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Streaked horned lark *Eremophila alpestris strigata* has distinct mitochondrial DNA

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Abstract

The Streaked Horned Lark (STHL; *Eremophila alpestris strigata*) is a federal candidate for listing under the Endangered Species Act. We evaluated the conservation status and level of genetic diversity of the STHL using the complete mitochondrial ND2 gene. We sampled 32 STHLs from the southern Puget Sound region, the Pacific coast, and Whites Island in the Columbia River of Washington, and additional 68 horned larks from Alaska, alpine and eastern Washington, Oregon, California, and Asia (outgroups). Our Maximum Likelihood analysis of 32 haplotypes identified three geographically concordant clades in Pacific coast states: Pacific Northwest (alpine and eastern Washington, Alaska), Pacific Coast (western Washington, California), and Great Basin (eastern Oregon). Each of the three clades was supported by bootstrap values ≥86%. The distance among them varied from 0.72 to 0.79% nucleotide divergence excluding intraclade variation. The relationship among the clades was not resolved. AMOVA also showed significant structuring of haplotypes among the three clades. Differences among clades accounted for 75.7% of sequence variation, differences among localities within clades accounted for 12.1%, and differences among individuals within localities accounted for the remaining 12.2%. Although STHL populations were closely related to the Californian sample, they appeared unique and isolated. All pairwise F_{st} values involving the STHL samples were significant (except between themselves). STHLs appear to have remarkably low genetic diversity; all 32 STHLs shared the same haplotype. Even with small sample sizes, all other localities had multiple haplotypes. Because the STHL appears to be unique and isolated, and to have little genetic diversity our data suggest it should be a conservation priority.

Introduction

The Streaked Horned Lark (STHL, *Eremophila alpestris strigata*) is a federal candidate for listing under the Endangered Species Act by the US Fish and Wildlife Service (Department of the Interior 2001). The Committee On the Status of Endangered Species in Canada lists the STHL as Endangered and it is on the Red List in British Columbia. It is listed as State Sensitive by the Oregon Department of Fish and Wildlife (Critical Status; Oregon Sensitive Species List 1997), and is a candidate for listing under the Washington State Endangered Species Act and has been proposed

as Endangered (Stinson 2005) and it is also considered a "priority species" for conservation by Oregon–Washington Partners in Flight (Altman 2000) and British Columbia Partners in Flight (Fraser et al. 1999).

The STHL once bred from southern British Columbia (Campbell et al. 1997) south through the Puget trough, on the outer coast of western Washington (Jewett et al. 1953), and in the Willamette Valley of Oregon south to the Rogue River Valley (Gabrielson and Jewett 1940; Gilligan et al. 1994). It no longer breeds in British Columbia, the northern Puget Sound, or the Rogue River Valley (Altman 1999; Rogers 2000; Beauchesne and Cooper 2003; Stinson 2005; Figure 1). Historically it was a common to abundant breeder in suitable habitats (Bowles 1900; Dawson and Bowles 1909; Gabrielson and Jewett 1940; Jewett et a1. 1953; Browning 1975; Campbell et al. 1997). Surveys conducted in the late 1990s detected only 49–58 birds in western Washington (Rogers 1999; MacLaren 2000) and approximately 150 singing males in Oregon (Altman 1999). However, recent research by S.F.P. suggests that the total number of breeding birds in Washington and Oregon may be as many as 800. From 0% to 58% of STHL nests fledge at least one young depending on the year and location (Altman 1999; Pearson 2003; Pearson and Hopey 2004, 2005), Nest success for other horned lark populations in North America range from 63% to 86% (Beason 1995). The apparent population decline and low nest success (Altman 1999; Pearson 2003; Pearson and Hopey 2004, 2005) raises concerns about the viability of its small populations.

Habitat loss is apparently the main reason for the population decline of the STHL at least in some parts of its historic range. The STHL was a common breeder in the glacial outwash or Puget prairies of the south Puget Sound area (Dawson and Bowles 1909; Bowles 1898, 1900; Suckley and Cooper 1860). Crawford and Hall (1997) estimated the historic distribution of grasslands in the southern Puget Sound region by mapping grassland soils. Currently, grasslands occupy approximately 22% of their historic distribution and prairies dominated by native species occupy approximately 3% of the historic grassland distribution. The loss of these grasslands has been attributed to urban development (33%), forest invasion or conversion (32%) and agriculture

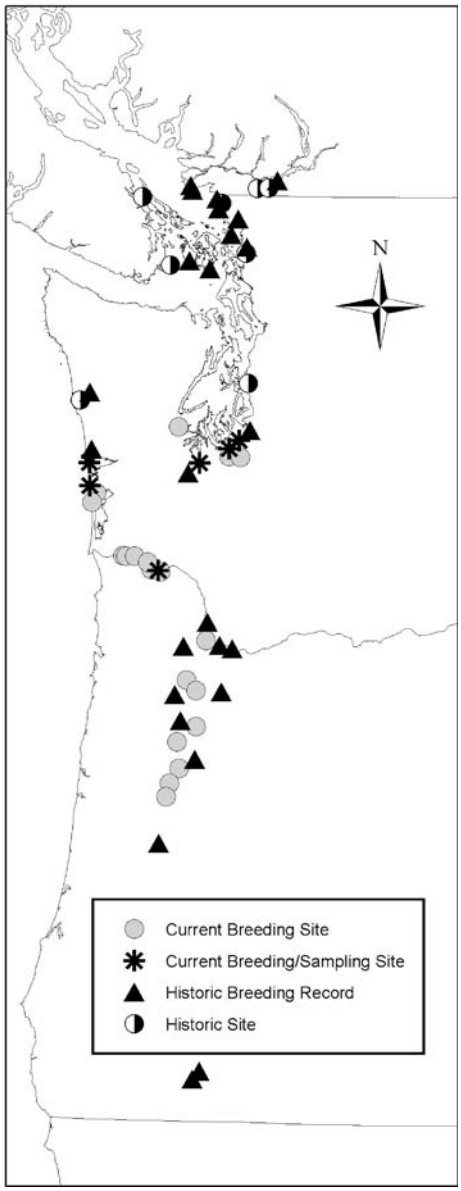

Figure 1. Locations of current and historic breeding sites of Streaked Horned Lark, its current breeding sites where tissue samples were also collected, and "historic sites" which consist of possible nesting sites or uncertain Streaked Horned Lark locations.

conversion (30%; Crawford and Hall 1997). Currently, there are only six known sites in the southern Puget Sound region inhabited by STHL, and four of these are associated with airports (Rogers 1999, 2000; MacLaren and Cummins 2000; Pearson 2003; Pearson and Hopey 2004, 2005). Even more dramatic grassland losses have occurred in the Willamette Valley of Oregon, where more than

99% of the pre-settlement grasslands have been destroyed (Johannessen et al. 1971; Towle 1982).

In our study, we use sequences of the complete mitochondrial ND2 gene (1041 bp) to address two aspects of population genetics of the STHL. First, we examine the relationship between this subspecies and neighboring populations of horned lark.

Although the STHL is relatively well defined phenotypically (Behle 1942; AOU 1957; Beason 1995), its evolutionary relationships with other populations/subspecies are unknown. Numerous population genetics studies recently reviewed by Zink (2004), showed that 97% of continentally distributed avian subspecies are not evolutionary distinct units, a finding that challenges whether they should be given management or conservation status. Thus, determining management priorities for the STHL might be affected by knowing whether it is an isolated, declining population with a unique evolutionary history, or merely a peripheral population of an abundant, widely distributed Holarctic species. Second, we assess the effects of the population decline on genetic variability in STHLs by comparing levels of mtDNA diversity in STHLs from the southern Puget Sound to that of horned larks from areas to the north (Alaska), east (eastern and alpine Washington, eastern Oregon), and south (California).

Methods

Ninty-eight horned lark samples were obtained from 9 localities across the US Pacific Coast States (Table 1). We used two additional horned larks from Asia, one from Central Asia (UWBM: cds4912) and one from Yamal Peninsula (UWBM: svd1427) as outgroups for phylogenetic analysis of Nearctic haplotypes. Samples of STHL were obtained during breeding seasons in 2002–2004 in the Puget lowlands ($n = 22$), on Pacific coast ($n = 5$), and Whites Island in the Columbia River ($n = 5$), all in Washington. In 2002 S.F.P. salvaged an unhatched egg or a dead chick from eight STHL nests after broods were fledged, and in 2003 he pulled 1–2 growing contour feathers from a single chick from an additional 13 STHL nests, Finally, in 2004 S.F.P, collected 11 samples of feathers from chicks (no more than one per nest) or blood samples from adults. These samples are housed at the University of Washington Burke Museum. Samples from other localities in Washington, Alaska, Oregon, and California were supplied by the University of Washington Burke Museum, US National Museum of Natural History, University of Alaska Museum, and Museum of Vertebrate Zoology (Table 1).

We sequenced the complete mitochondrial ND2 gene (1041 bp) for all individuals in both directions. We chose the ND2 gene because it harbors enough

Table 1 Localities, sample sizes, institutions that supplied samples, sample identification numbers, nucleotide diversity (π_n) and its standard deviation (sd) for each locality

Locality	Coordinates	N	Institution(N)	Tissue numbers	$\pi_n \times 10^3$	sd$\times 10^3$
AK_i	65.1°N 146.1°W	18	USNMNH(8), UWBM(3), UAM (7)	B13413, 13421-2, 13425-6, 13485, 13492-3, dab686, 688-9, uamx14, 775, 790, ksw1478-9, 1485, 1500	0.747	0.638
AK_c	65. 8°N 165.8°W	5	UWBM (5)	svd2365-9	1.729	1.388
CA	37.6°N 121.5°W	19	MVZ (19)	nkj5923-33, nkj6003-10	1.775	1.190
OR	42.6°N 120.2°W	5	UWBM (5)	svd2356-60	5.187	3.515
WA_o	47.1°N 122.6°W	22	UWBM (22)	evl481-4, gkd375-7, rcf2596, smb397-8, 401-8, 410-412	0.000	n/c
WA_c	46.8°N 124.1°W	5	UWBM(5)	smb413-7	0.000	n/c
WA_i	46.1°N 123.3°W	5	UWBM(5)	smb418-422	0.000	n/c
WA_e	47.4°N 119.4°W	16	UWBM (16)	bks1405, 1417, 1419, csw5140a-1a, 5717-8, 6422-3, plg216, dab411, svd2197-200, 2207	2.626	1.646
WA_a	48.8°N 121.4°W	4	UWBM (4)	sar7267, svd999-1001	0.961	0.952

Localities: AK_i – interior AK; AK_c –Bering Sea coast, AK; WA_o – vicinity of Olympia, WA; WA_c – Pacific coast of WA; WA_i – White island, WA; WA_e – eastern WA; WA_a – alpine tundra, Cascade mountains, WA. Institutions: UWBM – University of Washington Burke Museum, USNMNH – US National Museum of Natural History, MVZ – Museum of Vertebrate Zoology, UAM – University of Alaska Museum.

variation for population level comparisons and has been used successfully for phylogeographic studies (e.g. Drovetski et al. 2004a). Variation in ND2 is evenly distributed along the entire length of the gene (unlike Control Region), resulting in many haplotypes and reducing the probability of multiple substitutions at a single position; the latter often results in the clock-like phylogenetic trees based on ND2 sequences. DNA extraction, PCR profile, primers, sequencing, sequence alignment, and maximum likelihood (ML) phylogenetic analysis are described elsewhere (Drovetski et al. 2004b). Nucleotide diversity (π_n) and pairwise F_{st} values were calculated in Arlequin 2.000 (Schneider et al. 2000). The same program was also used for AMOVA (Excoffier et al. 1992) and Mantel test (Mantel 1967). We used DnaSP 4.01 (Rozas et al. 2003) to calculate tests for detecting population growth, Fu's F_s (Fu 1997) and the R_2 (Ramos-Onsins and Rozas 2002), and McDonald-Kreitman neutrality tests (McDonald and Kreitman 1991).

Results

Phylogenetic analysis of haplotypes

Among 98 individuals of horned lark from Pacific Coast states, 30 unique ND2 haplotypes were distinguished based on 43 substitutions (39 transitions and 4 transversions) at 42 variable sites. Guanine was underrepresented, and cytosine was overrepresented in these sequences (A = 29.89%, C = 36.92%, G = 10.49%, T = 22.70%; *G*-test $P < 0.0001$). All haplotypes shared this nucleotide bias and there was no evidence of base composition heterogeneity among them. GeneBank accession numbers for the ND2 sequences are DQ187388–DQ187487.

The Akaike information criterion (AIC; Akaike 1974) implemented in Modeltest 3.06 (Posada and Crandall 1998) selected the TIM + *I* model for the ML analysis of phylogenetic relationships among haplotypes. TIM is a submodel of the GTR model (Rodriguez et al. 1990) in which transitions are weighted differentially, A–C and G–T transversions are the only transversions present and are weighted equally, and *I* identifies the proportion of invariable sites in the dataset (Posada and Crandall 1998). This analysis resulted in a single tree (Figure 2). The molecular clock assumption for this tree was not rejected ($-\ln L$ without molecular clock enforced = 1789.79818, $-\ln L$ with molecular clock enforced = 1799.89069; $-2\Delta\ln L = 20.18502$, d.f. = 30, $P = 0.9116$).

Our horned lark haplotypes formed three geographically concordant clades. A northern clade combined all but a single individual from eastern Washington, all four individuals from Washington alpine habitats, all individuals from Alaska, and a single individual from eastern Oregon (Pacific Northwest clade; Figure 2). A Pacific Coast clade included all of the STHLs and 17 of 19 birds from the San Joaquin Valley of California and a single eastern Washington bird. A Great Basin clade included the other two Californian larks and 4 of 5 eastern Oregon birds.

Only four individuals (three haplotypes) were out of place geographically (Figure 2). Two of these three haplotypes were found to the south of their area of dominance: a northern haplotype from eastern Washington in the Great Basin of Oregon (svd2358), and a Great Basin haplotype from Oregon in two western California birds (nkj5930 and nkj6005). The third was found to the east of its typical range, a STHL haplotype in eastern Washington (p1g216), which historically may have been a part of the wintering range of STHL (AOU 1957). This bird (plg216) had the same haplotype that was shared by all 32 STHLs, but did not have the color characters of STHL.

The monophyly of the three western Nearctic clades was strongly supported by bootstrap analysis (96%), and each clade was supported by bootstrap values ≥86%. However, the relationship among the three clades was unresolved. ML divergence between clades (excluding intraclade variation) varied from 0.72% to 0.79%. ML pairwise differences among haplotypes within clades and their standard deviations (±s.d.) were 0.29 ± 0.12% for the Pacific Northwest clade, 0.28 ± 0.10% for the Pacific Coast clade, and 0.13 ± 0.06% for the Great Basin clade. The latter is likely an underestimate due to small sample size.

Population genetics analysis

The McDonald–Kreitman test (McDonald and Kreitman 1991) which compares the proportion of replacement substitutions in fixed differences between clades with that in intraclade polymorphisms did not detect a significant deficit or an

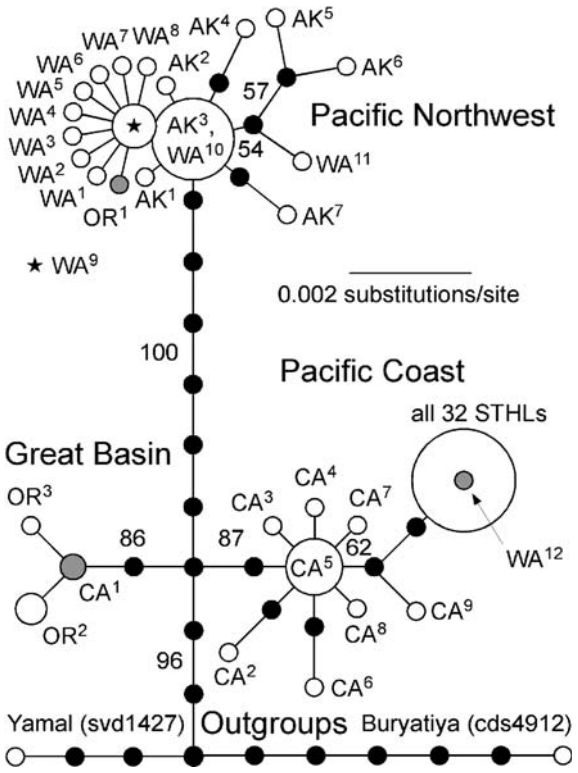

Figure 2. ML phylogram of horned lark haplotypes. Black circles – unsampled haplotypes, white – observed haplotypes, gray – haplotypes found outside of their range. Numbers next to branches are bootstrap values (1000 replicates with random addition of taxa). Circle area reflects number of individuals. AK^1 = B13425; AK^2 = uamx790; AK^3 = uamxl4, 775, B13413, 13422, 13426, 13485, 13492-93, ksw1478, 1485, 1500, dab686, 688-9; AK^4 = ksw1479; AK^5 = B13421; AK^6 = svd2369; AK^7 = svd2368; CA^1 = nkj5930, 6005; CA^2 = nkj6009; CA^3 = nkj6008; CA^4 = nkj6007; CA^5 = nkj5923-5, 5927, 5929, 5931-2, 6003-4, 6006; CA^6 = nkj5926; CA^7 = nkj5933; CA^8 = nkj6010; CA^9 = nkj5928; OR^1 = svd2358; OR^2 = svd2356, 2359-60; OR^3 = svd2357; WA^1 = bks1405; WA^2 = bks1419; WA^3 = csw5140a; WA^4 = csw5717; WA^5 = csw6422; WA^6 = dab411, WA^7 = svd2199; WA^8 = svd2207; WA^9 = bks1417, csw5141a, 5718, svd2197-8, 2200; WA^{10} = csw6423, sar7267, svd999-1000, 2365-7; WA^{11} = svd1001; WA^{12} = p1g216.

excess of replacement substitutions in fixed differences (Fisher's exact *P*-values varied from 0.534 to 1.000), a result suggesting that differences among clades are not the result of strong selection on ND2 sequences. Fu's F_S (Fu 1997) and R_2 (Ramos-Onsins and Rozas 2002) tests were significant only for the Pacific Northwest clade ($P = 0.003$ and $P < 0.0001$, respectively), which is likely reflects a recent demographic expansion of this lineage related to recolonization of glaciated areas in Washington, Canada, and Alaska. The other two clades that inhabited ice-free areas during the last glacial maximum do not appear to carry a signature of recent demographic expansion. Fu's F_S (Fu 1997) and R_2 (Ramos-Onsins and Rozas 2002) *P*-values for the Pacific Coast clade were $P = 0.130$ and $P = 0.276$, and for the Great Basin clade were $P = 0.434$ and $P = 0.550$, respectively.

Like the preceding phylogenetic analysis, AMOVA (Excoffier et a1. 1992) indicated that most of the sequence variation was due to differences among the clades (75.7% of observed sequence variation). Differences among individuals within localities accounted for 12.2% of the sequence variation, and differences among localities within clades accounted for 12.1% of the sequence variation. These data indicate a high degree of mtDNA differentiation among clades and among some localities.

The high level of differentiation among clades was also evident in the pattern of pairwise F_{st} values (Table 2). All of the pairwise comparisons of samples from different clades resulted in significant F_{st} values. Most of the pairwise comparisons within clades resulted in non-significant F_{st} values with a few exceptions: comparisons between the California sample and all three STHL localities and between eastern Washington and each of the two Alaska localities. A Mantel test (Mantel 1967) showed no significant relationship between pairwise F_{st} values and straight-line distances between localities ($r = -0.074$, $P = 0.513$) indicating that the observed population structuring was not due to isolation by distance.

Nucleotide diversity varied between $0.747 \times 10^{-3} \pm 0.638 \times 10^{-3}$ in the interior Alaska to $5.187 \times 10^{-3} \pm 3.515 \times 10^{-3}$ in Oregon (Table 1). All 32 individuals of STHL sampled in three localities shared a single haplotype (Figure 2), which is uncharacteristic for other sampled localities. Even in alpine Washington, coastal Alaska, and eastern Oregon, we found multiple haplotypes despite sample sizes 6–8 times smaller than our combined sample of STHLs.

Discussion

Relationship between STHL and other extreme western Nearctic larks

Our data identified the presence of geographically concordant phylogenetic structuring of haplotypes

Table 2. Pairwise F_{st} values (below diagonal) and their *P*-values (above diagonal)

	AK_i	AK_c	CA	OR	WA_o	WA_c	WA_i	WA_e	WA_a
AK_i	–	0.054	0.000	0.000	0.000	0.000	0.000	0.000	0.459
AK_c	0.120	–	0.000	0.018	0.000	0.000	0.000	0.000	0.748
CA	0.866	0.825	–	0.000	0.000	0.000	0.000	0.000	0.000
OR	0.785	0.617	0.549	–	0.000	0.009	0.009	0.000	0.036
WA_o	0.971	0.976	0.770	0.885	–	0.991	0.991	0.000	0.000
WA_c	0.950	0.931	0.647	0.707	0.000	–	0.991	0.000	0.000
WA_i	0.950	0.931	0.647	0.707	0.000	0.000	–	0.000	0.018
WA_e	0.311	0.226	0.786	0.630	0.906	0.837	0.837	–	0.117
WA_a	−0.006	−0.016	0.831	0.613	0.989	0.966	0.966	0.194	–

and some population differentiation within clades. The 30 haplotypes identified among 98 western Nearctic horned larks formed three geographically concordant clades: Pacific Northwest, Pacific Coast, and Great Basin. Only four individuals carried three haplotypes typical of other geographic regions.

In their review, Funk and Omland (2003) identified five main causes of species-level paraphyly and polyphyly: inadequate phylogenetic information, imperfect taxonomy, interspecific hybridization, incomplete lineage sorting, and unrecognized paralogy. These same causes may account for paraphyly of the intraspecific lineages observed in our study. It appears that ND2 sequences provide enough phylogenetic information for identifying clades within western horned larks and resolving relationships among them. S.F.P. and S.R. determined that two (svd 2358 and plg 216) of the four individuals caring "misplaced" haplotypes had phenotypes appropriate for their location excluding the possibility of misidentification. The other two individuals (nkj 5930 and nkj 6005), although prepared as skeletons, were originally identified as *E. a. actia*, a subspecies inhabiting the area where they were collected. The observed pattern of substitutions and clock-like evolution of ND2 sequences is inconsistent with the presence of nuclear copies (paralogy) in our dataset (see also below). Therefore, either incomplete lineage sorting or hybridization is likely to account for the three "misplaced" haplotypes.

The STHL haplotype appeared to be distantly related to the haplotypes found in the three closest localities – alpine and eastern Washington and eastern Oregon. It was imbedded within the western California sample, where it appeared to be one of the most recently evolved haplotypes. Our finding of the recently evolved STHL haplotype in an eastern Washington individual is inconsistent with incomplete lineage sorting because STHL haplotype apparently evolved after the Pacific Northwest and Pacific Coast clades diverged (Figure 2). On the other hand, our tree is similar to that expected for introgression of mtDNA of one lineage into the other (Funk and Omland 2003; Figure 1g) in respect to all three "misplaced" haplotypes. The presence of the STHL haplotype in eastern Washington could have resulted from a STHL female wintering in eastern Washington pairing with a local male. Horned larks breed much earlier in eastern than in western Washington, presumably because there is so much spring rain in the west (Rohwer et al. unpublished data). A similar mechanism may be responsible for our finding of two other haplotypes south of their respective breeding ranges.

All pairwise F_{st} values involving STHL samples were significant, indicating that the STHL population is differentiated and well isolated from all other sampled localities, including western California. Although the STHL was historically a part of a larger Pacific Coast lineage of horned larks, it has been evolving independently for some time and can be considered a distinct evolutionary unit, making it more deserving of conservation attention.

Alternative explanations for low mtDNA diversity in STHL

Our data showed the overall diversity in horned lark mtDNA to be similar to that in other Holarctic passerines. For example, mean ML pairwise distances among haplotypes within horned lark clades (0.13–0.29%) were comparable to that of

winter wren *Troglodytes troglodytes* clades (0.20–0.34%; Drovetski et al. 2004b) and Rosy-finches *Leucosticte* (0.19–0.43%; Drovetski et al. unpublished) indicating similarity in intraclade mtDNA variation among these taxa. Despite the normal levels of overall mtDNA variation, we found no variation in STHL ND2 sequences; all 32 individuals shared the same haplotype. All other localities had multiple haplotypes, including alpine Washington, where only four individuals were sampled. Such severe reduction of mtDNA diversity may have several explanations: (1) a nuclear copy of ND2 was sequenced for STHLs, (2) selective sweep in STHL, (3) recent northward expansion from California along the Pacific coast, or (4) a severe bottleneck caused by the range contraction and habitat loss due to human activity.

It is unlikely that we sequenced a nuclear copy of ND2. There are no stop colons or indels in our sequences. The samples came from different sites and were sequenced in different laboratories using different sequencing machines. Furthermore, the tree is consistent with the molecular clock and the STHL haplotype is one of the most recently evolved. Nuclear copies are likely either to be distantly related to mtDNA haplotypes, or to introduce branch length heterogeneity in the tree. Neither is true for the STHL haplotype.

The selective sweep hypothesis has little support. The McDonald–Kreitman (McDonald and Kreitman 1991), Fu's F_s (Fu 1997), and R_2 (Ramos-Onsins and Rozas 2002) tests found no significant deviation from genetic variation expected under neutrality in the Pacific Coast clade. Also, a selective sweep within a single population should cause noticeable branch length heterogeneity.

The hypothesis of recent northward expansion from California is also inconsistent with our data because we found evidence of a recent demographic expansion only in the Pacific Northwest clade, and not in the Pacific Coast clade. In the Pacific Northwest clade, all four populations share the same haplotypes despite very large geographic distances separating these samples. Although the distances between the California and STHL localities are much smaller than the distances between localities in the Pacific Northwest clade, the California sample does not have the STHL haplotype. Furthermore, pairwise F_{st} values were not significant in the Pacific Northwest clade, but they were significant between California and the STHL localities. These contrasts provide evidence for continuing or recent gene flow between localities within the Pacific Northwest clade and for lack of gene flow between the California and STHL localities.

The lack of evidence for selection, expansion, or gene flow in the Pacific Coast clade is consistent with the bottleneck hypothesis. Furthermore, the data on STHL population declines and habitat loss during last 100–130 years (Figure 1 and Introduction) provide independent support for the bottleneck hypothesis and are inconsistent with a hypothesis of recent northward expansion. Thus, our mtDNA data support recent conservation concerns about the STHL and its conservation status by federal and state Fish and Wildlife authorities. Indeed, the combination of its small population size (~800 individuals), severe loss of suitable habitat (97% for Puget lowlands and Willamette Valley), independent evolutionary history, current isolation, and possible high level of inbreeding may warrant an upgrade of the STHL's conservation status.

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