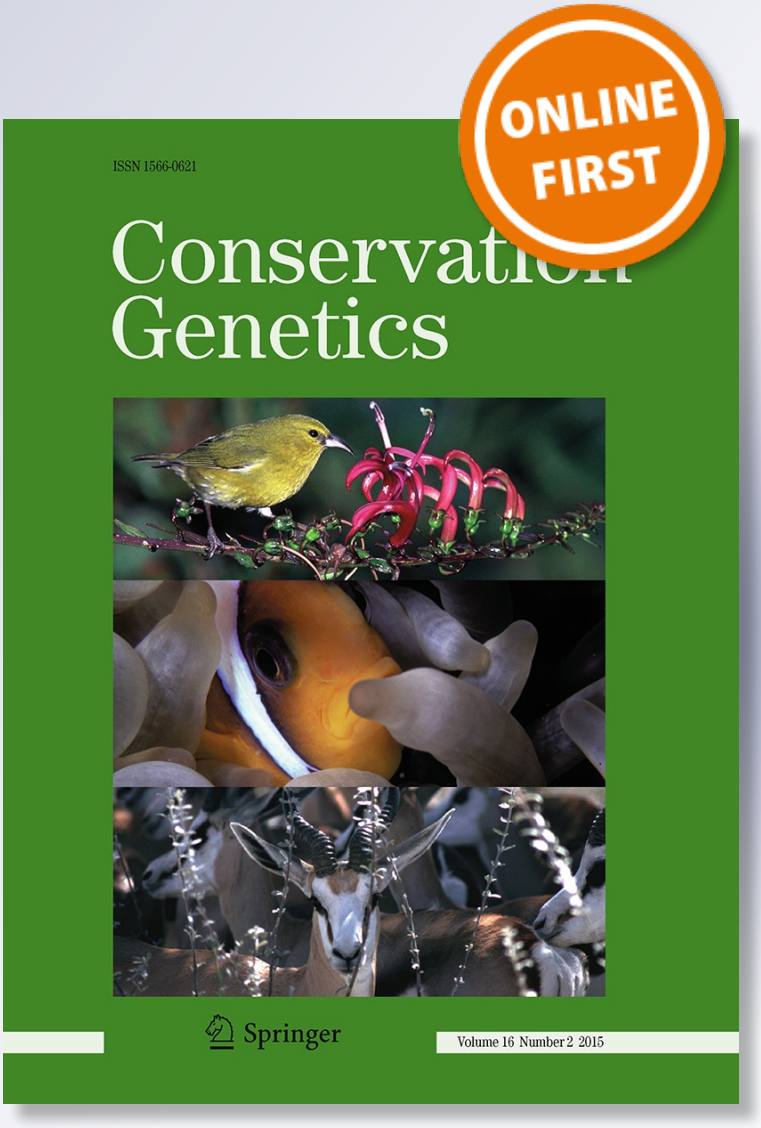

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RESEARCH ARTICLE

Spatial genetic structure among bat hibernacula along the leading edge of a rapidly spreading pathogen

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Abstract Viral, bacterial, parasitic and fungal pathogens pose a significant, current threat to global biodiversity. A virulent fungal pathogen (*Pseudogymnoascus destructans; Pd*) emerged in hibernating bats in eastern North America in 2006. In this paper, we seek to inform epidemiological models of the progression of Pd into populations of the little brown bat (*Myotis lucifugus*) in central Canada by characterizing the spatial genetic structure of the host ahead of the imminent arrival of *Pd*. We sampled 242 bats from eight hibernacula spanning 92,623 km^2 and two ecozones. We genotyped all individuals at eight microsatellite loci and sequenced 300 bp of HVII in a subset ($n = 72$) to test the null hypothesis of contemporary panmixia. We found evidence of spatial genetic structure associated with ecozone boundaries, and a predominant north–west to south–east directionality of bat movements among hibernacula, which opposes the current approach of the pathogen. Our large study area (larger than the dispersal distance of individual bats) allowed us to detect the first evidence of contemporary population structure among hibernacula of *M. lucifugus*. Our results suggest that the potential spread of *Pd* into north-central Canada may be retarded by the opposing direction of gene flow of the host species, and our findings of directional gene flow can be used to inform management strategies for the spread of *Pd* into the area.

Keywords White nose syndrome · Little brown bat · Central North America · Spatial genetic structure · Hibernacula · Ecozones

Introduction

Infectious disease caused by viral, bacterial, parasitic or fungal pathogens pose a significant threat to global biodiversity (Daszak et al. 2000). In recent years, many emerging diseases have been associated with fungal pathogens and pathogenic fungi are implicated in the majority of documented pathogen-driven extinctions of plants and animals (Fisher et al. 2012). Paradoxically, the loss of biodiversity from an area can further increase disease incidence in some cases, thereby increasing the risk of pathogen-driven extinctions for remaining, susceptible taxa (Keesing et al. 2010). This makes understanding and predicting the spread of fungal and other pathogens critical both for wildlife epidemiology and conservation of biodiversity.

The genetic structure of a host population can inform epidemiological models, in particular when pathogens are transmitted via direct contact between hosts (Blanchong et al. 2008; Biek and Real 2010). Pathogens that are transmitted via direct contact may move rapidly through areas of high host dispersal and panmixia, or be limited by barriers to dispersal and gene flow (Cullingham et al. 2009). Thus, the spatial genetic analyses of host populations can indicate the direction and magnitude of host

Christina M. Davy and Felix Martinez-Nunez have contributed equally to this work.

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dispersal across a landscape, and be combined with observed patterns of pathogen spread to generate and test hypotheses about pathogen transmission dynamics and host risk of disease and mortality (Biek and Real 2010). This approach has been successfully used to explain the distribution and incidence of diseases such as chronic wasting disease in deer, rabies in raccoons, tuberculosis in elk, and *henipaviruses* in fruit bats (Blanchong et al. 2008; Cullingham et al. 2009; Vander Wal et al. 2012; Peel et al. 2013). The generalizability of such hypotheses can then be more rigorously tested by tracking pathogen dispersal into naïve, genetically structured populations. Additionally, pre- and post-exposure sampling of host populations can facilitate studies of potential adaptation to a pathogen (Manel et al. 2010).

A particularly virulent fungal pathogen (*Pseudogymnoascus destructans; Pd*) emerged in hibernating bats in eastern North America in 2006 (Blehert et al. 2009; Minnis and Lindner 2013). Likely an introduced species to North America (Warnecke et al. 2012), *Pd* has spread rapidly since its discovery. In the laboratory, infection is spread by physical contact between bats (i.e. direct transmission), and bats may also become infected via contact with infected substrates in hibernacula (i.e. vehicle-borne transmission; Lorch et al. 2013) that also serve as non-zoonotic reservoirs for the pathogen. Cutaneous infection with *Pd* causes white nose syndrome (WNS), and has resulted in staggering mortality of hibernating bats across eastern North America (reviewed by Cryan et al. 2013).

Models based on the spread of WNS from its epicentre in the north-eastern United States suggest that the future spread of the pathogen can be largely predicted by ecological traits of its hosts (bats) and the availability of suitable habitat (Maher et al. 2012). However, these models did not account for effects of spatial genetic structure of host species on pathogen dispersal. A recent study characterized genetic structure in one of the most severely affected host species (the little brown Myotis, *Myotis lucifugus*) near the epicenter of WNS infection (Miller-Butterworth et al. 2014). Historic genetic structure of *M. lucifugus* (inferred from mtDNA) was correlated with the distance between hibernacula, topographic features and the spread of WNS infection across an 840 km distance spanning the Appalachian Mountains. However, inference of contemporary genetic structure based on nuclear markers indicated that bats overwintering in these hibernacula belong to a single, panmictic population, without significant barriers to gene flow (Miller-Butterworth et al. 2014). Therefore, the hypothesis that barriers to gene flow might affect transmission of *Pd* could not be tested in this study system. Similarly high connectivity was documented among swarming sites on the Atlantic coast of Canada across distances of up to 860 km (Burns et al. 2014). *Myotis lucifugus* can disperse over 800 km between summer and winter roosts, or among swarming sites, and individual movements >500 km are not uncommon (Fenton 1969; Norquay et al. 2013). As a result, very large spatial scales may be required to detect contemporary genetic structure among hibernacula, and the potential influence of host gene flow on pathogen transmission.

Here, we characterized genetic structure in *M. lucifugus* populations in a large study area that borders the current range limit of *Pd*. We quantified the strength and directionality of gene flow among hibernacula likely to be colonized by *Pd* in the near future to assess the potential for dispersal and seasonal movements of bats to slow or accelerate the spread of WNS. Specifically, we tested the hypothesis that *M. lucifugus* exhibits significant genetic structure when sampled across an area larger than individual dispersal distances. Our results identify the geographic range of biologically relevant genetic populations that could be used to test for signals of selection following the selective sweep of a virulent disease like WNS, and can be combined with data on future pathogen spread to test hypotheses about the role of host gene flow on pathogen spread in this system.

Methods

Collection and analysis of DNA

We took 2 mm sterile wing biopsy punches (Miltex Instrument Co.; Worthington-Wilmer and Barratt 1996) from 242 bats over the course of 2 years (2010 and 2011) from *M. lucifugus* at ten hibernacula in central Canada, located to the north of the most north-westerly current record of *Pd* (Fig. 1). Other survey work at these sites has confirmed that they are also used as swarming sites. Biopsy samples were preserved in 100 % ethanol and transported to the University of Winnipeg, where they were stored at 4 °C until analysis [see Electronic Supplementary Material (ESM) for details of sampling and subsequent DNA extraction methods]. Distance among sites ranged from 1.3 to 988 km, and the study area spans approximately 92,623 km^2 and two ecozones, prairie and boreal forest.

DNA was extracted from the 242 biopsy samples for amplification of microsatellites to investigate contemporary gene flow, and a subset of these samples (n = 72) were used to amplify a section of the mtDNA genome to investigate historic gene flow. We screened 22 microsatellite markers previously developed for bats (Castella and Ruedi 2000; Trujillo and Amelon 2009), and selected twelve loci for genotyping based on product quality and reliability (Table S1). We sequenced 300 bp of the non-coding hyper-variable domain II (HVII) from the mtDNA

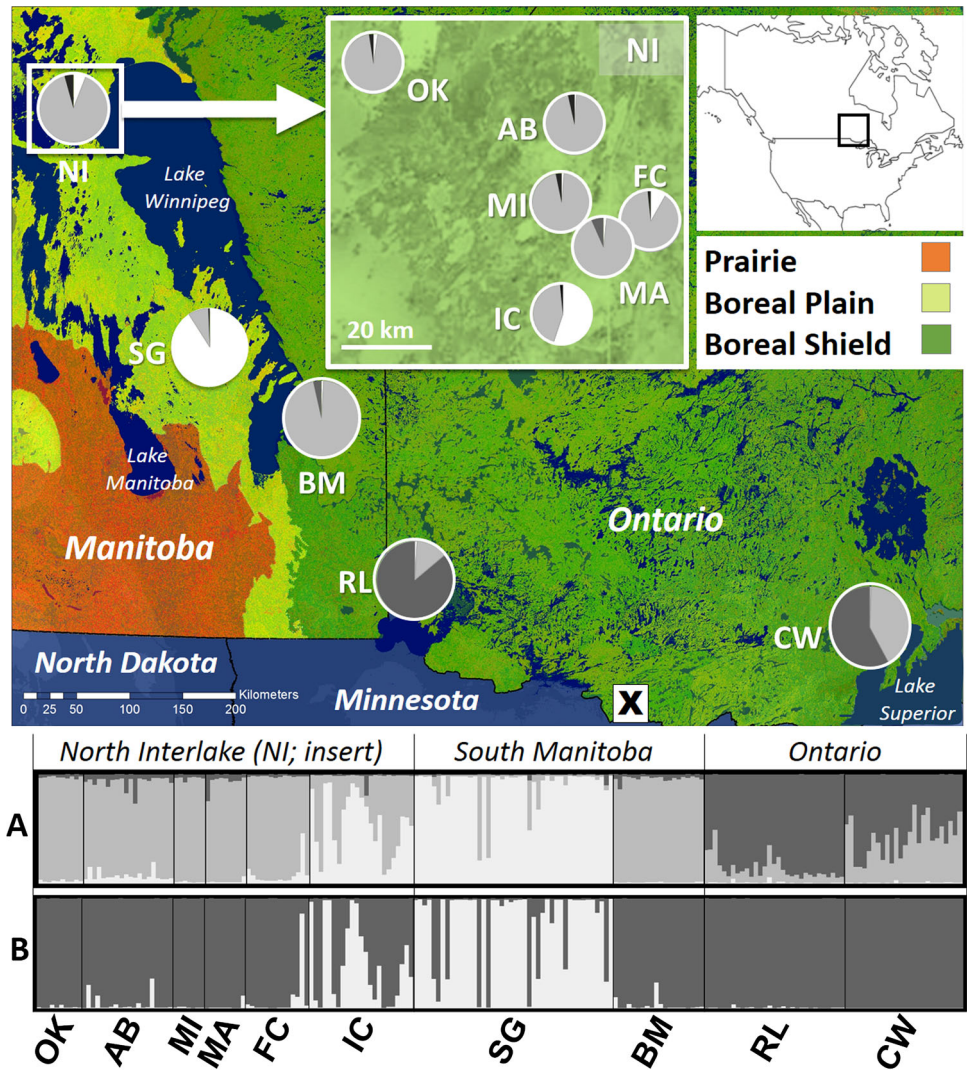

Fig. 1 Estimated genetic structure among 204 *Myotis lucifugus* based on microsatellite genotypes (8 loci). The study area is indicated on the inset map of North America, and the "X" in Minnesota indicates the nearest location at which samples have tested *Pd*-positive at the time of writing. Pie-charts on map indicate approximate locations of sampled hibernacula and show the overall q-values for each sampled site based on STRUCTURE results. Q-values for the cluster of hibernacula in the Northern Interlake Region is presented both as a single "site" (Northern Interlake; NI) on the larger map, and by individual hibernacula (see *inset map*: *OK* Okaw, *AB* Abyss, *MI* Microwave, *MA* Moosearm Pit, *FC* Firecamp, *IC* Iguana Crypt, *SG* St. George, *BM* Bissett Mine, *RL* Richard Lake, *CW* Caribou West). *Barplots* below map show individual q-values inferred by **a** STRUCTURE, and **b** TESS

control region, using the primers L16517 (Fumagalli et al. 1996) and sH651 (Castella and Ruedi 2000) in 72 bats, randomly selecting one bat per cluster per cave. Amplification, genotyping and sequencing methods and test for deviations from Hardy–Weinberg equilibrium (HWE) are provided in the ESM.

Analyses of mtDNA: historical population structure and gene flow

Detailed methods for mtDNA analyses are provided in the ESM. Briefly, once sequences were concatenated and edited we grouped individuals from the two nearby Northern Interlake hibernacula (Abyss and Firecamp). Thus, we considered six groups in our mtDNA analyses: Caribou West (CW), Richard Lake (RL), Bissett Mine (BM), St. George (SG), Iguana Crypt (IC), and Northern Interlake (NI; Fig. 1); estimates of mtDNA variation within groups were estimated in DnaSP v.5.0. (Librado and Rozas 2009). Levels of genetic differentiation among groups were estimated using GammaSt (an Fst analogue based on comparing levels of heterozygosity per nucleotide site) and Fst while the mean number of historical migrants per generation (N_m) was inferred from these estimates. We then performed a rank permutation test to test whether existing levels of population differentiation were significant based on the K_{ST}* statistic described of Hudson et al. (1992) using 1000 permutations. Estimates of gene flow and genetic differentiation were obtained using DnaSP v.5.0 (Librado and Rozas 2009).

We performed a spatial analysis of molecular variance in SAMOVA v.1.0 (Dupanloup et al. 2002) to identify the number of genetically homogeneous groups without assuming a priori population structuring using 10,000 permutations. The number of groups, k, was set from 2 to 4, and the value of k generating the highest value of F_{CT}, or

the amount of genetic variation among groups, chosen as the optimal number of groups and population configurations (Dupanloup et al. 2002). To further assess the relationship among haplotypes, we eliminated all missing data and then constructed a minimum spanning network using a median joining algorithm implemented in Network (Bandelt et al. 1999). Additionally, we used Maximum-likelihood (ML) methods to determine the best model of sequence evolution using the Akaike information criterion (AIC), and then reconstructed the phylogenetic relationship among haplotypes using ML (please see ESM for details).

Analyses of microsatellites: contemporary population structure and gene flow

We used GENEPOP 4.0.10 (Raymond and Rousset 1995; Rousset 2008) to test for linkage disequilibrium and deviations from HWE. We estimated genetic variation within each site using SPAGEDI 1.3 (Hardy and Vekemans 2002) and calculated D_{est} among sites (absolute genetic differentiation among samples; Jost 2008) using SMOGD (Crawford 2010). Estimates of D_{est} and F_{ST} can be biased by small sample sizes ($n < 10$) so we made two calculations. The first considered each site separately and the second considered the cluster of six hibernacula <80 km apart in the NI region as a single group.

Available methods for estimating population structure each have their own biases and assumptions (reviewed in François and Durand 2010). We therefore applied three methods and used congruence among results to assess the strength of the signal in the data. First, we used STRUCTURE v. 2.3.4 (Pritchard et al. 2000) to infer the number of genetic clusters (K) represented by the data. Second, we incorporated spatial data (sampling locations) by conducting a similar analysis in TESS v.2.3.1 (Chen et al. 2007; ESM). Third, we conducted a principal coordinates analysis (PCoA) in Genalex v. 6.501 (Peakall and Smouse 2006, 2012) based on pairwise D_{est} values among sites. PCoA can detect subtle population structure even in datasets with missing data and small numbers of loci (Bauchet et al. 2007). The PCoA analyses first considered each site separately, and then considered the individuals from the NI region as a single group. We tested for isolation by distance using a Mantel test of geographic distance and genetic distance (Jost's D) among sites, considering NI as a single site.

We estimated proportional migration among identified genetic clusters and the inbreeding coefficient (F_{IS}) for each cluster using BAYESASS v.3.0, which estimates gene flow over the last "several generations" (Wilson and Rannala 2003). This is typically interpreted as 1–5 generations (Chiucchi and Gibbs 2010; van der Meer et al. 2013), which represents approximately 3–50 years for *M. lucifugus* (COSEWIC 2012). BAYESASS runs followed the recent recommendations to ensure convergence (Meirmans 2014; ESM).

Results

We calculated indexes of genetic diversity among hibernaculum for 242 bats genotyped at 8 microsatellites and for the 300 bp of HVII mtDNA sequence obtained for a subset of 72 bats (Table 1).

Historical population structure and gene flow

Analysis of mtDNA differentiation across all hibernacula based on GammaSt and F_{ST} revealed low levels of population structure with moderate to high historical migration rates (overall GammaSt = 0.074, $N_m = 6.24$; $F_{ST} = 0.032$, $N_m = 15.29$); only CW showed moderate levels of population differentiation from other sites with most F_{ST} and GammaSt values ~0.2 (ESM). CW also displayed lower levels of nucleotide diversity than the other six groups, perhaps partly due to the small sample size (6) at that site (Table 1). However, the rank permutation test did not find significant levels of genetic differentiation after randomization across all sites (rank permutation test: Kst* = 0.017, p = 0.259).

SAMOVA indicated optimal support for two groups. The first group included CW and BM, and the second included RL, SG, IC and NI (ESM). This grouping accounted for ~21 % of the variation between the two groups while ~78 % of the variation occurred within groups. Permutation tests revealed marginally significant historic differentiation between these two groups ($F_{CT} = 0.214$, p = 0.050).

The minimum spanning network reconstructed two clusters of haplotypes separated by four mutational steps. The two haplotype clusters were broadly, but not exclusively, concurrent with a northeast-southwest split. The first cluster (on the left, Fig. 2) was dominated by individuals sampled at western locations (NI, IC and SG), while the second cluster of haplotypes (on the right, Fig. 2) largely represented individuals sampled in the eastern part of the range (CW, RL and BM). Samples from caves in the western part of Manitoba contained higher haplotype diversity, with haplotypes differing by fewer mutational steps than those from the eastern part of the study area. The most common haplotype in the right-side of the network (predominantly eastern) was found in eleven individuals, seven of which were found in eastern hibernacula, and all other haplotypes were close mutational derivatives of this most frequent haplotype.

Table 1 Sample sizes and genetic diversity of hibernacula based on mtDNA and nuclear markers, showing census number of bats using each sampled hibernacula (census), sample size (n; n differs for nuclear and mtDNA analyses); haplotype diversity (Hd), mean number of pairwise differences (k), nucleotide diversity (π), Allelic richness (Ar), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}). Site abbreviations correspond to Fig. 1

		Mitochondrial (HVII) diversity					*Nuclear diversity (12 microsatellite loci)*				
Site	**Census count**	**n**	**No. of haplotypes**	**Hd**	**K**	**Π**	**n**	**Ar**	**H_O**	**H_E**	**F_{IS}**
CW	--	6	5	0.933	1.533	0.005±0.0015	33	14	0.775	0.837	0.139
RL	>1000	23	16	0.964	4.95	0.0205±0.0017	40	18	0.771	0.880	0.145
BM	158	3	3	1.00	2	0.0177±0.009*	20	22	0.934	0.906	-0.019
SG	9015	18	13	0.954	6.20	0.021±0.0015	53	14	0.931	0.849	-0.049
IC	264	8	7	0.964	5.57	0.018±0.003	30	15	0.913	0.860	-0.016
AB	250	*AB and FC combined for mtDNA analysis:*					23	15	0.930	0.881	-0.056
FC	50	14	11	0.956	5.51	0.0195±0.0023	15	11	0.900	0.860	-0.044
MP	30	—	—	—	—	—	10	10	0.900	0.863	-0.024
MA	68	—	—	—	—	—	8	9	0.858	0.850	0.033
OK	7471	—	—	—	—	—	10	6	0.887	0.838	-0.028
All sites	N/A	72	44	0.97	5.12	0.02±0.00082	242				

* Sample size too small to be a reliable estimate

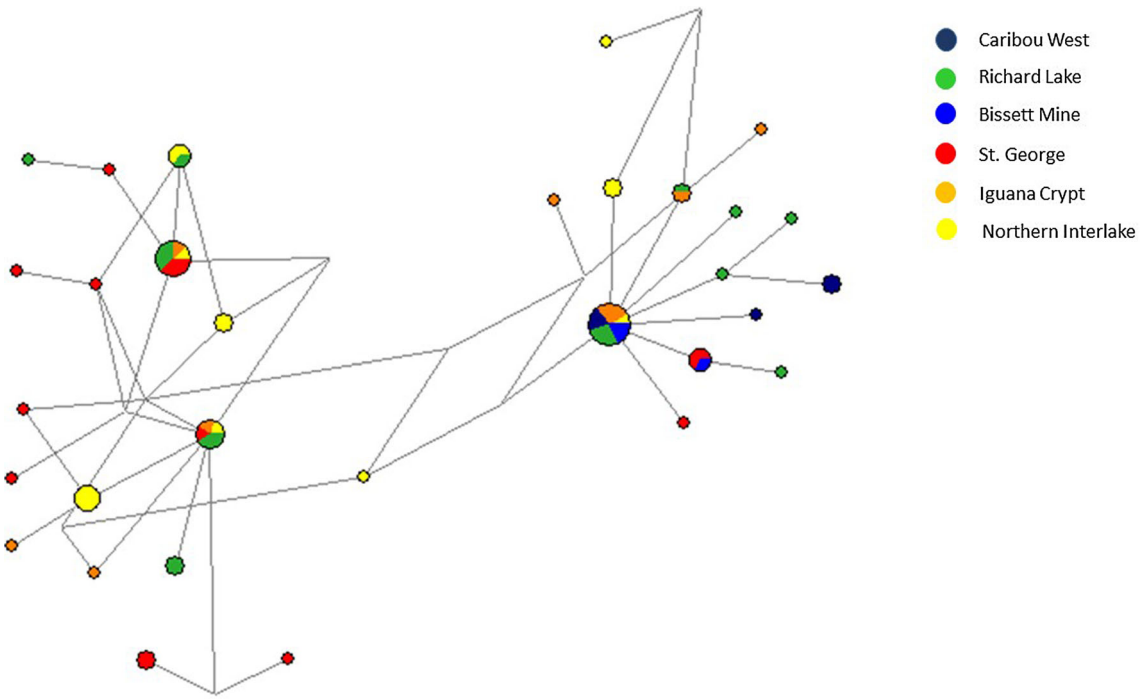

Fig. 2 Minimum spanning tree based on 300 bp of HVII region for 72 *Myotis lucifugus* sampled from hibernacula in central Canada shows weak geographic partitioning of haplotypes into an eastern and western cluster, and indicates recurrent contact among lineages

Table 2 Pairwise genetic differentiation among Myotis lucifugus sampled at ten sites, based on eight microsatellite loci

	CW	RL	BM	SG	NI: IC	NI: FC	NI: MA	NI: MW	NI:AB	NI: OK	NI: (All Sites)
Caribou West (26)	–	0.016	0.035	0.171	**0.071**	**0.110**	**0.016**	**0.156**	**0.055**	**0.057**	0.046
Richard Lake (31)	0.053**	–	0.107	0.162	**0.157**	**0.122**	**0.092**	**0.123**	**0.102**	**0.098**	0.096
Bissett Mine (20)	*0.025***	*0.028***	–	0.105	**0.017**	**0.018**	**0.000**	**0.017**	**0.001**	**0.001**	0.000
St. George (44)	*0.026***	*0.024***	0.001*	–	**0.071**	**0.155**	**0.091**	**0.168**	**0.095**	**0.121**	0.096
NI: Iguana Crypt (23)	**0.038*** **	**0.045*** **	**0.016****	**0.011****	–	**0.095**	**0.032**	**0.080**	**0.022**	**0.046**	–
NI: Fire camp (14)	**0.034*** **	**0.035*** **	**0.007**	**0.029*** **	**0.002***	–	**0.002**	**0.059**	**0.049**	**0.003**	–
NI: Moosearm Pit (9)	**0.024****	**0.030*** **	**0.000**	**0.028*** **	**0.031****	**0.006**	–	**0.010**	**0.001**	**0.000**	–
NI: Microwave (7)	**0.059*** **	**0.044*** **	**0.013**	**0.050*** **	**0.048***	**0.022***	**0.017**	–	**0.034**	**0.028**	–
NI: Abyss (20)	**0.023*** **	**0.027*** **	**0.005**	**0.023*** **	**0.001***	**0.024***	**0.186****	**0.006***	–	**0.064**	–
NI: Okaw Cave (10)	**0.055*** **	**0.054*** **	**0.012**	**0.045*** **	**0.041****	**0.016***	**0.000**	**0.007**	**0.025****	–	–
NI: All sites (83)	*0.021*** *	*0.022*** *	0.008	*0.016*** *	–	–	–	–	–	–	–

D_{est} above diagonal; F_{ST} below diagonal. Significant F_{ST} values based on 999 permutations are indicated with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Pairwise D_{est} and F_{ST} values are shown for the northern Interlake region (NI) as a whole as well as for separate sites within NI, but values calculated separately for sites within NI are bold to re-iterate that these results are less robust due to the smaller sample sizes at each site. For the five unbold sites, F_{ST} values in italics are significant after Bonferroni correction for multiple comparisons

Contemporary population structure and gene flow

We did not detect deviations from HWE, or evidence of linkage disequilibrium among loci. Removal of loci and individuals with >80 % missing data left 204 individuals genotyped at 8 microsatellite loci for subsequent analyses. Pairwise D_{est} ranged from 0 (NI and BM) to 0.171 (SG and CW; Table 2). After Bonferroni correction, F_{ST} values for microsatellites were significantly different from zero ($p < 0.05$) for all pairwise comparisons except CW and RL, BM and SG, and BM and NI (Table 2). A Mantel test detected no significant correlation between geographic and genetic distance ($Z = 354.869$, $r = -0.113$; $p = 0.557$).

STRUCTURE identified $K = 3$ as the most likely model of contemporary population structure. RL and CW were grouped into a single cluster ("Ontario," mean q ± standard deviation = 0.732 ± 0.199). STRUCTURE identified SG as genetically differentiated from all other sampled locations ("St. George," mean $q = 0.910 \pm 0.180$) and five of the NI hibernacula formed a cluster with BM ("Manitoba North," $q = 0.810 \pm = 0.252$). Winter aggregation of bats belonging to different genetic clusters (putative populations) was evident at some sites (Fig. 1). In particular, IC contained an approximately equal mixture of individuals assigned strongly to either the St. George or Manitoba North clusters, as well as individuals with admixed ancestry. TESS also identified IC as a contact zone between two clusters: 1) SG and approximately half of the individuals from IC, along with a few individuals from other NI sites; and 2) all other sites (Fig. 1).

The PCoA of 10 hibernacula inferred similar patterns to STRUCTURE and TESS (Fig. 3a). The first axis divided SG from eight other sites, with IC intermediate to these two groups. The second axis divided SG and the two Ontario sites from BM and the other NI sites, while the third axis divided RL and SG from CW. Cumulatively, these axes accounted for 92.62 % of the variation in the data. A second PCoA that grouped the northern Interlake hibernacula together showed a similar pattern (Fig. 3b). Based on these results, we considered the three clusters identified by STRUCTURE and the PCoA (Manitoba North including IC, St. George, and Ontario) in the subsequent analyses.

BAYESASS analyses detected recent directionality in gene flow among the three clusters, with stronger gene flow from northwest to southeast than in the opposite direction (Fig. 4). BAYESASS also estimated a high inbreeding coefficient for the Ontario cluster ($F_{IS} = 0.172 \pm 0.031$), with lower values for the St. George (0.0144 ± 0.0105) and Manitoba North clusters (0.0078 ± 0.006).

Discussion

Contrary to the hypothesis of contemporary panmixia, we found high levels of structure across a study area that exceeds the known dispersal distance of individual *M. lucifugus*. South-eastward gene flow in these *Pd*-naive host populations opposes the westward and north-westward approach of the oncoming pathogen. Opposing directionality of movement in this host-pathogen system highlights the value of our study area for testing impacts of gene flow on pathogen spread as *Pd* moves into central Canada, and has important implications in the broader context of species conservation. The genetic structure we identified among

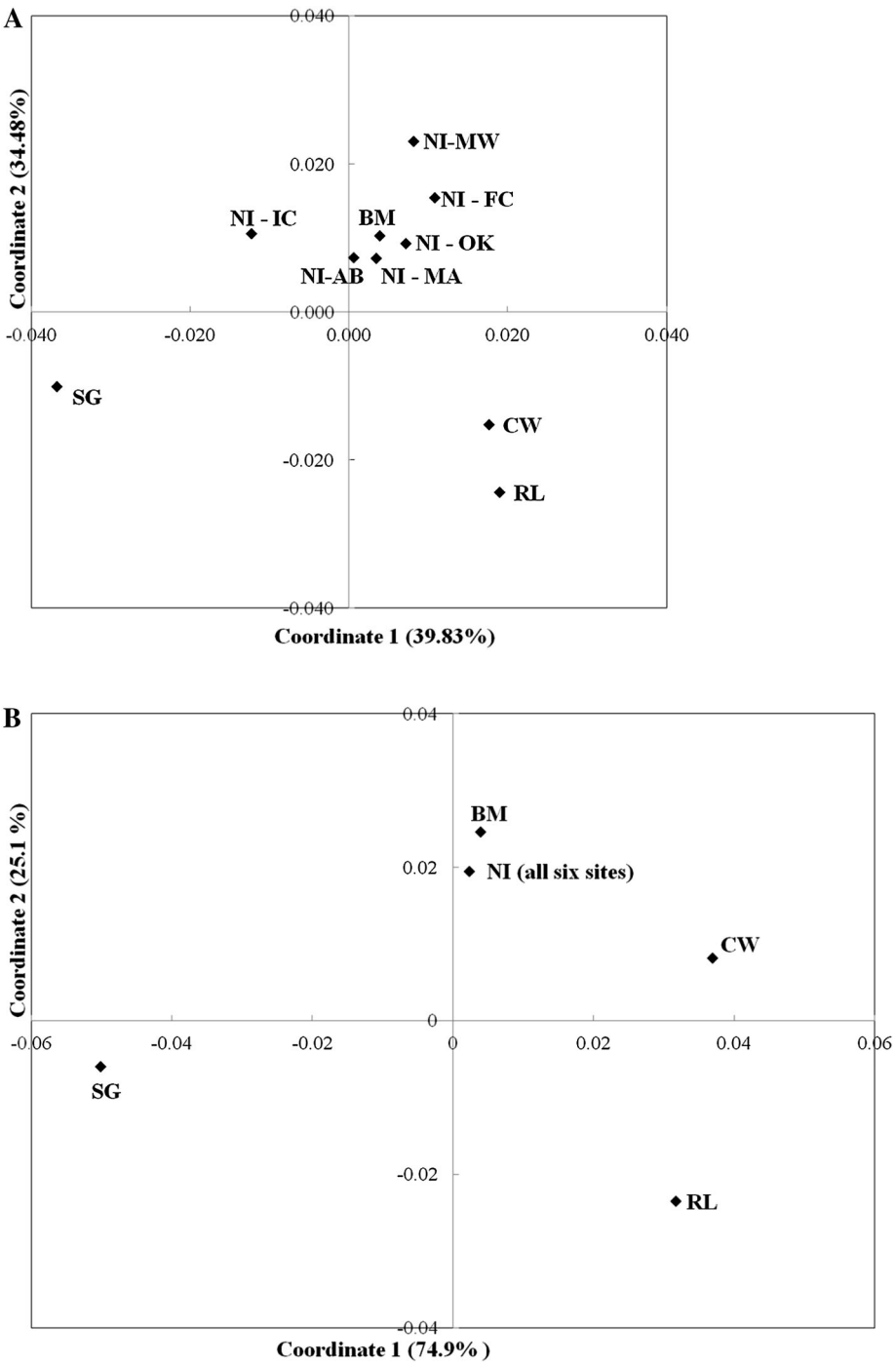

Fig. 3 Principal coordinates analysis of genetic distance (Jost 2008) among ten hibernacula, based on 204 individual *Myotis lucifugus* genotyped at eight microsatellite loci. **a** All hibernacula considered separately, **b** hibernacula in the North Interlake region (NI) considered as a single site. The percent of variation explained by each axis is indicated in parenthesis

hibernacula expected to be impacted by WNS in the near future provides a unique system for testing pathogen-mediated selection and potential adaptation of bat populations to WNS as *Pd* arrives.

Contrary to previous analyses (Miller-Butterworth et al. 2014, Burns et al. 2014), we found significant, contemporary genetic structure among hibernacula in this widespread and highly vagile species. The diversity of ecozone-level variation on our study landscape is one possible driver of the observed structure, although further study over a larger area is required to test this hypothesis. On a longer time-scale, our mtDNA results show only weak partitioning into eastern and western groups. The contrasting results based on microsatellites and mtDNA may be explained by the different effective size and mutation rates of both types of markers: maternally inherited mtDNA has half the effective population size and a slower mutation rate than the biparentally inherited microsatellite

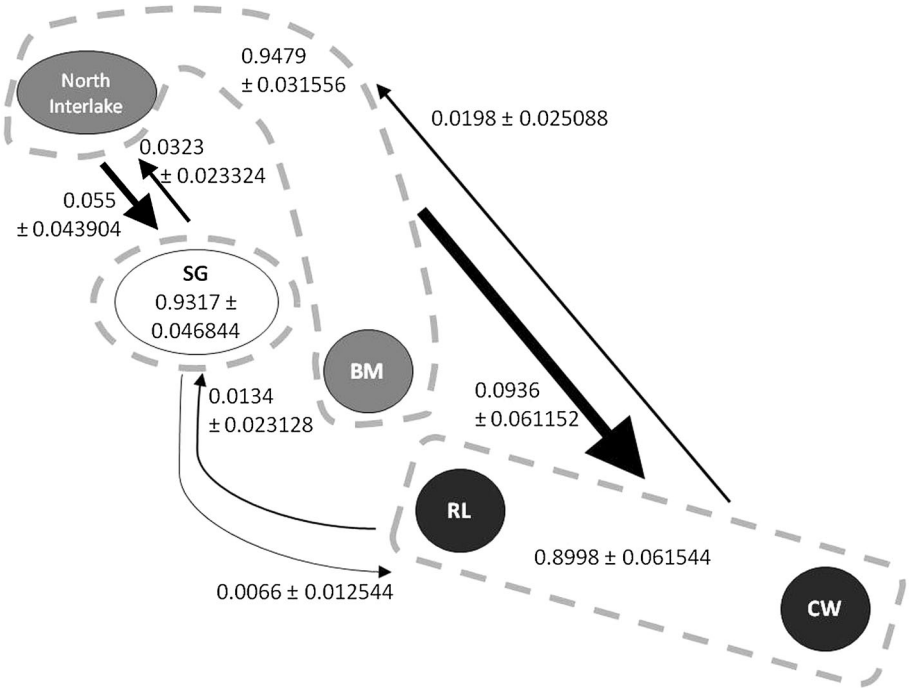

Fig. 4 Contemporary migration rates among genetic subpopulations of *Myotis lucifigus* inferred through Bayesian inference in BAYESASS v.3.0.3 (see text for details). *Dashed lines* delimit genetic clusters ("subpopulations") identified by STRUCTURE, but are not meant to indicate the true geographic boundaries of each genetic subpopulation. Values within *dashed lines* indicate the proportion of non-migrant individuals in each subpopulation, and values *above arrows* indicate the magnitude of directional gene flow among subpopulations (the proportion of migrants from each population that are derived from another). *Arrow thickness* is proportional to directional gene flow

markers (Ruedi and Castella 2003; Wan et al. 2004). Thus, levels of genetic variation at mtDNA loci reflect those in the more distant past while variation at microsatellite loci reflects more recent demographic and ecological events, especially when multilocus genotypic information is used. The weakly structured distribution of haplotypes among hibernacula for the mtDNA data suggests a complex history of post-Pleistocene expansion of *M. lucifugus* into Canada, with multiple post-glacial colonization events into the study area and low but recurring contact among lineages. This hypothesis could be tested with a larger mtDNA sample size sampled over an even broader geographic scale.

Gene flow among genetically differentiated populations in our study area was low (Fig. 4) and cannot be explained by distance among sites. The most important insight of our analysis may be the contemporary differentiation of SG from the other hibernacula, with IC representing a zone of admixture between SG and NI/BM. The mtDNA analyses suggest that SG, IC and NI are derived from the same historical haplotypes. Combined with the nuclear data, this implies that recent environmental factors play a stronger role than post-glacial colonization history in shaping contemporary population structure in this system. The SG hibernaculum houses approximately 12,000 bats and is the largest known hibernaculum in our region. To our knowledge there are no *M. lucifugus* hibernacula for nearly 1000 km between our sites in central Manitoba and the foothills of the Rocky Mountains in Alberta. We hypothesize that structure among hibernacula in central Canada may be partly shaped by large-scale landscape features related to the boundaries between prairie and boreal ecozones. If so, one possible explanation for the detection of a differentiated genetic cluster among bats hibernating at SG is that this site is used by bats migrating east from summer roosts in the prairies. These bats could form a separate population from *M. lucifugus* in the adjacent boreal plains, but would need to enter the boreal ecozones in the winter to access suitable hibernacula. Similar correlations between physiographic regions and historic genetic structure have been documented for *M. lucifugus* and a variety of other taxa (e.g. Miller-Butterworth et al. 2003, 2014; Sork and Werth 2014). Another possible influence on population structure is that bats use some cue to actively select mates from their genetic source populations during swarming. For example, echolocation calls vary on a continental scale in *M. lucifugus* (Veselka et al. 2013) raising the possibility of an acoustic signal of origin that could affect mate choice. Similar cultural acoustic influences on mate choice are well known for

other species, including orcas (*Orcinus orca,* Riesch et al. 2012) and cowbirds (*Molothrus ater,* Freeberg et al. 2002). Where hibernation sites are also the site where swarming occurs (as in our study area), selection for mates exhibiting particular acoustic cues or other behaviours could maintain differentiation among genetic populations.

A surprising result of our analyses was the inference of relatively high levels of inbreeding at the RL and CW sites. This result is most likely due ato a Wahlund effect rather than true inbreeding. Both sites support large numbers of bats, making inbreeding unlikely. Furthermore, our clustering analyses assigned individuals hibernating at RL and CW to two distinct genetic clusters, thus confounding calculation of *F*-statistics for these sites and potentially causing over-estimation of inbreeding (Excoffier 2001). Spurious inferences of inbreeding can also occur when the sampled populations include genotypes from other, unsampled populations, something that could easily occur in our study area since it is in the centre of the range of this highly mobile species (Hedrick 2012).

Comparing population structure based on mtDNA and nuclear markers has two major benefits. First, as mentioned, the different mutation rates of these markers allow inference of historic and contemporary population structure, respectively (e.g. Ruedi and Castella 2003). In this framework, our results contrast with previous studies of genetic structure in temperate bats, which typically exhibit some mtDNA (historic) population structure but little or no nuclear (contemporary) structure (e.g.; Ruedi and Castella 2003; Bryja et al. 2009; Burns et al. 2014; Miller-Butterworth et al. 2014). While we cannot explicitly test the causes of the observed structure with the current data, our hypothesis that ecozone boundaries mediate population structure provides a testable potential explanation for the patterns we observed in central Canada.

The second benefit of comparing nuclear and mtDNA markers stems from their inheritance. Nuclear markers are biparentally inherited and subject to recombination, while mtDNA is maternally inherited as a single unit. Thus, these markers can be used together to test hypotheses about sex-specific dispersal patterns. For example, genetic profiling of bat maternity colonies typically shows mtDNA differentiation among colonies, indicating female philopatry to maternity sites. However, nuclear differentiation among maternity colonies is usually low, indicating high gene flow among male and female mating pairs (during mating at swarming sites or in hibernacula; e.g. Dixon 2011; Kerth and van Schaik 2012; Patriquin et al. 2013, Ruedi and Castella 2003, Bryja et al. 2009). This is supported by the high gene flow observed among temperate swarming sites where most mating is thought to occur (e.g. Veith et al. 2004; Furmankiewicz and Altringham 2007; Burns et al. 2014; but see Rivers et al. 2005). Even though there is high gene flow at swarming sites, which are often located at the entrances to hibernacula, bats may show fidelity to hibernacula because of selective forces influencing hibernation strategies such as possible over-wintering with kin. Miller-Butterworth et al. (2014) detected a weak signal of female philopatry to hibernacula in *M. lucifugus* in the north-eastern United States (Miller-Butterworth et al. 2014). However, the low mtDNA structure in our data is not consistent with sex-biased dispersal among hibernacula, and our parallel finding of significant genetic differentiation among hibernacula at nuclear markers suggests that there may be fidelity to hibernacula within our study area, but that it is not sex-biased. Interestingly, mark-recapture data from our study area show that the few bats that disperse among hibernacula are as likely or more likely to be female than male (Norquay et al. 2013). Overall, our results contribute to a growing body of data on the genetic structure of temperate bat populations that demonstrates that bat population structure cannot be described solely based on data from a single season, but requires joint consideration of summer maternity roosts, swarming sites and hibernacula.

The hibernacula we sampled lie north–west of current records of *Pd*, and are expected to be *Pd*-positive within the next few years (https://www.whitenosesyndrome.org/resources/map). Our data highlight the value of this study system for testing the hypothesis that contemporary genetic structure predicts the spread of WNS as Pd arrives. Contemporary gene flow (a proxy for dispersal) opposes the approach of *Pd* in our system, suggesting that west-to-east patterns of movements by individual bats across the landscape may slow transmission of the fungus relative to patterns of spread in eastern North America (Miller-Butterworth et al. 2014). Host dispersal mediates the spread of many diseases, including chronic wasting disease, rabies and West Nile virus (Cullingham et al. 2009; Vander Wal et al. 2012; Venkatesan and Rasgon 2010). WNS differs from these diseases because *Pd* can persist in substrate reservoirs in the cave environment (Lorch et al. 2013) so declines in host populations may not reduce *Pd* prevalence in hibernacula. Nevertheless, *Pd* dispersal is mediated by its hosts and we recommend that the genetic structure of affected bat populations (especially measures of contemporary gene flow) be incorporated into existing epidemiological models (e.g. Maher et al. 2012). Spread of *Pd* through areas with multiple *Pd*-susceptible species will be complex to predict. An added benefit of our study area is that it is overwhelmingly dominated by only two susceptible (hibernating) bat species (*M. lucifugus* and *M. septentrionalis*), providing a simplified system in which we can understand how bat gene flow affects the spread of WNS.

Limited gene flow and the maintenance of distinct genetic populations of bats at smaller scales have implications for assessing impacts of other sources of mortality.

Although migratory bat species are most heavily impacted by wind energy, an estimated 51,617–106,925 *M. lucifugus* were killed by North American wind turbines from 2000 to 2011, and this number is expected to increase because the capacity of wind energy has continued to increase (Arnett and Baerwald 2013). This level of mortality appears low relative to historic population sizes, or when each year's mortality is viewed as a discrete event. However, bats are long-lived, exhibit low, density-independent fecundity, and cannot recover rapidly from cumulative increases in mortality and subsequent population declines. Even a small, persistent increase in adult mortality could cause extirpation in just a few decades when combined with mortality from WNS (Arnett and Baerwald 2013). Low gene flow compounds this problem, as metapopulation theory predicts a low probability of recolonization of extirpated subpopulations when dispersal among neighbouring subpopulations is low (Hanski 1982). Moreover, dependence on coloniality for reproduction in temperate, hibernating bats creates strong potential for Allee effects as populations decline (Gregory and Jones 2011).

Characterizing genetic structure of host populations at the leading edge of an oncoming pathogen can also facilitate studies of host-pathogen co-evolution. Biologically relevant populations delimited using neutral markers are appropriate units within which to test functional genes for signatures of selection (Manel et al. 2010; Keller et al. 2011; Kyle et al. 2014). When virulent and rapidly spreading wildlife diseases cannot be artificially controlled or treated, adaptation to the pathogen becomes the host population's only chance of endurance (Laine et al. 2010; Thrall et al. 2012). No effective treatments for WNS are currently available and it is unlikely that *Pd* can be removed from infected hibernacula. However, a suite of bat species in Europe have apparently adapted to *Pd* (Puechmaille et al. 2011), presumably via co-evolution with the fungus and selection for *Pd*-resistant individuals. Similarly, the evolutionary potential of affected North American species to adapt to the pathogen will depend on the presence of *Pd*-resistant individuals and the time-scale on which selection can occur. Systems such as ours provide an opportunity to investigate effects of a massive, pathogen-mediated selective sweep within multiple differentiated sub-populations, and study "real-time" evolution of a susceptible host in the wild.

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