Subspecific relationships and genetic structure in the spotted owl

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Abstract

Hierarchical genetic structure was examined in the three geographically-defined subspecies of spotted owl (*Strix occidentalis*) to define relationships among subspecies and quantify variation within and among regional and local populations. Sequences (522 bp) from domains I and II of the mitochondrial control region were analyzed for 213 individuals from 30 local breeding areas. Results confirmed significant differences between northern spotted owls and the other traditional geographically defined subspecies but did not provide support for subspecific level differences between California and Mexican spotted owls. Divergence times among subspecies estimated with a 936 bp portion of the cytochrome *b* gene dated Northern and California/Mexican spotted owl divergence time to 115,000–125,000 years ago, whereas California/Mexican spotted owl divergence was estimated at 15,000 years ago. Nested clade analyses indicated an association between California spotted owl and Mexican spotted owl haplotypes, implying historical contact between the two groups. Results also identified a number of individuals geographically classified as northern spotted owls (*S. o. caurina*) that contained haplotypes identified as California spotted owls (*S. o. occidentalis*). Among all northern spotted owls sampled (n = 131), 12.9% contained California spotted owl haplotypes. In the Klamath region, which is the contact zone between the two subspecies, 20.3% (n = 59) of owls were classified as California spotted owls. The Klamath region is a zone of hybridization and speciation for many other taxa as well. Analyses of population structure indicated gene flow among regions within geographically defined subspecies although there was significant differentiation among northern and southern regions of Mexican spotted owls. Among all areas examined, genetic diversity was not significantly reduced except in California spotted owls where the southern region consists of one haplotype. Our results indicate a stable contact zone between northern and California spotted owls, maintaining distinct subspecific haplotypes within their traditional ranges. This supports recovery efforts based on the traditional subspecies designation for the northern spotted owl. Further, although little variation was found between California and Mexican spotted owls, we suggest they should be managed separately because of current isolation between groups.

Introduction

The vagility of birds has frequently given rise to hybridization among closely-related species. Use of mitochondrial markers has been helpful in resolving these relationships (e.g., Avise et al. 1990; Tegelstrom and Gelter 1990; Seutin et al. 1993; Zink 1994; Lovette et al. 1999; Sattler and Braun 2000; Rohwer et al. 2001). The implications of this hybridization to speciation and conservation status can be profound (Rhymer and Simberloff 1996; Wolf et al. 2001; Allendorf et al.

2001; Haig et al. 2004; Haig and Allendorf 2004). While commonly occurring, less attention has been directed at intraspecific hybridization, introgression, and genetic mixing in birds (although see Fleischer et al. 1991; Zink et al. 1991, 2001; Goodstrey et al. 1998; Bensch et al. 1999; Rhymer et al. 2001; Glenn et al. 2002). Furthermore, a recent decision by the U.S. Fish and Wildlife Service to list distinct population segments under the U.S. Endangered Species Act has stimulated greater interest in these topics. To date, most of these cases have involved non-avian taxa (e.g., Allendorf and Leary 1988; Bernatchez 1995; Firestone et al. 1999; Parker et al. 1999; Roman et al. 1999; although see Avise and Nelson 1989; Young and Allard 1997; Nelson et al. 2000; Zink et al. 2000). In this study, we examine population structure as well as relationships within and among geographically-defined subspecies for a threatened avian species with little morphological variation yet historical divergence among subspecies. Subspecific definition and identification of contact among subspecies has immediate consequences for their listing and/or delisting under the U.S. Endangered Species Act.

The spotted owl (*Strix occidentalis*) is one of the USA's most controversial threatened species. Issues surrounding the species have arisen from their association with declining, fragmented, late-successional forests in the Pacific Northwest and Southwestern U.S. (Thomas et al. 1990; U.S. Forest Service and U.S. Bureau of Land Management 1994; U.S. Fish and Wildlife Service 1995). Spotted owls are long-lived, primarily non-migratory, have high site fidelity, low reproductive rates, and large home ranges (summarized in Forsman et al. 1984, 2002; Franklin et al. 2004; Gutiérrez et al. 1995, Miller et al. 1997).

Spotted owls have a locally fragmented, but almost continuous distribution from British Columbia to central Mexico (Figure 1, Gutiérrez et al. 1995). Currently, there are three recognized subspecies (American Ornithologists' Union 1957, Figure 1). Northern spotted owls (NSO, *S. o. caurina*) are resident from southern British Columbia to central California. California spotted owls (CSO, *S. o. occidentalis*) occur in the Sierra Nevada Mountains in eastern California and coast ranges in southern California. Mexican spotted owls (MSO, *S. o. lucida*) are distributed in a network of island populations in the

canyonlands of the southwest U.S. through central Mexico (Gutiérrez et al. 1995). Initial subspecies designations were based on plumage color and body size (Oberholser 1915), although significant differences have not been described (Gutiérrez et al. 1995).
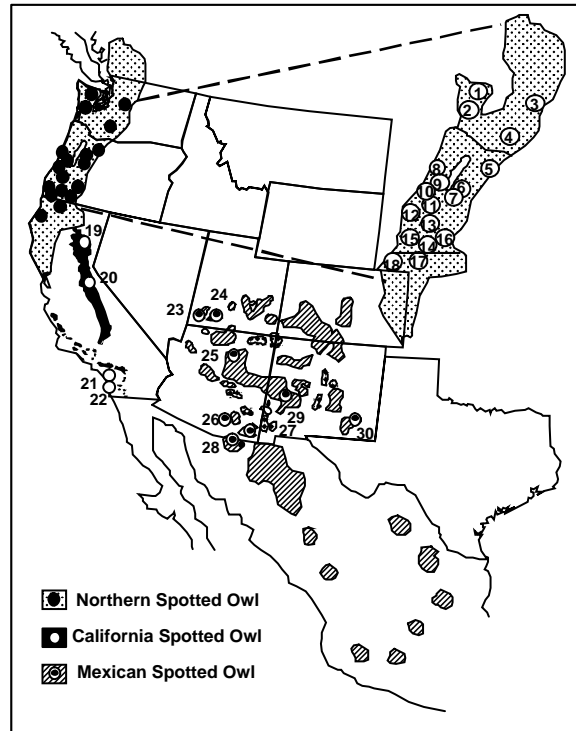

Figure 1. Range of the spotted owl (modified from Gutiérrez et al. 1995) and locations of sampling sites in Washington (WA), Oregon (OR), California (CA), Utah (UT), Arizona (AZ), and New Mexico (NM). 1. QUIL – Olympic National Forest, Quilcene, WA; 2. QUIN – Olympic National Forest, Quinault, WA; 3. WENA – Wenatchee National Forest, WA; 4. YAKI – Yakima Indian Reservation, WA; 5. WSPR – Warm Springs Indian Reservation, OR; 6. EUCA – Eugene BLM district (Cascades), OR; 7. WLNF – Willamette National Forest, OR; 8. WALD – Siuslaw National Forest, Waldport, OR; 9. ALSE – Siuslaw National Forest, Alsea, OR; 10. MAPL – Siuslaw National Forest, Mapleton, OR; 11. EUCO – Eugene BLM District (Coast Range); 12. COOS – Coos Bay, OR; 13. ROSE – Roseburg BLM District, OR; 14. JACK – Jackson Co., OR; 15. JOSE – Josephine Co., OR; 16. KLCO – Klamath Co., OR; 17. KLNF – Klamath National Forest, OR/CA; 18. HUMB – Humboldt Co, CA; 19. LASS – Lassen National Forest, CA; 20. FRES – Fresno, CA; 21. SBER – San Bernadino Range, CA; 22. SJAC – Mount San Jacinto, CA; 23. ZION – Zion National Park, UT; 24. CAPR – Capitol Reef National Park, UT; 25. COPL – Coconino Plateau, AZ; 26. PIMA – Pima Co., AZ; 27. COCH – Cochise Co., AZ; 28. SCRZ – Santa Cruz Co., AZ; 29. TULA – Tularosa Mts, NM; 30. SACR – Sacramento Mts, NM.

Recent mitochondrial analyses support the historic presence of three evolutionary lineages concordant with current geographically-defined subspecies designations in spotted owls (Barrowclough et al. 1999). However, more detailed sampling including the contact zone between northern and California spotted owls using random amplified polymorphic DNA did not support these divisions (Haig et al. 2001). In this study, we use more extensive sampling to further examine population structure and relationships among traditionally (geographically) defined spotted owl subspecies to better clarify these issues. Currently, northern and Mexican spotted owls are listed as threatened (55 Federal Register 11413, 55 Federal Register 26114) and the northern spotted owl is currently undergoing a federal status review. The California spotted owl was recently rejected for listing under the U.S. Endangered Species Act, although additional petitions have been filed. Clarification of taxonomic status and population structure is essential in all of these deliberations.

Methods

Sample collection

Blood samples were collected from individuals captured at 22 local breeding areas among the three geographically-defined subspecies of spotted owls during routine field work (Figure 1). No known close relatives (e.g., parent/offspring, siblings, etc.) were included in the sampling. Following guidelines approved by the American Ornithologists' Union (Oring et al. 1988), blood samples (0.5 ml) were collected from the brachial vein with a 26 gauge needle. Blood was stored in cryogenic tubes containing a buffer solution (100 mM Tris—HCl, pH 8.0, 100 mM EDTA pH 8.0, 10 mM NaCl, 0.5% SDS) and frozen at $-80\ °C$ until analysis.

In addition to samples collected by the authors, eight additional breeding areas were included in the study via use of sequence data from Barrowclough et al. (1999). These included owls from the Willamette National Forest, OR; San Bernadino Mountains, CA; Mount San Jacinto, CA; Zion National Park, UT; Capitol Reef National Park, UT; Coconino Plateau, AZ; Tularosa Mountains, NM; and Sacramento Mountains, AZ (Figure 1).

Aside from subspecies designations outlined by the American Ornithologists' Union (1957), there are no previously published *a priori* groupings (i.e., in recovery plans, metapopulation discussions, etc.) of spotted owls. Thus, we grouped samples by geography and potential geographic barriers to dispersal at three levels: *local breeding areas, regions*, and *subspecies. Breeding areas* were classified by the study area in which samples were collected. *Regional groups* were defined by significant geographic features (e.g., Olympic Range, Cascade Range, Sierras) or by extreme geographic distances between areas. The Klamath region was defined by the geographic provinces known as Oregon Klamath and California Klamath Provinces. Subspecies were geographically defined by the American Ornithologists Union (1957).

Molecular methods

DNA was obtained by a standard phenol/chloroform extraction as previously reported (Haig et al. 1994). Briefly, 10 μl of blood was digested in 400 μl of extraction buffer (50 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, 200 mM NaCl, 2% SDS) with Proteinase K (20 mg/ml) added to a final concentration of 600 μg/ml. Samples were then vortexed and incubated overnight ($\sim$18 h) at 50 °C. If blood clots were not fully dispersed, a second aliquot of Proteinase K was added and the samples were incubated an additional 2 h. Samples were extracted with equal volumes of phenol (saturated with 10 mM Tris, pH 8.0) and then chloroform/isoamyl alcohol (25:1). DNA was precipitated by adding a 1/10 volume of 3 M sodium acetate and two volumes of cold 95% ethanol and pelleted at approximately 15,000 g for 20 min in a microcentrifuge. Pellets were washed once with 70% ethanol, dried under vacuum and resuspended in 30 μl TE (10mM Tris, pH 8.0, 0.1 mM EDTA, pH 8.0). DNA concentration of samples was quantified with a Hoefer TKO 100 fluorometer and working solutions of 100 ng/μl were prepared for experimental use.

A $\sim$1.1 kb fragment of the rapidly evolving mitochondrial control region was obtained using the PCR primers N1 and D16 (Barrowclough et al. 1999). Amplifications were performed using a PTC 100 thermal cycler (MJ Research). A total

reaction volume of 50 μl was used with the following concentrations: 10 mM Tris–HCl at pH 8.3; 50 mM KCl; 0.001% gelatin; 3.5 mM MgCl$_2$; 100 μM for each of the dNTPs; 0.2 μm of each primer; 100 ng of template; and 1.5 U AmpliTaq Gold Polymerase (Perkin Elmer). The following parameters were used for amplifications: 12 min denaturation at 93 °C, followed by 35 cycles of 30 s at 93 °C, annealing at 50 °C for 30 s, and elongation at 72 °C for 1 min. A final 10 min period of elongation at 72 °C followed the last cycle. PCR amplification quality was assessed by visualizing 10 μl of the product with ethidium bromide on 1% agarose gels. Successful PCR reactions were cleaned and concentrated by centrifugation dialysis using Microcon 30,000 MW cutoff filters (Amicon Bioseparations). Internal primers D11, D12 and D24 (Barrowclough et al. 1999) were used to generate bidirectional DNA sequence of domain I and II of the control region. The resulting sequence contains 85% of the variation present in the amplified region. Sequences were generated using ABI Prism Big Dye Terminator Cycle Sequencing chemistry on an automated sequencer (377 DNA Sequencer ABI Prism 377XL Collection Software) located in the Central Services Laboratory at Oregon State University. Ambiguities were resolved by comparing light and heavy-strand sequences or from overlap of different fragments. Sequences were aligned by eye.

Use of avian blood as a DNA source may increase the incidence of nuclear homologue amplifications. Thus, our amplification of a large fragment (1.1 Kb) of the mitochondrial genome as well as bi-directional sequencing assured that DNA sequences were mitochondrial in origin. Samples containing ambiguous sequence (i.e., double peaks) were excluded from further analyses.

To estimate divergence times among subspecies, a 936 bp portion of the cytochrome b gene was sequenced from five individuals from each subspecies, using the primer set L14996/H16064 (Sorenson et al. 1999) and PCR conditions as indicated previously.

Phylogenetic analyses

The program PAUP 4.0b10 (Swofford 2002) was used to generate tree topologies representing phylogenetic relationships among control region haplotypes using maximum parsimony, maximum likelihood criteria, and the neighbor joining approach. ML and NP analyses were preformed using 100 replicate heuristic searches with random taxon addition (10 replicates) and tree bisection–reconnection (TBR) branch swapping. Reliability of support for tree nodes was assessed for each method by bootstrap consensus (1000 replicates for MP and NJ, 100 replicates for ML; Felsenstein 1985). The optimum maximum likelihood model of evolution was inferred using the program Modeltest v.3.06 (Posada & Crandall 1998) which compares different nested models of DNA substitution in a hierarchical hypotheses testing framework. Estimated likelihood values were then analyzed to determine optimum models. This likelihood test was used for nested hypotheses and Akaike information criterion for non-nested hypotheses (Akaike 1974). Maximum likelihood searches were run using the resulting optimal Tamura–Nei (1993) substitution model plus gamma (TrN + I + G), gamma shaped parameter 0.0191. Barred owl (*Strix varia*) sequences were used as an outgroup for each tree.

Nested clade analysis

We used nested clade analysis (NCA) for the analysis of phylogenetic patterns and to discriminate between recurrent gene flow and historic events (Templeton et al. 1993, 1998). NCA is a statistical method that can detect and test evolutionary processes responsible for the spatial distribution of observed genetic variation. A haplotype tree with 95% parsimonious connections was constructed using the program TCS 1.13 (Clement et al. 2000). This tree was then converted into a series of nested clades following the nesting rules in Templeton et al. (1987). Ambiguities in the network were resolved using guidelines provided in Templeton and Sing (1993). The program GeoDis 2.0 (Posada et al. 2000) was used to implement the nested cladistic analysis. Two statistics were calculated: clade distance (D_C) represented the geographical spread of the clade and nested clade distance (D_N) represented distance of the clade from the geographical center of the nested clade. An interior-tip statistic was also estimated within each nested category as the average interior distance minus the average tip distance. This contrast represents an approximation of the distribution of young versus old haplotypes. Clades

were tested against their geographical locations using a permutational contingency analysis. The null hypothesis tested is random geographic distribution of all clades within a nested clade.

Molecular clock

A log-likelihood ratio test (TrN model with gamma correction) was used to test the control region sequences for departure from a molecular clock, using the difference in likelihood from a best fit haplotype tree, with and without the molecular clock enforced (Haulsenbeck & Crandall 1997). We applied the two-cluster test (Takezaki et al. 1995) using the program LINTREE to determine whether individual lineages within spotted owls were evolving in a clock-like fashion. The two-cluster test examines the hypothesis that a molecular clock is in effect along two lineages (b_A, b_B) diverging from an interior node of a generated neighbor-joining tree (Tamura–Nei plus gamma distance). The test assesses whether the difference (δ) in length between daughter lineages is significantly different from zero. The confidence probability (CP = 1 – P) is determined with a value of 95% or higher rejecting the molecular clock for that node. If a molecular clock can be inferred at a node linking two daughter-lineages, a 2% sequence divergence per million years can be applied to derive dates. There is no fossil record to provide a good calibration for divergence rates in these birds, thus we used a calibration rate of 2% as has been applied in many other North American avian species assessments (see Brown et al. 1979; Klicka & Zink 1997; Avise & Walker 1998). The mean branch length between daughter-lineages (h) is the expected value after correction for the assumption of 2% per million year divergence (i.e., 1% along each lineage).

For comparison, cytochrome *b* divergence times were estimated using Arlequin (version 2.001; Schneider et al. 2000) to generate mean pairwise differences between subspecies which were then used to calculate % sequence divergence values. The 2% sequence divergence per million years was then applied to these values to derive dates of divergence.

Population structure and diversity

Summary statistics within and among breeding areas were derived using ARLEQUIN (version 2.001; Schneider et al. 2000) and included mean ($\pm$SD) haplotype diversity (Nei 1987) and mean nucleotide diversity ($\pm$SD; Tajima 1983; Nei 1987). Pairwise sequence divergence within regions and subspecies was calculated using the methods of Tamura & Nei (1993). Deviations from the assumptions of neutrality (Kimura 1983) were calculated using Tajima's (1989) *D*-statistic. The *D*-statistic is based on the fact that under the neutral model there is a correlation between estimates of the number of segregating sites and the average number of nucleotide differences. In addition to testing for selection, significant *D* values can be indicative of factors such as bottlenecks, population increases, or heterogeneity of mutation rates (Tajima 1993; Aris-Brosou & Excoffier 1996; Tajima 1996). Examination of demographic equilibrium was carried out via comparison of mismatch distributions to a sudden population expansion model (Rogers & Harpending 1992; Rogers 1995) using the generalized non-linear least-squares approach of Schneider & Excoffier (1999). Populations that have been stable for a long period of time or are slowly declining have ragged and multimodal distributions whereas populations that have undergone a recent expansion or bottleneck are represented by a single smooth Poisson distribution. DnaSP v.3.5.1 (Rozas & Rozas 1999) was used to compare results with ARLEQUIN and compute Fu & Li's (1993) F-test for selective neutrality.

Population differentiation and gene flow

Hierarchical analysis of molecular variance (AMOVA; Schneider et al. 2000) was used to assess structure within and among subspecies. Total sequence variation was partitioned into within and among subdivision components at three levels: *geographically described subspecies*, *regions*, and *breeding areas*. Significance of *F* was tested using nonparametric permutations (1000 replications). We calculated mean Tamura-Nei corrected sequence divergence (Tamura & Nei 1993) and F_{ST} values (Slatkin 1995) which were correlated with geographic distance using a Mantel test (Mantel 1967) among all breeding areas. Finally, estimates of gene flow, expressed as the estimated number of female migrants per generation ($N_{ef} m_f$, where N_{ef} is the genetic effective population size of females and m_f is the female migration rate per generation)

were derived from $F_{ST} = 1/(1 + 2 N_{ef} m_f)$ (Slatkin 1991) as a means of relative comparison among regions and subspecies. A sequential Bonferroni test was used to estimate p for multiple comparisons of F_{ST} (Rice 1989). Maximum likelihood estimates of $N_{ef} m_f$ were also calculated in MIGRATE v.1.6.5 (Beerli & Felsenstein 1999, 2001). This Markov Chain Monte Carlo (MCMC) approach estimates the effective number of migrants (haplotypes) exchanged between sampling areas each generation over the entire coalescent time. Results account for unequal migration rates and different population sizes and assumed recombination or selection had not occurred. Although these estimates may not be accurate measures of movement among breeding areas, their relative estimation provides insight into population isolation.

Results

Sequence diversity

Control region sequences were generated from a large 1.1 kb fragment obtained from primers located within the flanking tRNA fragment and from a highly conserved region within the spotted owl control region. The pattern of variability across sequences paralleled that reported in previous spotted owl mtDNA analyses (Barrowclough et al. 1999). Therefore, we assumed sequences were mitochondrial in origin, rather than nuclear. To verify the absence of heteroplasmic individuals, clone libraries were generated from two spotted owl samples. Ten individual clones were sequenced from each library and in each case only one haplotype was recovered.

Among 213 individuals sampled in 30 breeding areas, we found 63 unique haplotypes (approximately one for every three individuals sequenced) in a 522 bp portion that spanned domain I and a portion of domain II of the avian mitochondrial control region (Table 1; Baker and Marshall 1997). Sequences resulted in identification of 59 transitions, 2 transversions, and 2 deletions occurring between base pairs 194 and 700 (Table 1). Among traditional geographically-defined subspecies, there were 29 distinct haplotypes in northern spotted owls, 11 in California spotted owls, and 23 in Mexican spotted owls. Two of the three most widespread haplotypes (*Quin3*, n = 32 birds; and *Wspr5*, n = 25 birds) were found throughout the range of northern spotted owls. The other common haplotype, *Lass1*, was the most common California spotted owl haplotype and was found in 31 birds within the range of northern and California spotted owls.

Relationships among subspecies

Phylogenetic analyses based on aligned control region sequences of unique haplotypes indicated similar topologies for all methods of tree inference. For maximum parsimony analyses, 4270 most parsimonius trees were found, each comprised of 114 steps based on 43 parsimony-informative characters. Maximum likelihood bootstrap analyses yielded a –ln likelihood score of 1384.51, allowing for rate heterogeneity among codon positions and using distances generated with the Tamura–Nei substitution model. The neighbor joining tree, using the Tamura–Nei plus gamma model, indicated a northern spotted owl clade (bs = 85) and a California and Mexican spotted owl clade: (Figure 2). The northern clade was basal to the California spotted owl and Mexican spotted owl clade and contained only haplotypes identified as northern spotted owls. The California spotted owl/Mexican spotted owl clade further segregated into California and Mexican clades, although with weak support. California spotted owls formed a sister clade to the Mexican spotted owl clade and also contained haplotypes that, based on geographic location, would be identified as northern spotted owls. Mexican spotted owls formed a monophyletic group, which was reciprocally monophyletic in two of the three methods. However, depending on the inference method used, the Mexican group containing the *Pima1* haplotype segregated unstably between the California and Mexican clades. There was weak support for structure within the three clades which did not appear to correspond to any geographic predictions.

Haplotypes of northern spotted owls and Mexican spotted owls were not found outside of their predicted range (Table 1). However, California spotted owl haplotypes were found in the traditional geographic ranges of both northern and Mexican spotted owls. California spotted owl haplotypes were found in 15/116 (12.9%) owls that
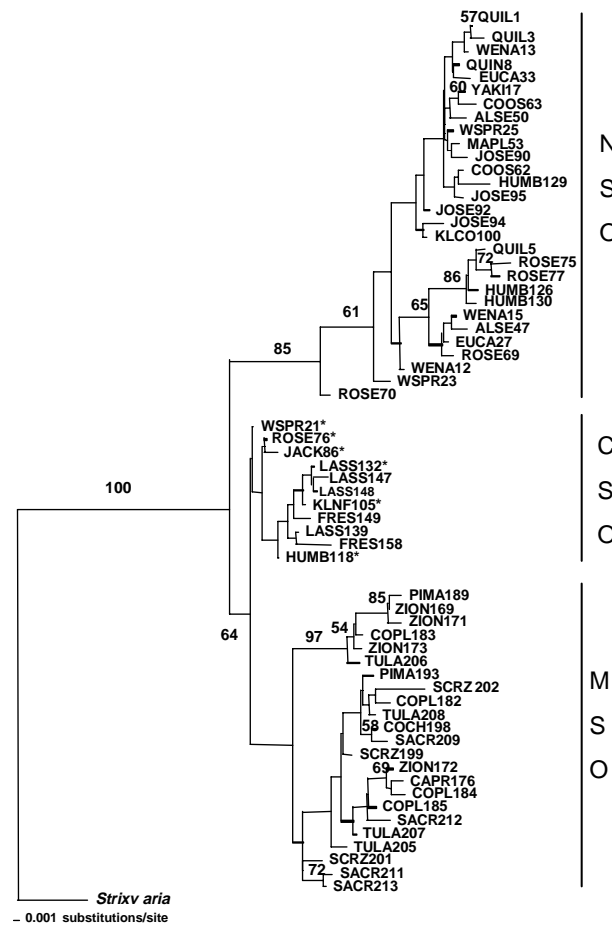
Table 1. Mitochondrial DNA control region haplotype and sequences (522 bp) for three geographically defined Spotted Owl subspecies. *indicates deletion.

Figure 2. Neighbor-joining tree for spotted owls based upon sequences (522 bp) in domains I and II of the mitochondrial control region. Bootstrap values of 50 or more are reported. Asterisks (*) indicate California spotted owl (CSO) haplotypes found in the range of northern spotted owls (NSO). MSO = Mexican spotted owl.

occupied the traditional geographic range of the northern spotted owl (Table 2). These haplotypes were most common among owls in the Klamath Mountains (12/59 birds; 20.3%), with a single haplotype (*Wspr1*) occurring as far north as Waldport, Linn Co., OR, in the Coast range and the Warm Springs Indian Reservation, Jefferson Co., OR, in the Cascade range. This may indicate the presence of a stable northern spotted owl/ California spotted owl hybrid zone throughout the Klamath region with the *Wspr1* haplotype representing a deep penetration arising from a rare introgression or founder event.

Haplotype network and nested design

Nested clade analyses resulted in a maximum number of nine mutational steps between haplo-

types that allowed parsimonious connections at a probability equal or greater than 95% (Figure 3). More than one most parsimonious solution was possible, as indicated by the presence of loops in the resulting cladogram. Thus, ambiguities were addressed as recommended by Templeton et al. (1987). Ambiguities which could not be resolved were included in the next nesting level.

The cladogram nesting procedure resulted in three distinct level 4 clades. Clade 4-1 was composed entirely of haplotypes identified as northern spotted owl and was separated from the remaining cladogram by seven mutational steps. Clade 4-3 was separated from Clade 4-2 by three mutational steps and contained only Mexican spotted owl haplotypes. Clade 4-2, contained all California spotted owl haplotypes in addition to haplotypes that geographically would be considered northern

Table 2. Occurrence of northern and California spotted owl haplotypes found in the traditional geographic range of northern spotted owls

Region	Breeding area	Subspecies	
		Northern spotted owl	California spotted owl
Olympic Range		10	–
	Quilcene, WA	5	–
	Quinault, WA	5	–
Cascade Range		29	1
	Wenatchee, WA	5	–
	Yakima, WA	5	–
	Warm Springs, OR	4	1
	Eugene BLM, OR	10	–
	Willamette NF, OR	5	–
Coast Range		18	2
	Waldport, OR	4	1
	Alsea, OR	5	–
	Mapleton, OR	5	–
	Eugene Coast, OR	4	1
Klamath Region		59	12
	Coos Bay, OR	6	1
	Roseburg, OR	9	1
	Josephine Co., OR	6	2
	Klamath Co., OR	6	–
	Jackson Co., OR	7	3
	Humboldt Co., CA	18	2
	Klamath NF, OR/CA	7	3
Total		116	15

Values represent the number of birds in each region and breeding area that contain haplotypes from each subspecies.

Table 3. Inference chain for nested geographical analysis of spotted owl cladograms using the inference key given in Templeton (1998)

Clades nested in	Inference chain/populations involved	Inference
1-1	1-2-11-17-4-9-10; Quil, Quin, Wena	AF
1-11	1-2-3-4-9-10; NO; Jack, Klnf, Lass, Fres	IS
2-1	1-2-11-12-13; Quil, Quin, Wena, Wspr, Jose, Mapl	LDC
2-2	1-2-3-4-9 NO; Quil, Wena, Euca, Alse, Rose, Humb	PF
2-4	1-2-3-4-9 NO; Quin, Euca, Coos, Jose, Humb	PF
2-9	1-2-3-4-9-10; Zion, Capr, Cocc, Tula, Sacr	AF
3-4	1-2-11-12-13-14 YES	LDC
3-7	1-2-11-17-4-9-10 YES	AF
4-2	1-2-11-17-4 NO	IBD
4-3	1-2-11-12 NO/1-2-11-17	CRE/IO
Entire cladogram	1-2-3-4 NO	IBD

spotted owl and Mexican spotted owl. This cladogram showed little variation from the haplotype structure presented in the neighbor-joining tree (Figure 2). The Mexican spotted owl haplotypes

from the *Pima1* clade, which grouped with clade 4-2, also segregated with the California spotted owl clade in the neighbor-joining tree under certain inference conditions (Table 3). In one variation,

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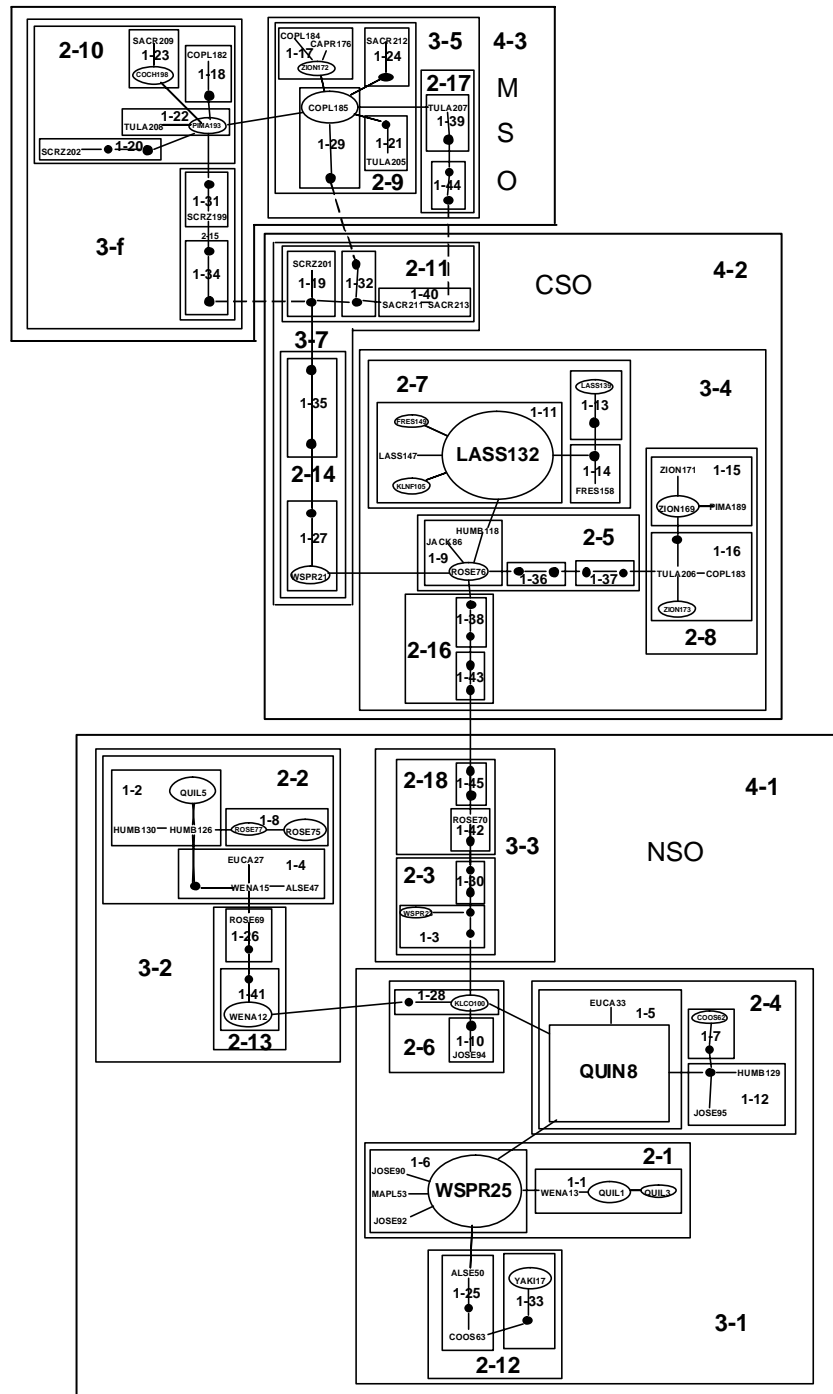
Figure 3. Minimum-spanning haplotype network nested following the procedures of Templeton et al. (1987). Connections <9 substitutions have a 0.95 probability of being parsimonious. Zeros indicate ancestral haplotypes that were not sampled. Solid line represents a single mutational step which connects two haplotype states. Dashed lines represent a connection for which parsimony is not supported at the 95% level. The level of the nested clade is given: 1-x for 1-step clades, 2-x for 2-step clades, etc.

Mexican spotted owl haplotypes from Santa Cruz, AZ, and the Sacramento Mts, NM segregated into clade 4-2. While this association was not present in the neighbor-joining tree, there was little bootstrap support for their segregation. Only two Mexican spotted owl breeding areas from distinctly separate parts of the range (Capitol Reef National Park, UT and Cochise Co., AZ) did not contain haplotypes which associated with the California spotted owl group in the NCA. As determined by permutational contingency analyses, geographic structure was significant in two of the 1-step clades (1-1, 1-11), three of the 2-step clades (2-1, 2-2, 2-9), the higher level clades 3-4, 3-7, 4-2, and the entire cladogram 5-1 (Table 4). *Quin8* was determined to be the oldest haplotype observed from frequency and nesting structure. It contained samples from throughout the traditional range of the northern spotted owl.

The log-likelihood ratio test (lrt) of the best-fit tree, with and without a molecular clock enforced, rejected the simpler (clock-like) tree on a significance level of 5% (1447.08 versus 1384.51, lrt = 62.57, $P < 0.01$, df = 63) suggesting the overall spotted owl control region was not evolving in a clock-like manner. However, 37 of 62 nodes of the constructed neighbor-joining tree contained daughter lineages evolving in a manner consistent with a molecular clock. Using the two-cluster test and a calibration rate of 2% per million years, approximate dates can be inferred at each of the 37 nodes and estimated separation dates among the subspecies can be established: northern spotted owl versus California spotted owl/Mexican spotted owl (1.9 mya), California spotted owl versus Mexican spotted owl (1 mya). Calculation of divergence times using cytochrome *b* data suggested more recent divergence times between the subspecies: NSO versus MSO/CSO (115,000-125,000 yrs), CSO versus MSO (15,000 yrs).

Population structure and variation

Overall levels of sequence variation were typical of those found in other avian studies (Table 5, Baker and Marshall 1997). Haplotype diversity was high within breeding areas with the exceptions of northern spotted owls in the Willamette National Forest and all California spotted owls. Among all owls, mean genetic variation within breeding areas (nucleotide diversity, π) was 0.024 ± 0.012,

Table 4. Permutational chi-square probabilities for geographical structure of spotted owl clades identified in Figures 2 and 3 from 1000 resamples. Clades with probability values less than 0.05 suggest significant geographical structure. Clades with no genetic or geographical variation are excluded

Clade	Permutational	
	Chi-square statistic	Probability
1-1	10.40	0.04*
1-2	8.00	0.87
1-4	6.00	1.00
1-5	5.14	0.77
1-6	31.36	0.57
1-9	8.25	0.37
1-11	47.14	0.02*
1-12	2.00	1.00
1-15	8.40	0.53
1-16	10.00	0.21
1-17	5.56	0.69
1-22	4.00	0.54
1-23	1.88	1.00
1-25	2.00	1.00
2-1	35.00	0.00*
2-2	30.00	0.04*
2-4	50.68	0.06
2-6	4.00	1.00
2-7	5.17	0.78
2-8	2.74	0.90
2-9	32.74	0.03*
2-10	20.90	0.17
2-11	3.00	0.33
2-12	6.00	0.29
2-13	8.00	0.48
3-1	52.61	0.41
3-2	12.61	0.55
3-3	2.00	1.00
3-4	106.67	0.00*
3-5	5.29	0.60
3-6	2.18	1.00
3-7	6.00	0.45
4-1	37.02	0.31
4-2	66.00	0.00*
4-3	12.33	0.06
Entire cladogram	292.09	0.00*

although it varied widely. The highest nucleotide diversity occurred in the Klamath Mountains. The lowest nucleotide diversity occurred among California spotted owls; northern spotted owls from Mapleton, OR, and the Willamette National Forest, OR; and Mexican spotted owls from

Table 5. Genetic variability within subspecies, regions, and breeding areas of spotted owls

Subspecies	Region	Breeding area	Individuals	Haplotypes	Haplotype diversity (h)	Nucelotide diversity (π)	Tajima's D (p)
Northern spotted owl			131	35	0.88 ± 0.02	0.0114 ± 0.0060	−0.38 (0.38)
	Olympic Range		10	4	0.73 ± 0.12	0.0071 ± 0.0044	0.17 (−0.43)
		Quilcene, WA	5	3	0.80 ± 0.16	0.0080 ± 0.0056	−0.89 (0.27)
		Quinault, WA	5	3	0.70 ± 0.22	0.0073 ± 0.0051	−0.86 (0.28)
	Cascades Range		30	11	0.83 ± 0.05	0.0068 ± 0.0040	−1.37 (0.08)
		Wenatchee, WA	5	4	0.90 ± 0.16	0.0073 ± 0.0051	−0.07 (0.52)
		Yakima, WA	5	4	0.90 ± 0.16	0.0084 ± 0.0058	−0.60 (0.37)
		Warm Springs, OR	5	5	1.00 ± 0.13	0.0142 ± 0.0093	−0.27 (0.47)
		Eugene BLM, OR	10	5	0.76 ± 0.13	0.0051 ± 0.0034	−0.71 (0.27)
		Willamette NF, OR	5	2	0.40 ± 0.24	0.0008 ± 0.0010	−0.82 (0.30)
	Coast Range		20	7	0.75 ± 0.08	0.0079 ± 0.0046	−0.88 (0.21)
		Waldport, OR	5	3	0.70 ± 0.22	0.0115 ± 0.0077	−1.22 (0.09)
		Alsea, OR	5	4	0.09 ± 0.16	0.0077 ± 0.0054	−0.29 (−0.38)
		Mapleton, OR	5	3	0.70 ± 0.22	0.0019 ± 0.0018	0.24 (−0.39)
		Eugene, OR	5	3	0.70 ± 0.22	0.0112 ± 0.0079	−1.02 (0.21)
	Klamath Region		71	24	0.91 ± 0.02	0.0142 ± 0.0074	0.38 (−0.34)
		Coos Bay, OR	7	5	0.90 ± 0.10	0.0177 ± 0.0106	−0.09 (0.49)
		Roseburg, OR	10	7	0.91 ± 0.08	0.0138 ± 0.0080	0.09 (−0.45)
		Jackson Co., OR	10	8	0.96 ± 0.06	0.0189 ± 0.0107	0.57 (−0.30)
		Josephine Co., OR	8	7	0.96 ± 0.08	0.0146 ± 0.0087	0.52 (−0.31)
		Klamath Co., OR	6	3	0.73 ± 0.16	0.0089 ± 0.0059	1.10 (−0.17)
		Klamath NF, OR/CA	10	7	0.91 ± 0.08	0.0189 ± 0.0107	1.13 (−0.12)
		Humboldt Co., CA	20	9	0.82 ± 0.07	0.0101 ± 0.0057	−0.84 (0.22)

Species	Region	Location	n			
California spotted owl	Sierra Range		37	0.38 ± 0.10	0.0015 ± 0.0012	−1.36 (0.09)
			27	0.50 ± 0.11	0.0020 ± 0.0015	−1.12 (0.14)
		Lassen NF, CA	17	0.42 ± 0.14	0.0017 ± 0.0014	−1.04 (0.16)
		Fresno, CA	10	0.64 ± 0.15	0.0025 ± 0.0019	−1.04 (−0.17)
	Southern California		10	0.00 ± 0.00	0.0000 ± 0.0000	N/A
		San Bernadino Mts, CA	5	0.00 ± 0.00	0.0000 ± 0.0000	N/A
	Mt. San Jacinto,	CA	5	0.00 ± 0.00	0.0000 ± 0.0000	N/A
Mexican spotted owl			45	0.93 ± 0.02	0.0098 ± 0.0054	−0.96 (0.18)
	Northern MSO		11	0.80 ± 0.10	0.0086 ± 0.0052	0.24 (−0.40)
		Zion NP, UT	5	0.90 ± 0.16	0.0092 ± 0.0063	−0.65 (0.35)
		Capitol Reef NP, UT	3	0.73 ± 0.16	0.0084 ± 0.0056	−0.52 (0.36)
	Southern MSO		35	0.90 ± 0.04	0.0077 ± 0.0044	−1.47 (0.07)
		Coconino Plateau, AZ	10	0.84 ± 0.10	0.0077 ± 0.0047	−0.88 (0.22)
		Pima, AZ	5	0.70 ± 0.22	0.0077 ± 0.0054	−1.92 (0.11)
		Cochise, AZ	5	0.80 ± 0.16	0.0019 ± 0.0018	0.24 (−0.39)
		Santa Cruz, AZ	5	0.90 ± 0.16	0.0088 ± 0.0061	−0.30 (−0.46)
		Tularosa Mts, NM	5	1.00 ± 0.13	0.0080 ± 0.0056	−0.89 (0.27)
		Sacramento Mts, NM	5	1.00 ± 0.13	0.0107 ± 0.0007	0.60 (−0.31)

Means are presented ± SD, N/A = not applicable.

Cochise, AZ. A Tajima's test on all groupings, assuming equilibrium and unstructured populations, resulted in values of D that did not significantly vary from expectations of the neutral mutation model. Thus, we cannot reject the hypothesis that sequence variation is selectively neutral or that populations are stable or expanding. Although not significant, D values for northern spotted owls in the Oregon Cascades and in the Coast Range near Waldport, California spotted owls, and southern Mexican spotted owls were quite negative. Overall D for spotted owls was 0.16 ($P = -0.42$).

Mismatch distributions of pairwise substitution differences between pairs of haplotypes were plotted for spotted owl subspecies grouped as they have been traditionally defined geographically (Figure 1). None of the analyses resulted in a unimodal distribution (Figure 4) and all resulted in non-significant ($P > 0.10$) Fu & Li F-statistics (northern spotted owl $= -0.44$, California spotted owl $= -0.83$, Mexican spotted owl $= -1.90$). Thus, we found no evidence for recent or extensive population growth.

Population differentiation and gene flow

Hierarchical comparisons of genetic variation within groups to among groups was investigated via AMOVA (Table 6). Significant variation was found among regions in each geographically-defined subspecies. Further, in all comparisons except between geographically-defined subspecies, most variation was attributed to variation among individuals within breeding areas.

This included significant differentiation (56.2%) within breeding areas of Mexican spotted owls. Maximum likelihood estimates of gene flow among suggested little genetic exchange among geographically-defined subspecies with the exception of from California spotted owls to northern spotted owls and a small amount from Mexican to California spotted owls (Table 7).

A Mantel test suggested a significant relationship between geographic distance and pairwise F_{ST} values when all breeding areas were compared ($r = 0.74$, $P < 0.01$) and within Mexican spotted owl breeding areas ($r = 0.54$, $P < 0.01$; Table 8). However, there was not a significant relationship within northern or California spotted owl breeding areas ($r = 0.15$ and -0.98, respectively). Compar-
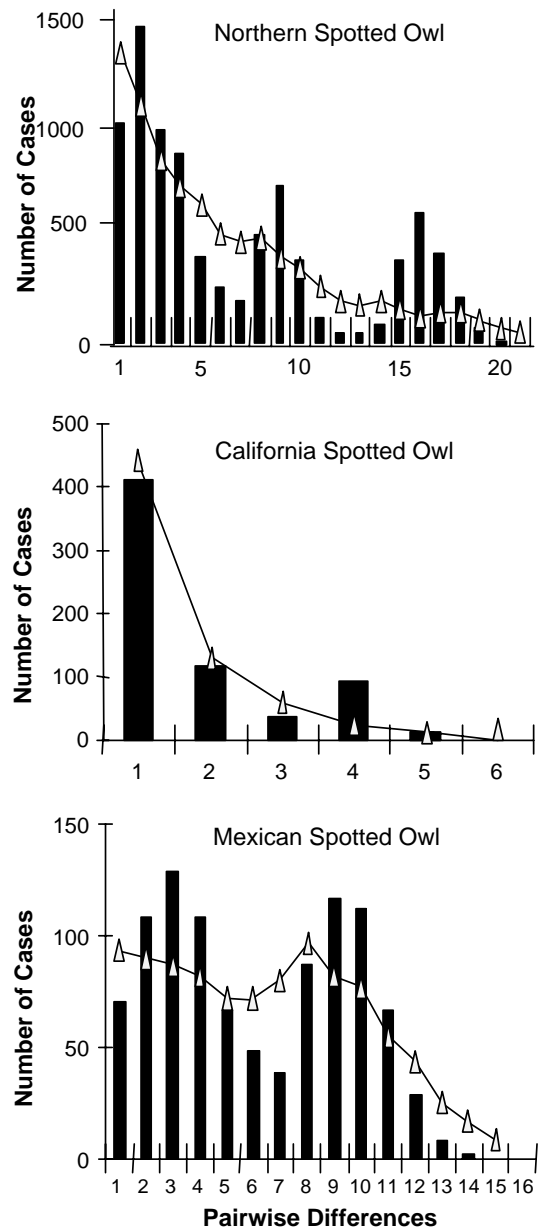
Figure 4. Observed mismatch distributions for the number of differences among mitochondrial control region haplotypes in spotted owls. Distributions presented represent geographical (i.e., not phylogenetic) groupings of subspecies. Triangles with connecting lines indicate expected distribution for a sudden population expansion (Rogers & Harpending 1992; Rogers 1995).

ison of pairwise F_{ST} values among all breeding areas within geographically-defined subspecies indicated no significant differentiation (all $P < 0.05$), except for one comparison in northern spotted owls and in 3 of 28 paired comparisons

Table 6. Analysis of molecular variance (AMOVA) of control region mitochondrial DNA haplotypes within and among the traditional ranges of three geographically-defined spotted owl subspecies

Source of variation	Variance components				
	d.f.	Variance	% of total	F	$P \le$
Spotted owls					
Among subspecies	2	5.74	69.32	$F_{CT} = 0.69$	0.00
Among breeding areas within subspecies	27	0.18	2.14	$F_{SC} = 0.07$	0.01
Among individuals within subspecies	184	2.36	28.53	$F_{ST} = 0.71$	0.00
Among subspecies	2	5.27	67.16	$F_{CT} = 0.67$	0.00
Among regions within subspecies	5	0.30	3.76	$F_{SC} = 0.11$	0.00
Among individuals within regions	205	2.28	29.08	$F_{ST} = 0.71$	0.00
Northern spotted owls					
Among regional groups	3	0.15	4.68	$F_{CT} = 0.05$	0.01
Among breeding areas within regional groups	14	−0.07	−2.19	$F_{SC} = -0.02$	0.62
Among individuals within breeding areas	113	3.04	97.51	$F_{ST} = 0.02$	0.31
California spotted owls					
Among regional groups	1	0.01	3.46	$F_{CT} = 0.03$	0.00
Among breeding areas within regional groups	2	−0.02	−5.58	$F_{SC} = -0.06$	0.48
Among individuals within breeding areas	33	0.37	102.12	$F_{ST} = -0.02$	0.66
Mexican spotted owls					
Among regional groups	1	1.63	44.39	$F_{CT} = 0.44$	0.00
Among breeding areas within regional groups	6	−0.02	−0.62	$F_{SC} = -0.01$	0.51
Among individuals within breeding areas	38	2.06	56.23	$F_{ST} = 0.44$	.00

Table 7. Maximum likelihood estimates of asymmetric migration (2 N_{ef} m_f) among geographically-defined spotted owl subspecies

To	From		
	Northern spotted owl	California spotted owl	Mexican spotted owl
Northern spotted owl	–	1.6	6.9×10^{-10}
California spotted owl	2.7×10^{-16}	–	0.07
Mexican spotted owl	2.3×10^{-15}	8.4×10^{-10}	–

among Mexican spotted owl breeding areas (Table 8). However, comparisons between breeding areas of different geographically-defined subspecies indicated significant differentiation in 82% of northern versus California spotted owl comparisons, 72% of California versus Mexican spotted owl comparisons, and 70% of northern versus Mexican spotted owl comparisons. All comparisons of F_{ST} between regions of different geographically-defined subspecies were significantly different (Table 9). Within subspecies, Mexican spotted owls from the northern portion of their range (i.e., Utah) were significantly different from Mexican spotted owls in more southern regions. Subsequent estimates of gene flow between all

regions within subspecies, while most valuable in a comparative sense only, exceeded Wright's (1931) recommendation of one migrant per generation with the exception of Mexican spotted owls where Nm between regions was 0.55 (Table 9).

Discussion

Results from this work suggest a different view of spotted owl genetic structure than has previously been reported. We investigated these issues to further resolve spotted owl genetic structure, integrating a complex history of range fluctuation, introgression, and vicariance biogeography (Avise

Table 8. Geographic distance (in km; above diagonal) and pairwise F^{st} values (below diagonal) for 30 populations of spotted owls

	1 QUIL	2 QUIN	3 WENA	4 YAKI	5 WSPR	6 EUCA	7 WLNF	8 WALD	9 ALSE	10 MAPL	11 EUCO	12 COOS	13 ROSE	14 JACK	15 JOSE	16 KLCO	17 KLNF	18 HUMB	19 LASS	20 FRES	21 SBER	22 SJAC	23 ZION	24 CAPR	25 COPL	26 PIMA	27 COCH	28 SCRZ	29 TULA	30 SACR
1 QUIL		75	125	250	350	200	335	375	350	225	200	475	500	500	550	545	650	700	750	1125	1500	1580	1345	1395	1490	1750	1900	2025	1890	2150
2 QUIN	-0.18		175	250	350	350	325	300	300	375	350	400	450	460	500	500	625	675	700	1100	1495	1575	1375	1420	1515	1750	1900	1760	1925	2170
3 WENA	0.02	-0.07		150	275	350	365	400	375	450	400	500	475	450	550	525	625	700	700	1100	1500	1580	1310	1330	1465	1700	1825	1850	1835	2085
4 YAKI	0.14	0.07	-0.13		100	250	240	300	250	325	300	375	350	325	425	400	475	550	550	900	1330	1415	1150	1175	1300	1550	1675	1700	1675	1920
5 WSPR	0.12	0.07	-0.05	-0.05		175	70	275	250	300	225	350	275	225	350	290	375	475	425	800	1165	1250	1045	1090	1175	1425	1550	1575	1585	1845
6 EUCA	0.14	0.03	-0.11	0.02	0.06		60	125	90	110	80	150	100	125	175	150	260	340	340	750	1145	1235	1085	1140	1205	1450	1600	1590	1630	1900
7 WLNF	0.3*	0.22	0.06	0.18	0.12	0.05		160	125	155	95	210	175	210	255	220	295	450	445	860	1165	1250	1020	1130	1200	1565	1720	1690	1625	1890
8 WALD	0.13	0.06	-0.07	-0.02	-0.17	0.02	-0.03		40	50	50	100	150	225	200	210	325	350	420	825	1195	1280	1175	1230	1285	1550	1700	1700	1720	1990
9 ALSE	0.11	0.03	-0.17	-0.09	-0.03	-0.06	0.1	-0.05		60	30	110	125	200	190	200	310	350	400	800	1185	1270	1145	1205	1265	1500	1675	1675	1690	1965
10 MAPL	0.2	0.09	0.05	0.2	0.11	-0.02	0.08	0.02	0.08		40	50	90	200	135	160	275	300	375	750	1160	1245	1145	1215	1255	1500	1650	1490	1685	1970
11 EUCO	0.07	0.00	-0.08	0.00	-0.17	-0.02	0.02	-0.21	-0.05	-0.03		90	100	175	150	175	280	310	350	775	1140	1225	1097	1165	1210	1475	1625	1625	1645	1915
12 COOS	0.08	0.03	-0.04	-0.08	-0.09	0.08	0.09	-0.08	-0.04	0.1	-0.07		90	200	100	175	260	250	340	750	1115	1200	1125	1200	1235	1500	1625	1625	1675	1950
13 ROSE	0.08	0.02	-0.09	-0.05	-0.1	0.02	0.08	-0.07	-0.06	0.06	-0.09	-0.05		150	90	75	200	240	275	675	1075	1160	1060	1140	1170	1400	1550	1550	1600	1875
14 JACK	0.12	0.07	0.01	0.00	-0.12	0.11	0.13	-0.08	0.02	0.12	-0.09	-0.06	-0.05		150	75	150	250	225	625	985	1065	965	1045	1020	1325	1460	1460	1505	1785
15 JOSE	0.12	0.07	-0.01	0.03	-0.14	0.07	0.08	-0.14	0.00	0.06	-0.16	-0.04	-0.04	-0.07		90	160	160	240	640	1050	1130	1075	1155	1170	1375	1525	1525	1615	1900
16 KLCO	0.16	0.09	-0.06	-0.08	0.02	0.08	0.16	0.03	-0.03	0.19	0.06	-0.02	-0.05	0.04	0.08		120	190	200	490	955	1035	905	985	1025	1330	1475	1475	1450	1725
17 KLNF	0.15	0.11	0.04	0.02	-0.07	0.16	0.19*	0.00	0.05	0.18	-0.01	-0.03	-0.03	-0.07	0.00	0.05		125	80	600	960	1040	1000	1090	1090	1225	1350	1350	1540	1815
18 HUMB	0.09	0.01	-0.05	0.00	-0.03	-0.02	0.00	-0.07	-0.03	-0.03	-0.1	0.02	0.00	0.04	-0.01	0.06	0.1		150	500	865	950	970	1090	1055	1275	1425	1425	1505	1785
19 LASS	0.91*	0.91*	0.9*	0.9*	0.81*	0.9*	0.95*	0.85*	0.9*	0.94*	0.84*	0.77*	0.77*	0.64*	0.75*	0.89*	0.64*	0.78*		400	745	810	780	1000	870	1180	1380	1320	1336	1610
20 FRES	0.87*	0.88*	0.87*	0.86*	0.75*	0.88*	0.94*	0.8*	0.87*	0.93*	0.79*	0.7*	0.72*	0.57*	0.69*	0.85*	0.56*	0.75*	-0.02		390	455	955	835	690	800	850	900	1125	1415
21 SBER	0.88	0.89	0.88*	0.86	0.72*	0.89*	0.95*	0.79*	0.88	0.97*	0.77*	0.65*	0.68*	0.51*	0.65*	0.85*	0.5	0.73*	-0.07	-0.03		90	510	660	430	505	760	495	810	1085
22 SJAC	0.88	0.89	0.88*	0.86	0.72	0.89*	0.99	0.79*	0.88*	0.97*	0.77*	0.65*	0.68*	0.51*	0.65	0.85	0.5	0.73*	-0.07	-0.03	0.00		555	685	430	465	715	610	775	1050
23 ZION	0.78*	0.78	0.77*	0.75	0.62*	0.82*	0.87*	0.68*	0.77	0.85*	0.67*	0.3*	0.64*	0.51*	0.6*	0.76*	0.53*	0.71*	0.8*	0.73*	0.74*	0.74*		155	165	550	650	635	545	820
24 CAPR	0.79*	0.79*	0.78*	0.76*	0.64*	0.82*	0.86*	0.7*	0.77*	0.85*	0.69*	0.61*	0.65*	0.52*	0.61*	0.77*	0.54*	0.72*	0.8*	0.73*	0.73*	0.73*	-0.19		265	610	665	675	495	745
25 COPL	0.79*	0.79*	0.77*	0.76*	0.65	0.81*	0.95*	0.84*	0.77*	0.97*	0.7*	0.62*	0.66*	0.54*	0.63*	0.76*	0.56*	0.72*	0.79*	0.73*	0.73*	0.73*	0.34	0.36		390	520	495	455	740
26 PIMA	0.79*	0.79	0.77*	0.75*	0.61	0.82	0.87	0.67*	0.77	0.97*	0.67	0.57*	0.63*	0.49*	0.59*	0.75	0.51	0.7*	0.83*	0.77*	0.79	0.79	0.29	0.38	-0.11		150	150	390	615
27 COCH	0.86	0.87	0.86	0.84	0.71*	0.87*	0.96	0.77	0.85*	0.94	0.77*	0.63*	0.69*	0.56*	0.66*	0.83	0.57*	0.74*	0.91*	0.88*	0.95*	0.95	0.61	0.62	0.07	0.02		50	220	370
28 SCRZ	0.78*	0.78*	0.76*	0.74	0.61	0.81*	0.86	0.66	0.76	0.85	0.67*	0.55*	0.63*	0.5*	0.59*	0.75	0.52*	0.71*	0.84*	0.78*	0.78	0.78	0.47*	0.49*	0.15	0.07	0.03		330	505
29 TULA	0.77	0.78*	0.76	0.74	0.59	0.81*	0.87	0.66	0.75	0.85	0.66	0.55*	0.61*	0.47*	0.57	0.74	0.49	0.69*	0.82*	0.76*	0.77*	0.77*	0.31	0.34	-0.08	-0.15	0.06	0.09		290
30 SACR	0.75*	0.75*	0.73*	0.7	0.57*	0.79*	0.83*	0.63	0.72*	0.82	0.63*	0.53	0.6*	0.47*	0.56*	0.72*	0.49*	0.69*	0.82*	0.75*	0.74	0.74*	0.38	0.41*	0.08	0.01	0.07	-0.02	0.01	

Locations identified (by number) in Figure 1. Boxes indicate traditional subspecies boundaries (NSO, CSO, MSO, respectively). *Significant differentiation using sequential Bonferroni test ($P < 0.05$).

Table 9. Estimates of pairwise population differentiation (F_{ST}; below diagonal) and gene flow (Nm; above diagonal) within and among regional populations of geographically-defined northern spotted owls (NSO), California spotted owls (CSO), and Mexican spotted owls (MSO)

Region	1	2	3	4	5	6	7	8
1. NSO – Olympic	—	4.74	3.66	5.58	0.02	0.03	0.03	0.03
2. NSO – Cascades	0.10	—	inf	inf	0.04	0.06	0.04	0.05
3. NSO – Coast Range	0.12	−0.03	—	inf	0.06	0.08	0.05	
4. NSO – Klamath	0.08	−0.00	−0.02	—	0.19	0.23	0.11	0.13
5. CSO – Northern	0.96*	0.92*	0.90*	0.73*	—	9449	0.13	0.13
6. CSO – Southern	0.94*	0.90*	0.86*	0.68*	0.00	—	0.19	0.17
7. MSO – Northern	0.94*	0.93*	0.90*	0.82*	0.80*	0.73*	—	0.55
8. MSO – Southern	0.94*	0.91*	0.90*	0.80*	0.79*	0.74*	0.47*	—

Boxes indicate geographical (i.e., traditional) subspecies delineation. *Indicates significant population differentiation ($P < 0.05$).

2000). This clarification will help resolve current debates regarding ESA listing of various subspecies and populations of the spotted owl.

Relationships among subspecies

Analyses at the subspecies level revealed several important changes in our understanding of these groups. For the three geographically-defined subspecies, historic events such as allopatric fragmentation and long distance colonization appeared to be predominant processes for shaping clade structure. Among subspecies, control region and cytochrome *b* divergence times suggest a Pleistocene origin with northern spotted owls. Control region dates would put this initial divergence at 1.9 mya, while cytochrome *b* places the split at a more recent 150,000 yrs. The cytochrome *b* dates should reflect a more accurate timeframe due to the clock-like manner of the gene, however both methods reinforce the initial divergence occurring between the Northern Spotted Owls and California and Mexican Spotted Owls, with the split between California and Mexican Spotted Owls coming at a much later date.

The original divergence between northern and California spotted owls is perplexing as their current ranges are adjacent or overlapping and there appears to be more potential geographic or habitat barriers to dispersal within these subspecies ranges than between them. Further, the most notable potential habitat barriers within subspecies (e.g., Puget Sound, WA, or Willamette Valley, OR) have been breached numerous times indicating spotted owls are effective dispersers (Forsman et al. 2002).

Insight into the radiation of northern and California spotted owls may be gained by examination of phylogeographic patterns of other species in the Klamath region, the area in which a zone of hybridization has occurred on the north-south border between northern and California spotted owls. This region is also the north-south border and often the hybrid zone between numerous other plant and animal subspecies or species (reviewed in Soltis et al. 1997). Multiple hypotheses have been proposed to explain this apparent phenomenon that occurred before and after Pleistocene glaciation. The north–south recolonization hypothesis (Soltis et al. 1997), where well separated northern and southern populations undergo genotype fixation followed by secondary contact, may best explain the structure observed between northern and California spotted owls. For example, clade 4-1, which represents northern spotted owls, indicates strong evolutionary clustering and is separated from the remaining cladogram by a long branch length with missing intermediates. This structure implies past fragmentation which may support the unglaciated northern refugia theory in which northern and southern haplotypes became fixed due to isolation by glaciation (Avise 1994). Following glaciation, a continuous geographic range was reestablished, but with the genetic discontinuity now present.

An alternative hypothesis is that deep phylogeographic breaks can occur in a continuously dispersing species if individual dispersal distances and/or population sizes are low (Irwin 2002). This would result from low dispersal and modes of mtDNA inheritance. This hypothesis could be true

for all three subspecies of spotted owls given their demography and relatively short dispersal distances (Forsman et al. 2002) and would not exclude elements of hypotheses introduced above.

Somewhat different processes may have shaped the relationship between California and Mexican spotted owls. Their overall structure appears to reflect repeated post-glacial range expansion by long-distance colonization (e.g., clade 3-4) on the part of California spotted owls, followed by fragmentation and secondary contact with Mexican spotted owls. Clade 3-7 appears to be influenced by fragmentation events whereas clade 2-11 is at the extreme eastern end of the species distribution and a great distance from the geographic center of the clade.

The significance of Pleistocene glaciation on avian speciation and population structure is currently under debate. Our results support the idea that biogeographic factors during the Pleistocene promoted substantial microevolutionary genetic diversification in birds (Avise & Walker 1998). We do not know the pre-Pleistocene history of the species and cannot speculate on its significance. Therefore, we cannot evaluate the hypothesis of Klicka & Zink (1997) that the Pleistocene was not the prime force in bird speciation and subsequent structure.

Given the range overlap and lack of phenotypic differences among spotted owl subspecies, it is not surprising that we found California spotted owl haplotypes in the geographic range of northern spotted owls. These results are supported by previous genetic studies by Barrowclough et al. (1999) and Haig et al. (2001). Historically, the Pit River (east of Redding, CA) is considered the subspecies boundary, although there has always been an assumption that there was mixing in this contact zone and that reproductive isolation was not a criteria for defining these subspecies (Verner et al. 1992). Our study supports the presence of a hybrid zone in the Klamath region of southern Oregon and northern California. This zone is narrow relative to owl dispersal rates (σ), yet does not appear to be situated on a sharp ecotone. Since these subspecies have been in contact for some time, it appears that selection against hybrids is occurring, which would prevent dispersal from increasing the width of the zone. Thus, this stable hybrid zone appears to be a tension zone that remains narrow due to a balance between selection and dispersal.

Although apparently a widespread phenomena and resulting in viable intraspecific hybrids, the genetic effect of intraspecific hybridization is not clear for spotted owls. There is no evidence that Haldane's rule (1922) has taken effect. That is, there have been no observations of reduced fitness in the heterogametic sex (females) in the areas of widespread hybridization, although there needs to be closer examination of this issue with the extensive comparative demographic data collected from throughout the species range (e.g., Forsman et al. 1996; Seaman et al. 1999; Blakesley et al. 2001). Further, it can take many years for these effects to become obvious (Price & Bouvier 2002).

Fitness due to intraspecific hybridization may be reduced and local adaptations may be lost over time, however, these local adaptations may not be recognized if they are essential only during occasional extreme conditions (Allendorf et al. 2001). For example, while California spotted owls in northern Califonia and northern spotted owls in southern Oregon occupy the same or similar habitats, one striking difference between the subspecies is that some California spotted owls in the Sierras undertake an altitudinal migration in extreme winters (Laymon 1988). The effect of the potential loss of this adaptation in hybrid owls in Oregon is unknown but should be considered. Finally, in addition to intraspecific hybridization, interspecific hybridization with barred owls is occurring in both northern and California subspecies (Hamer et al. 1994; Dark et al. 1998; Kelly et al. 2003; Kelly & Forsman 2004; Haig et al. 2004). These hybrids produce viable offspring, but rates of hybridization appear to be low (Kelly & Forsman 2004), so it is not clear how extensively this will effect subsequent gene pools.

Our data suggest weak support for the traditional Mexican and California spotted owl relationship at the subspecies level. Barrowclough et al. (1999) reported three separate subspecies based on primary differentiation of northern spotted owls from a California and Mexican spotted owl sister group, although evidence for gene flow was found across the three subspecies. Haig et al. (2001) concluded that Mexican spotted owls were an evolutionary significant unit relative to California and northern spotted owls. However, there were only three Mexican spotted owl and two California spotted owl breeding areas examined. Neither previous study contained the

extensive geographic sampling presented in the current study.

While there are no specific guidelines (e.g., rates of sequence divergence) for what level of differentiation constitutes an avian subspecies. One criteria suggested has been reciprocal monophyly which we do not have between California and Mexican spotted owls. Further, there are no morphological or other distinct differences between the groups. While lack of subspecies differences may be problematic from a current ESA listing perspective, Zink (2003) convincingly argues not to base units for listing on subspecific designation in the first place. Rather, identification of distinctive population structure is more relevant to conservation.

Population genetic structure and status

The genetic status of spotted owls is difficult to characterize as it varies tremendously among areas. Exact population estimates and overall population trends are not known for spotted owls although recent analysis of mark-recapture data suggest the spotted owl populations are declining in some areas and relatively stable in others (La Haye 1992; Forsman et al. 1996; Seaman et al. 1999; Franklin et al. 2000, 2004; Blakesly et al. 2001). However, we estimate there are about 18,000 owls (approximately 12,000 northern spotted owls, E. Forsman, unpub. data; 4000 California spotted owls, G. Gould, California Fish and Game, pers. comm; and perhaps 1500 Mexican spotted owls, Gutiérrez et al. 1995) distributed over a vast portion of western North America. Comparison of these data and other demographic data (i.e., Forsman et al. 2002; Franklin et al. 2004) with mismatch distributions suggest that a large degree of population expansion has not taken place. Moreover, considering that female spotted owls disperse further than males (Forsman et al. 2002) and the maternal inheritance of mitochondrial DNA, our results represent a more optimistic view of population status than if paternally inherited markers were considered as well.

Among our characterization of northern spotted owls, estimates of gene flow and genetic diversity are affected by inclusion of birds carrying California spotted owl haplotypes in all but one region (Olympic). Comparative RAPD data

suggest low levels of genetic diversity and significant differentiation among breeding areas and regions (Haig et al. 2001). This is corroborated by field data suggesting that most male spotted owls settle within four territory widths of their natal site whereas most females settle within seven territory widths of their natal site (Forsman et al. 2002). Finally, northern spotted owls occur in only 29% of their former range further suggesting reduced population viability (Gutiérrez 1994).

Among California spotted owls, there is a pronounced lack of genetic diversity, particularly in the southern California samples as evidenced in estimates of haplotype and nucleotide diversity. Lack of diversity in the southern California breeding areas may reflect a unique founding event by a few individuals and small population size. Recent population estimates do not exceed 300–350 pairs throughout the southern California "archipelago" of habitat (Noon & McKelvey 1992). Further, La Haye (1992) estimated that spotted owls in the San Bernadino Mountains declined significantly between 1987 and 1993 and no dispersal was observed between the San Bernadino and San Jacinto mountains (La Haye et al. 1994). Rapid habitat alteration and fragmentation in southern California may also contribute to this lack of diversity. Sierra breeding areas also indicated signs of declining abundance or a recent bottleneck. Recent demographic studies (1990–1999) corroborated these results for at least the Lassen National Forest, where data indicated a significant annual decline from 1990 to 2000 (Blakesley et al. 2001; Franklin et al. 2004). Thus, this may never have been a large population and it is distributed over a large geographic region with significant historic habitat gaps and more recent fragmentation in the Sierras and southern California.

An alternative hypothesis proposed by Barrowclough et al. (1999) suggests that there may be a recent advantageous mutant haplotype that has swept through these areas. This would result in elimination of some previous haplotype diversity. While possible, consideration should be made of the means and speed by which this mutant haplotype would be transmitted over such a sparse and widely distributed range in a subspecies with limited dispersal.

Mexican spotted owls have a far more fragmented distribution than the other two subspecies,

with an estimated 1500 individuals found in the isolated mountains and canyonlands of the arid southwestern U.S. and Mexico. Genetic data reflected this dispersion with a F_{ST} that was 22 times larger than estimates for the other subspecies. Further, over one third of between-breeding area comparisons resulted in a significant differentiation. Breeding areas in Utah were often significantly different from breeding areas further south. Dispersal data support these results where, similar to northern spotted owls, most juveniles observed in Arizona did not disperse more than a few territories from their natal sites (Ganey et al. 1998). The median dispersal distance in northern Arizona was 16.6 km (Ganey et al. 1998) and 25.7 km in Utah (Willey & van Riper 2000). Finally, owls in the Coconino Plateau and Tularosa Mountains have undergone significant annual declines further suggesting genetic and/or demographic issues of concern (Seaman et al. 1999).

Conservation and population management in spotted owls

This comprehensive assessment of spotted owl population genetic structure adds critical information to be considered in developing management actions to benefit the species. Despite mixing in a hybrid zone in southern Oregon, the clear genetic distinctions between California and northern spotted owls suggest that it is appropriate to continue recovery efforts for northern spotted owls based on traditional geographic subspecies. Similarly, we do not believe that lack of significant genetic differences between California and Mexican spotted owls is a reason for abandoning conservation efforts. Rather, lack of genetic diversity in the highly fragmented and declining southern California breeding areas and high population fragmentation among Mexican spotted owls suggests the need for close attention to the management of owls in both regions.

Acknowledgements

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704

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