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RESEARCH PAPER

# Evidence for recent population bottlenecks in northern spotted owls *(Strix occidentalis caurina)*

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Abstract The northern spotted owl *(Strix occidentalis caurina)* is one of the most controversial threatened subspecies ever listed under the US Endangered Species Act. Despite protection of its remaining forest habitat, recent field studies show continued declines of northern spotted owls. One potential threat to northern spotted owls which has not yet been shown is loss of genetic variation from population bottlenecks. Bottlenecks can increase the probability of mating among related individuals, potentially causing inbreeding depression, and can decrease adaptive potential. Here we report evidence for recent bottlenecks in northern spotted owls using a large genetic dataset (352 individuals and 10 microsatellite loci). The signature of bottlenecks was strongest in the Washington Cascade Mountains, in agreement with field data. Our results provide independent evidence that northern spotted owls have recently declined, and suggest that loss of genetic variation is an emerging threat to the subspecies' persistence. Reduced effective population size ($N_e$) shown here in addition to field evidence for demographic decline highlights the increasing vulnerability of this bird to extinction.

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## Introduction

The debate over northern spotted owl *(Strix occidentalis caurina)* conservation and logging is one of the most famous chapters in conservation history. Population declines stemming from harvest of the northern spotted owl's old forest habitat in the US Pacific Northwest led to listing of the subspecies as threatened under the US Endangered Species Act in 1990 (US Fish and Wildlife Service 1990). In response, the percentage of federal forest land in the range of spotted owls allocated to reserves was increased to 77% under the Northwest Forest Plan in 1994, dramatically reducing timber harvest (Stokstad 2005; Noon and Blakesley 2006). Nonetheless, recent field studies indicate that northern spotted owls have continued to decline at an average rate of 3.7% per year and that declines are most severe in Washington state (Anthony et a1. 2006). Moreover, <4 pairs remain in the wild in British Columbia (Fenger et al. 2007).

Two possible causes for continued decline, in addition to habitat loss, is competition and hybridization with invasive barred owls *(Strix varia)* which have rapidly expanded into the range of northern spotted owls from their historic range in eastern North America (Kelly et a1. 2003; Haig et al. 2004b; Kelly and Forsman 2004; Olson et al. 2005; Anthony et a1. 2006; Funk et al. 2007). Barred owls first appeared in Washington in 1965 (Reichard 1974), in Oregon in 1974 (Taylor and Forsman 1976), and in California in 1981 (Dark et al. 1998). Another potential threat

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that has not yet been shown for northern spotted owls is loss of genetic variation when effective population size ($N_e$) decreases, known as a genetic bottleneck. The probability of mating with related individuals increases in small populations which can reduce survival and reproductive rates due to inbreeding depression (Crow and Kimura 1970). In turn, population growth rates can decline and extinction probabilities can increase (Saccheri et al. 1998). Decreased genetic variation also reduces adaptive potential (Burger and Lynch 1995). Thus, determining whether effective population sizes of northern spotted owls are decreasing is essential for fully understanding threats to the subspecies and possible reasons for its continued decline.

Genetic methods allow detection of population bottlenecks as well as population growth or expansion. Although these methods have tremendous potential for detecting changes in $N_e$, their power and limitations in natural populations are still being explored. One potential limitation is that bottleneck tests may not have the power to detect relatively slow, steady rates of population decline, as seen in northern spotted owls. There are several examples of the utility of genetic bottleneck tests for detecting catastrophic population crashes (Cornuet and Luikart 1996; Goossens et al. 2006), but their capacity to detect slower rates of decline is less clear. Nonetheless, it is critical to detect slow declines so that there is still time to act before populations decrease to the point where recovery is unlikely. Another potential limitation is that the genetic signal of historic population expansions may swamp the signal of recent declines, or vice versa, since expansions and declines have opposite effects on patterns of genetic variation. This problem may be particularly acute in temperate species such as northern spotted owls that are currently declining, but that likely expanded during Pleistocene interglacial cycles (as has been shown for many temperate species; e.g., Mila et al. 2000; Lessa et al. 2003).

Northern spotted owls provide an excellent opportunity to assess the utility of bottleneck tests to detect relatively slow declines because detailed demographic data is available for specific individuals and study areas from over 30 years of intensive capture-recapture analysis (Anthony et al. 2006). We genotyped 352 northern spotted owls across the subspecies' range at ten variable microsatellite loci to test for recent bottlenecks. We used two analyses with different temporal resolution and sensitivity to characterize timing and magnitude of bottlenecks: the Cornuet and Luikart (1996) method which is most effective at detecting very recent bottlenecks of low magnitude (Williamson-Natesan 2005; Beebee and Rowe 2001; Spear et al. 2006); and Garza and Williamson's (2001) M-ratio test which is best suited for detecting more severe, older bottlenecks (Williamson-Natesan 2005). Since field studies of northern spotted owls show recent declines of relatively low magnitude (Anthony et al. 2006), we predicted that bottlenecks would most likely be detected with the Cornuet and Luikart method. Using these analyses, our goal was to address two primary questions: (1) Is there a genetic signature of population decline in northern spotted owls despite relatively slow, steady declines?; and (2) If declines are detected, does the geographic pattern of decline identified with genetic data match the pattern observed in the field?

## Methods

### Sampling

We collected blood samples from 352 northern spotted owls from six regions across the subspecies' range from 1990 to 2006 following the American Ornithologists' Union protocol (Gaunt and Oring 1997; Fig. 1; Table 1). Ninety-four percent of these were collected from 1994 to 2006. We chose regions as an appropriate grouping for analysis because: (1) regions were genetically differentiated from each other (based on Funk et al. 2008 and the current analysis); (2) $N$ was large enough for each region to allow reasonable power to detect bottlenecks (mean = 59, range = 22-90; Table 1); (3) regions were biogeographically and ecologically distinct from each other; and (4) regions were separated from each other by habitat gaps (e.g., the Willamette Valley between the Oregon Coast Ranges and Oregon Cascade Mountains and the Umpqua Valley between the Oregon Coast Ranges and Klamath Mountains; Fig. 1), geographic features (e.g., the Columbia River between Washington and Oregon regions and Puget Sound between the Olympic Mountains and Washington Cascade Mountains), and distance. No known close relatives (parent-offspring or siblings) were included. Relatedness within regions, estimated using program KINSHIP (Goodnight and Queller 1999), tended to be slightly larger than zero (mean = 0.044, SD = 0.032), as expected in a random sample from a randomly mating population.

### Microsatellite data

DNA extraction, PCR, and fragment analysis were performed as described previously (Funk et al. 2007). All owls were genotyped at 10 variable microsatellite loci (Table 2) developed for Mexican spotted owls *(S. o. lucida;* loci: 6H8, 15A6, 13D8, 4El0.2; Thode et al. 2002), Lanyu scops owls *(Otus elegans botelensis;* loci: Oe3-7, Oe053, Oe128, Oe129, Oe149; Hsu et al. 2003, 2006), and ferruginous pygmy-owls *(Glaucidium brasilianum;* locus: FEPO5; Proudfoot et al. 2005). One of these loci (Oe128) and an additional microsatellite marker (Bb126) are diagnostic of

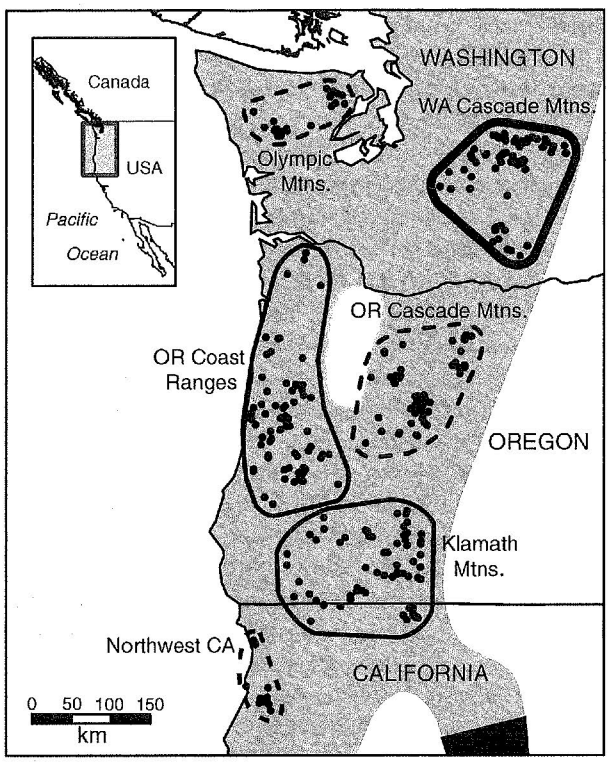


**Fig. 1** Recent population bottlenecks in northern spotted owls based on the Cornuet and Luikart method implemented in program BOTTLENECK. *Points* represent the 352 individual owls included in the analysis which are grouped into six regions. A statistically significant bottleneck ($\alpha = 0.05$) was detected in the Washington Cascades under 5, 10, and 15% multi-step mutation models (indicated by a *solid, bold outline*). Significant bottlenecks were detected in the Oregon Coast Ranges and Klamath Mountains under 10 and 15% multi-step mutation models (*solid outlines*). No significant bottlenecks were detected in the three remaining regions (*dashed outlines*). *Grey shading* shows the range of northern spotted owls and *black* the range of California spotted owls (*S. o. occidentalis*)

spotted versus barred owls (Funk et al. 2007) and were genotyped to assure that no barred owls or spotted owl-barred owl hybrids were included. One barred owl was detected which was removed from subsequent analyses.

**Table 2** Microsatellite loci used for population genetic analysis of northern spotted owls

| Locus | Repeat | Size range (bp) | No. alleles | $H_e$ |
|---|---|---|---|---|
| 6H8 | GATA | 86–114 | 8 | 0.80 |
| 15A6 | GATA | 142–166 | 7 | 0.67 |
| 13D8 | GATA | 167–195 | 8 | 0.73 |
| 4E10.2 | ATTTT | 196–246 | 11 | 0.83 |
| Oe3-7 | GATA | 118–138 | 6 | 0.77 |
| Oe053 | GATA | 200–224 | 7 | 0.73 |
| Oe128 | GATA | 315–327 | 4 | 0.64 |
| Oe129 | GATA | 253–285 | 9 | 0.75 |
| Oe149 | GATA | 236–276 | 10 | 0.73 |
| FEPO5 | AGAT | 258–286 | 8 | 0.80 |

$H_e$ = mean expected heterozygosity (Hardy–Weinberg heterozygosity). Primer sequences and annealing temperatures can be found in original references

## Standard population genetic analyses

Exact probabilities for Hardy-Weinberg (HW) proportions, linkage disequilibrium (LD), and allelic differentiation and $F_{ST}$ values (Weir and Cockerham 1984) were calculated using GENEPOP 3.4 (Raymond and Rousset 1995). Expected heterozygosities ($H_e$) and allelic richness ($R_S$) were calculated in MICROSATELLITE ANALYZER(Dieringer and Schlotterer 2003). Allelic richness is an index of the number of alleles corrected for sample size using rarefaction (El Mousadik and Petit 1996). In the Washington Cascades, Oregon Coast Ranges, Oregon Cascades, and Klamath Mountains, samples sizes were adequate (Table 1) to test whether genetic variation, measured as $H_e$ or $R_S$, decreased during the study period. For this analysis, owls were divided into those that hatched in 1999 or earlier versus those that hatched in 2000 or later, roughly the mid-point of the sampling period. Year of hatching was determined from long-term capture-recapture analysis of these owls (Anthony et al. 2006). Mann-Whitney tests were used to

**Table 1** Population genetic variation within northern spotted owl regions at ten microsatellite loci

| Region | $N$ | $H_e$ | $R_S$ |
|---|---|---|---|
| Olympic Mountains | 22 (21, 1) | 0.72 (NA) | 5.4 (NA) |
| Washington Cascade Mountains | 82 (61, 14) | 0.75 (0.74, 0.76) | 5.8 (5.4, 5.6) |
| Oregon Coast Ranges | 90 (30, 58) | 0.73 (0.73, 0.72) | 5.6 (5.3, 5.1) |
| Oregon Cascade Mountains | 57 (23, 18) | 0.74 (0.74, 0.75) | 5.9 (5.6, 5.4) |
| Klamath Mountains | 73 (49, 16) | 0.77 (0.77, 0.75) | 6.1 (5.7, 5.5) |
| Northwest California | 28 (28, 0) | 0.75 (NA) | 6 (NA) |

$N$ = sample size, $H_e$ = mean expected heterozygosity (Hardy–Weinberg heterozygosity), $R_S$ = mean allelic richness. $N$, $H_e$, and $R_S$ values in parentheses correspond to owls that hatched in 1999 or earlier (left) versus 2000 or later (right). Since some owls could not be positively assigned to a time period, total $N$ is greater than the sum of sample sizes for the 1999 or earlier and 2000 or later time periods. Sample sizes were insufficient in the Olympic Mountains and Northwest California to calculate $H_e$ or $R_S$ for different time periods (indicated by NA)

determine the significance of reductions in $H_e$ and $R_S$ between these two time periods.

### Cornuet and Luikart method for population bottleneck analysis

We tested for recent population bottlenecks in all six regions using an analysis developed by Cornuet and Luikart (1996). This method is based on the loss of rare alleles predicted in recently bottlenecked populations. It uses a single population sample to test whether there has been a recent reduction in allelic variation. Simulations (Cornuet and Luikart 1996; Williamson-Natesan 2005), theory (Garza and Williamson 2001), and case studies (Cornuet and Luikart 1996; Beebee and Rowe 2001; Goossens et al. 2006; Spear et al. 2006) all show that this is the best method available for detecting recent, low-magnitude declines in $N_e$. Simulation studies (Cornuet and Luikart 1996; Williamson-Natesan 2005) also demonstrate that this method has low type I error rates (i.e., falsely detecting a bottleneck when there is not one).

We used the program BOTTLENECK 1.2.02 (Piry et al. 1999) to implement this analysis. The stepwise mutation model (SMM) and the two-phase mutation model (TPM) were used to generate null distributions under mutation-drift equilibrium, as these are considered most appropriate for microsatellites (Shriver et al. 1993; Di Rienzo et al. 1994; Garza and Williamson 2001). A wide range of values was used for the percent of multi-step mutations (0, 1, 2, 3, 4, 5, 10, and 15%). The 0% multi-step mutation model is equivalent to a strict stepwise mutation model. The Wilcoxon signed rank test was used to determine significance of heterozygosity excess. A loss of rare alleles caused by a population bottleneck results in heterozygosity excess (defined as $H_e$, Hardy-Weinberg heterozygosity, minus $H_{eq}$, equilibrium heterozygosity at mutation-drift equilibrium). As explained by Piry et al. (1999), heterozygosity excess here never refers to the proportion of heterozygotes observed ($H_o$). Thus, BOTTLENECK does not test for an excess of heterozygotes ($H_o > H_e$), but rather heterozygosity excess ($H_e > H_{eq}$). Bottlenecks were also tested separately for owls that hatched in 1999 or earlier versus those that hatched in 2000 or later. A modified false discovery rate (FDR) method was used to correct for multiple comparisons (Narum 2006). Finally, to test the overall significance of bottlenecks across regions for each mutation model, combined P-values were calculated using Fisher's method and the Z-transform method (Whitlock 2005).

### Garza and Williamson;s *M-ratio* test

We also tested for bottlenecks using Garza and Williamson's (2001) M-ratio test. $M$ is the ratio of the number of alleles over the range in fragment sizes. After a severe bottleneck, $M$ is predicted to decline because the number of alleles should decrease faster than the range in fragment sizes (Garza and Williamson 2001). A comparison of Cornuet and Luikart's method with the M-ratio test using simulations demonstrated that the Cornuet and Luikart method is better at detecting more recent and less severe bottlenecks (Williamson-Natesan 2005). Thus we predicted, based on field data showing recent, slow rates of decline in northern spotted owls (Anthony et al. 2006), that the M-ratio test would be less likely to detect bottlenecks.

We estimated critical M-ratio values ($M_c$, below which bottlenecks are inferred) using the program *M-crit* (Garza and Williamson 2001). This program requires three parameters set by the user: theta (8) = 4 x effective population size ($N_e$) x mutation rate (?); proportion of mutations greater than one step; and average size of non one-step mutations. We used a strict stepwise mutation model and a two-phase mutation model with 15% multi-step mutations, spanning the full range of reasonable models. The average size of non one-step mutations was set to 2.8 which is the mean value for this parameter in the literature (Garza and Williamson 2001). Theta was estimated for each region using the Brownian micro satellite mutation model in program MIGRATE 2.4.1 (Beerli and Felsenstein 2001). For each run of MIGRATE,10 short chains of 10,000 sampled and 500 recorded trees, followed by three long chains of 100,000 sampled and 5,000 recorded trees were used. M-ratio tests were also conducted with small (half the estimated) and large (twice the estimated) values of theta to test the sensitivity of the results to this parameter.

## Results

### Standard population genetic analyses

Out of 60 tests for departures from Hardy-Weinberg (HW) proportions, only 3 (5%) were significant, as expected by chance. Out of 270 tests for LD, more were significant (28 tests) than expected by chance (13.5), but there was no evidence for consistent LD between any two loci, indicating that loci were independent. Mean pairwise $F_{ST}$ among regions was 0.024 (range = 0.012-0.041). Allelic differentiation was highly significant for all pairwise comparisons ($P < 0.001$).

Within regions, expected heterozygosity ($H_e$) ranged from 0.72 in the Olympic Mountains to 0.77 in the Klamath Mountains (Table 1). Allelic richness was also lowest in the Olympic Mountains ($R_S = 5.4$) and highest in the Klamath Mountains ($R_S = 6.1$). Owls that hatched in 2000 or later did not have significantly lower $H_e$ or $R_S$ than owls

that hatched in 1999 or earlier in the Washington Cascades ($N = 10$ loci for each time period, $P = 0.186$ for $H_e$ comparison, $P = 0.241$ for $R_S$ comparison), the Oregon Coast Ranges ($P = 0.678$ for $H_e$, $P = 0.734$ for $R_S$), the Oregon Cascades ($P = 0.970$ for $H_e$, $P = 0.910$ for $R_S$) or the Klamath Mountains ($P = 0.521$ for $H_e$, $P = 0.73$

Cornuet and Luikart method

We found a significant signature of population decline in the Washington Cascades using the Cornuet and Luikart method for the two-phase mutation model with 5, 10, and 15% of multi-step mutations and a significant signature of decline in the Oregon Coast Ranges and Klamath Mountains with 10 and 15% of multi-step mutations (based on Wilcoxon signed rank tests; Fig. 1; Table 3). Thus declines were significant in the Washington Cascades over a broader range of mutation models than in the Oregon Coast Ranges and Klamath Mountains. No significant declines were found in the other three regions (Olympics, Oregon Cascades, and Northwest California) under any mutation model. No bottleneck tests were significant under the stepwise mutation model or two-phase mutation model with 1-4% of multi-step mutations for any region. Combined P-values of the overall significance of bottleneck tests across regions were significant for two-phase mutation models with 3% or more multi-step mutations using Fisher's method and 2% or more multi-step mutations using the Z-transform method (Table 3).

Differences were observed between hatching time periods (1999 or earlier versus 2000 or later) in the significance of bottleneck tests for mutation models with low percentages of multi-step mutations (Table 3). Specifically, in the Washington Cascades, there was a significant bottleneck for owls that hatched in the later time period, but not for owls from the earlier time period under the two-phase mutation model with 5% multi-step mutations. Conversely, there were significant bottlenecks for owls from the earlier time period, but not for owls from the later time period in the Oregon Coast Ranges under 3-5% multi-step mutation models and in the Klamath Mountains under 2-5% multi-step mutation models. For these same three regions, bottlenecks were significant in both time periods for 10 and 15% multi-step mutation models. Bottlenecks were not significant for either hatching time period under any mutation model for the Oregon Cascade Mountains.

Garza and Williamson's M-ratio test

Bottlenecks were not detected using Garza and Williamson's M-ratio test which is most effective at detecting older,

**Table 3** *P*-values of bottleneck tests implemented in program BOTTLENECK for different northern spotted owl regions (top section of table) and combined *P*-values across regions for different mutation models (bottom section of table)

| Region | Percent multi-step mutations (under two-phase mutation model) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 10 | 15 | | |
| Olympic Mtns. | 0.216 | 0.188 | 0.188 | 0.188 | 0.161 | 0.138 | 0.080 | 0.065 | | |
| WA Cascade Mtns. | 0.278 (0.461, 0.097) | 0.138 (0.348, 0.097) | 0.080 (0.246, 0.065) | 0.065 (0.246, 0.053) | 0.065 (0.161, 0.042) | **0.009** (0.080, **0.016**) | **0.002** (**0.007**, **0.009**) | **0.001** (**0.005**, **0.005**) | | |
| OR Coast Ranges | 0.423 (0.053, 0.313) | 0.348 (0.053, 0.313) | 0.246 (0.053, 0.278) | 0.246 (**0.012**, 0.246) | 0.161 (**0.012**, 0.161) | 0.116 (**0.009**, 0.161) | **0.012** (**0.001**, **0.016**) | **0.003** (**0.001**, **0.009**) | | |
| OR Cascade Mtns. | 0.615 (0.116, 0.216) | 0.615 (0.097, 0.161) | 0.500 (0.097, 0.097) | 0.461 (0.097, 0.097) | 0.313 (0.065, 0.097) | 0.278 (0.080, 0.097) | 0.097 (0.053, 0.042) | 0.053 (0.053, 0.042) | | |
| Klamath Mtns. | 0.188 (0.097, 0.246) | 0.138 (0.053, 0.216) | 0.097 (**0.016**, 0.161) | 0.080 (**0.005**, 0.097) | 0.053 (**0.003**, 0.097) | 0.042 (**0.002**, 0.080) | **0.005** (**0.001**, **0.012**) | **0.002** (**0.001**, **0.005**) | | |
| Northwest CA | 0.246 | 0.246 | 0.216 | 0.188 | 0.138 | 0.138 | 0.080 | 0.065 | | |
| Combined *P*-value | | | | | | | | | | |
| Fisher's method | 0.272 | 0.144 | 0.061 | **0.043** | **0.015** | **0.002** | **0.000005** | **<0.000001** | | |
| *Z*-transform method | 0.122 | 0.058 | **0.019** | **0.013** | **0.003** | **0.0005** | **0.000001** | **<0.000001** | | |

In the top section of the table, *P*-values from Wilcoxon signed rank tests are the probability of observed heterozygosity excess under mutation-drift equilibrium and the given two-phase mutation model for each region (calculated using program BOTTLENECK). The model with 0 multi-step mutations is equivalent to a strict stepwise mutation model. *P*-values in parentheses correspond to bottleneck tests for owls that hatched in 1999 or earlier (left) versus 2000 or later (right). *P*-values of bottleneck tests significant (at the $\alpha = 0.05$ level) after correcting for multiple tests are in bold. In the bottom section of the table, *P*-values are combined probabilities to determine the overall significance of bottleneck tests across regions for each mutation model (calculated using Fisher's method and the *Z*-transform method). Combined *P*-values <0.05 are in bold

**Table 4** Results of *M*-ratio tests for population bottlenecks for different northern spotted owl regions

| Region | $\theta$ | $M$ | $M_c$ | |
|---|---|---|---|---|
| | | | SMM | TPM |
| Olympic Mountains | 5.19 | 0.957 | 0.858 | 0.716 |
| Washington Cascade Mountains | 7.33 | 0.986 | 0.867 | 0.742 |
| Oregon Coast Ranges | 5.69 | 0.965 | 0.900 | 0.772 |
| Oregon Cascade Mountains | 9.54 | 0.946 | 0.873 | 0.751 |
| Klamath Mountains | 8.63 | 0.940 | 0.882 | 0.764 |
| Northwest California | 7.21 | 0.926 | 0.854 | 0.721 |

$\theta = 4N_e\mu$; $M$ = the ratio of the number of alleles over the range in allele sizes; $M_c$ = critical value below which $M$ is significant at the $\alpha = 0.05$ level for the stepwise mutation model (SMM) and the two-phase mutation model with 15% multistep mutations (TPM)

more severe declines. Mean $M$ values ranged from 0.926 in Northwest California to 0.986 in the Washington Cascades (Table 4). All of these $M$ values were above the critical Me values regardless of the mutation model, and therefore were not statistically significant. Changing values of theta (by halving or doubling) used in M-ratio tests did not change the conclusion of no significant bottlenecks using this method.

## Discussion

### Recent bottlenecks in northern spotted owls

These results provide compelling evidence for recent population bottlenecks in northern spotted owls. Although the Cornuet and Luikart method does not provide an estimate of the timing of decline, two findings suggest that the genetic signal of decline reflects recent decreases in $N_e$, likely over the last few decades. First, the geographic pattern of decline detected with the Cornuet and Luikart method matches the overall pattern of demographic declines observed in a field study from 1985 to 2003 (Anthony et al. 2006). Like our study, Anthony et al. (2006) found that the most severe declines were in the Washington Cascades. Second, a significant bottleneck was detected for owls that hatched in the later part of the study (2000 or later), but not for owls that hatched in the first half of the study (1999 or earlier) in the Washington Cascades under the 5% multi-step mutation model (Table 3). This suggests that in this region, bottlenecks became increasingly severe later in the study. Interestingly, bottlenecks were detected in tile first half of the study, but not the second half in two other regions (Oregon Coast Ranges and Klamath Mountains) for some mutation models, suggesting more severe bottlenecks in the earlier part of the study in these regions. No significant reductions were observed in $H_e$ or $R_s$ between these sampling periods which is not particularly surprising given the short time interval (~10 years) relative to the life span of northern spotted owls (at least 16-17 years in the wild; Gutierrez et al. 1995). Additionally, as mentioned above, previous work (Cornuet and Luikart 1996; Beebee and Rowe 2001; Garza and Williamson 2001; Williamson-Natesan 2005; Goossens et al. 2006; Spear et al. 2006) demonstrates that the Cornuet and Luikart method is most effective at detecting recent changes in $N_s$.

Previous mitochondrial DNA (mtDNA) studies of northern spotted owls also provide some corroborating evidence for recent declines. In particular, Haig et al. (2004a) found positive Tajima's $D$ (Tajima 1989) values in some study areas, suggesting a decrease in population size. Although these values were not significant, this was likely due to much smaller sample sizes and the use of a single mitochondrial locus versus 10 microsatellite loci used here. Additionally, Barrowclough et al. (2005) were able to reject unimodal mismatch distributions, indicating that populations have not recently expanded, leaving stable or declining populations as remaining possibilities.

Our main finding of a significant bottleneck in the Washington Cascades agrees with Anthony et al.'s (2006) demographic study of northern spotted owls which also found that the most severe demographic declines were in this region (generally corresponding to the "Rainier", "Wenatchee", and "Cle Elum" study areas in Anthony et al. 2006). Our bottleneck tests for the other five regions agree with some of Anthony et al.'s (2006) demographic results, but not with other results. For example, as with Anthony et al.'s (2006) study, we found evidence for declines in the Oregon Coast Ranges ("Coast Ranges" and "Tyee" in Anthony et al. 2006) and Klamath Mountains ("Klamath" and "South Cascades" in Anthony et al. 2006). We did not, however, find evidence for bottlenecks in the Olympic Mountains (same in Anthony et al. 2006) or Northwest California ("NW California", "Hoopa", and "Simpson" in Anthony et al. 2006), regions in which Anthony et al. (2006) detected demographic declines. One reason for these discrepancies might be statistical. In particular, our sample sizes for the Olympics ($N = 22$) and Northwest California ($N = 28$) were relatively small, resulting in low power to detect bottlenecks. Another reason for these discrepancies might be biological. For example, the rate of demographic decline in Northwest California was relatively low, with annual population growth rates between 0.970 and 0.985 for the three Northwest California demographic study areas. Bottleneck tests are unlikely to detect low rates of population decline such as these. In addition, it is important to keep in mind that reductions in $N_e$ (detected with bottleneck tests) are

different than reductions in demographic population size (detected with demographic field studies) and reductions in one of these parameters does not necessarily result in a change in the other. Finally, our regions did not always perfectly overlap Anthony et al.'s (2006) demographic study areas. Nonetheless, our bottleneck tests and Anthony et al.'s (2006) demographic study both found striking evidence for declines in the Washington Cascades.

Bottleneck tests assume random mating (no population structure) and population closure (no gene flow). Non-random mating can produce genealogies that resemble bottlenecks, whereas gene flow is generally predicted to resemble recent expansion by introducing rare alleles (Cornuet and Luikart 1996; Goossens et al, 2006; Busch et al. 2007). Thus, an alternative explanation for the bottlenecks detected here is that they are artefacts of non-random mating (i.e., population structure) or gene flow. Agreement with HW proportions supported random mating within regions (i.e., lack of genetic substructure), but relatively low $F_{ST}$ values suggested gene flow among areas. Yet despite gene flow that can mimic recent expansion, we found a consistent signature of bottlenecks, providing even stronger evidence for recent reductions in $N_e$. Bottlenecks in northern spotted owls may therefore actually be more severe than they appear in our analyses.

Bottleneck tests were sensitive to the mutation model used, with more regions showing statistically significant bottlenecks with an increasing percent of multi-step mutations in the two-phase mutation model (Table 3). Previous studies support a variety of different mutational models for micro satellite loci, ranging from strict stepwise mutation models for some loci (Shriver et al. 1993) to two-phase models with 15-20% (Di Rienzo et al. 1994) of multi-step mutations. Perhaps the most thorough literature survey of fully resolved microsatellite mutations is that of Garza and Williamson (2001) which found that 12% of mutations were larger than one step. If a 12% multi-step mutation model is correct for northern spotted owls, then bottlenecks are significant for three regions: Washington Cascades, Oregon Coast Ranges, and Klamath Mountains. Although there is uncertainty regarding the correct mutation model, our results show evidence for significant bottlenecks in northern spotted owls for several reasonable mutation models.

As expected, bottlenecks were not detected using M-ratio tests, despite clear evidence for recent bottlenecks based on the Cornuet and Luikart method. Simulation tests of the relative performance of the Cornuet and Luikart method and M-ratio test show that the Cornuet and Luikart method has higher power to detect more recent, lower magnitude declines (Williamson-Natesan 2005). Since field data show that northern spotted owl declines have occurred recently (in the last few decades) at relatively slow, steady rates (Anthony et al. 2006), we predicted that there was a lower probability that we would detect these declines with M-ratio tests. Nonetheless, we observed many low frequency alleles (represented by only one or two copies) of intermediate size in several regions. Thus if northern spotted owls continue to decline, we would expect that many of these low frequency intermediate-sized alleles will drop out entirely in the neal' future, thereby reducing $M$ values and resulting in significant M-ratio tests.

More tests for LD were significant than expected by chance. Linkage disequilibrium can be caused by several different factors, including actual physical linkage between loci, population structure, hybridization, or genetic drift in small populations (Allendorf and Luikart 2007). We did not observe consistent LD between the same pair of loci across populations, suggesting that LD was not due to physical linkage between loci. Moreover, previous analyses showed that there is little population structure within regions (Funk et al. 2008), suggesting population structure is not the cause of LD. Hybridization between northern spotted owls and the other two subspecies of spotted owls (California spotted owls, S. *o. occidentalis,* and Mexican spotted owls, S. *o. lucida)* and/or genetic drift in small populations are more likely explanations for LD in northern spotted owls. Funk et al. (2008) demonstrated hybridization between northern spotted owls and the other two subspecies in some study areas, suggesting hybridization could contribute to LD. Additionally, evidence for bottlenecks presented above suggests that effective population sizes are decreasing which should in turn result in increased genetic drift and LD. Thus the finding of LD provides additional evidence for bottlenecks in northern spotted owls.

## Conservation implications

Evidence for genetic declines in northern spotted owls detected here with bottleneck tests indicates reduction in $N_e$ and genetic variation rather than demographic population size per se. In addition to habitat loss and the presence of invasive barred owls, northern spotted owls may become increasingly threatened by genetic factors stemming from reductions in $N_e$ such as inbreeding depression and loss of adaptive genetic variation. Inbreeding depression may reduce stage-specific survival and reproductive rates causing an increase in the rate of decline through a process termed an extinction vortex (Soule and Mills 1998). Anthony et al. (2006) found evidence for declines in survival and fecundity in some study areas which could be caused by the loss of genetic variation detected here. Thus it is possible that northern spotted owls are already caught in an extinction vortex. However, it is not possible to determine with our data whether inbreeding is contributing

to vital rate reductions, Regardless, future efforts to conserve northern spotted owl populations will require greater consideration of genetic threats to persistence,

Our results demonstrate the potential of genetic methods for monitoring changes in $N_e$ of threatened species (Schwartz et al. 2007). We detected declines in $N_e$ in spite of the relatively slow, steady rate of decline in northern spotted owl population sizes. For example, it is not surprising that the Cornuet and Luikart method was able to detect declines in orang-utans *(Pongo pygmaeus)* which have experienced declines of up to 33% in a single year (Goossens et al. 2006), In contrast, the average annual rate of decline in northern spotted owls from 1985 to 2003 across its range was only 3.7%, with a maximum average rate of 10.4% for a single study area (Anthony et al. 2006), This agrees with simulation studies showing that the Comuet and Luikart method is able to detect declines of relatively low magnitude (Williamson-Nates an 2005), Thus, genetic methods should become increasingly useful for population monitoring, especially as it becomes easier and less expensive to acquire large numbers of molecular markers which will provide greater power (Luikart et al. 2003),

## Conclusions

By combining different population genetic analyses, it was possible to arrive at a deeper understanding of genetic bottlenecks in northern spotted owls than would have been possible using a single approach, For example, only using the M-ratio test would have led to the conclusion that there are no population bottlenecks, despite field evidence to the contrary, On the other hand, only using the Cornuet and Luikart method would have revealed recent bottlenecks, but would not have provided any specific information on how recently these declines have occurred. By also conducting bottleneck tests between the earlier and later hatching time periods and using the M-ratio test, we now know that these genetic declines are very recent and of relatively low magnitude, as predicted based on field data, We recommend using a variety of analyses to extract as much information as possible out of genetic data about demographic trends of threatened and declining species,

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