Female natal philopatry and gene flow between divergent clades of pallid bats (*Antrozous pallidus*)

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In long-lived temperate bats, female philopatry can influence the genetic structure of roosting groups and the potential for individuals to interact across generations. Although direct observation of dispersal between social groups is difficult given the vagility and nocturnal activity of most bats, molecular markers can be used to infer dispersal and mating patterns. Here we report on female philopatry among pallid bats, *Antrozous pallidus*, a species that exhibits dynamic fission-fusion roosting behavior in which philopatry and familiarity with a roosting area may help individuals choose roost sites and gain benefits associated with social roosting. In addition, we use genetic data to draw inferences about how dispersal and mating relate to the presence of divergent mitochondrial clades, which have been previously reported for pallid bats in western North America. We found significant genetic structure among colonies in central Oregon based on sequence variation at the mitochondrial (mt) DNA control region (pairwise Φ_{ST} - 0.08 – 0.678) but very little structure among colonies for bi-parentally inherited nuclear microsatellites (pairwise F_{ST} - 0.004 – 0.01) indicative of female philopatry and male-mediated gene flow. Interestingly, some bats captured in the same colony had mtDNA haplotypes that differed by more than 12% yet failed to exhibit differences at nuclear markers. Thus, even though such divergence values are sometimes associated with species differences, evidence indicates that bats from these clades freely interbreed. This unusual pattern seems likely to be the result of post-glacial range expansions from separate southern refugia northwards followed by recent secondary contact.

Keywords: *Antrozous pallidus*, Chiroptera, fission-fusion, microsatellites, mitochondrial DNA, philopatry, phylogeography

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Patterns of genetic variation among natural populations are a result of historical patterns of vicariance and range expansion as well as contemporary factors such as mating behavior and dispersal resulting in gene flow among populations. At the microgeographic level, the degree to which females are philopatric and males disperse critically impact the social structure of animal groups. Consequently, estimating the relative strength of these two factors can help shed light on the evolution of sociality in a given species. In most mammals, females provide the majority of care for the young and may benefit by remaining on their natal territory through greater familiarity with foraging and roosting areas, as well as from increased acquisition of resources by kin (Handley and Perrin 2007). Such cooperative behaviors can be enhanced either actively through the establishment of a kin recognition system or passively through philopatry, which can lead to higher relatedness and a greater opportunity for social interactions among group members that remain at the natal territory (Lukas et al. 2005).

Direct observation of dispersal using traditional methods of mark-recapture or radio-telemetry is difficult and can often underestimate the degree to which dispersal is occurring, especially over large spatial scales. Thus, indirect estimation of these factors using molecular markers has been crucial to making inferences about how their relative strength and direction contribute to the social structure of mammalian groups (Handley and Perrin 2007). This is especially true for bats, which are a particularly interesting group of mammals to examine population dynamics and social organization given their unique capacity for powered flight and considerable ecological diversity. However their high mobility and nocturnal activity make direct observation difficult, highlighting the importance of molecular techniques to examine population dynamics (Burland and Wilmer 2001).

In most temperate bat species, females form maternity colonies in the summer to give birth and nurse their pups while males roost away from the maternity colony in smaller bachelor groups (McCracken and Wilkinson 2000). Recent work has shown that the majority of bats fit the mammalian pattern of female philopatry and male-biased dispersal (Castella et al. 2001; Kerth et al. 2002; Arnold 2007; Vonhof et al. 2008; Piaggio et al. 2009) although there are exceptions, such as the brown long-eared bat (*Plecotus auritus*) (Burland et al. 2006) and Schreibers' long-fingered bat (*Miniopterus schreibersii natalensis*) (Miller-Butterworth et al. 2003), where both sexes are philopatric, and the white-lined bat (*Saccopteryx bilineata*) (McCracken 1984; Nagy 2007) and Proboscis bat (*Rhynchonycteris naso*) (Nagy et al. 2013) in which females disperse.

There are several lines of evidence that suggest that female philopatry may be an important factor influencing bat sociality. Bats live more than three times as long as other placental mammals of similar body size (Wilkinson and South 2002), which along with female natal philopatry results in increased opportunity for social interactions among individuals in a maternity colony across multiple generations. In addition, female

philopatry may provide experience with potential roosting areas that can be subsequently used when selecting roosts for rearing young (Kerth and Reckardt 2003; Kerth et al. 2006).

Pallid bats (*Antrozous pallidus*) exhibit labile roosting behavior in which females in a maternity colony frequently switch among multiple rock crevice day roosts within larger rocky outcrops (Lewis 1996). Like most temperate vespertilionid bats, male and female pallid bats are spatially segregated during the maternity season, with males roosting in small groups (Hermanson and O'Shea 1983), although mixed sex maternity colonies have been observed (Orr 1954). Mating occurs in the fall when bats travel short distances away from their summer roosting areas prior to hibernation and females store sperm over the winter during hibernation (Barbour and Davis 1969). The effect of these seasonal movement patterns on the genetic structure of maternity colonies, including the degree to which females return to their natal roosting area after hibernation, is not well known. Female philopatry in pallid bat maternity colonies is of special interest because pallid bats emit individually specific vocalizations that assist in the formation of roosting groups (Vaughan and O'Shea 1976; Arnold and Wilkinson 2011). Thus, examining the genetic structure of pallid bat maternity colonies may reveal how these vocalizations could influence roosting associations that extend across generations.

In addition, while examining population structure on a microgeographic scale is informative for investigating the potential effects that biased dispersal and gene flow have on the genetic structure of social groups, analyzing molecular variation on a macrogeographic scale allows for greater inference about how these behavioral patterns shape genetic differences among populations over the range of a species (Ruedi and McCracken 2009). For example, sequence variation in the rapidly evolving mitochondrial DNA (mtDNA) control region has been used to infer long distance seasonal migration patterns (Wilkinson and Fleming 1996; Russell et al. 2005) as well as historical patterns of vicariance and gene flow in bats (Ruedi and Castella 2003; Martins et al. 2009; Turmelle et al. 2011). Analysis of mtDNA variation in pallid bats across North America revealed three distinct and deeply diverged clades corresponding to major desert regions (Fig. 1; Weyandt and Van Den Bussche 2007). In addition, Lack et al. (2010) utilized bi-parentally inherited nuclear amplified fragment length polymorphisms (AFLPs) to examine male-mediated gene flow among the diverged clades and found evidence for gene flow over broad geographic areas both within and among the diverged clades. However, these studies contained relatively few samples from the northern part of the range of pallid bats and did not include analysis of the genetic structure of maternity colonies.

Therefore, we had two goals for this study. First, to determine if females are philopatric to their natal maternity colony, we examined the genetic structure of pallid bats in central Oregon within and among roosting areas using both bi-parentally inherited microsatellite markers and maternally inherited mtDNA sequences. Following Castella et

al. (2001), if female philopatry and male-biased dispersal are strong factors affecting genetic variation at pallid bat maternity colonies, we expected to observe differentiation among colonies at the mitochondrial level and limited genetic structure at the nuclear level. Second, to determine how local patterns of mating and dispersal relate to historical patterns of gene flow and range expansion in the Pacific northwest, we examined mtDNA variation in a broader phylogeographic context by combining sequences from bats captured in Oregon with sequences from bats sampled throughout western North America (Weyandt and Van Den Bussche 2007).

MATERIALS AND METHODS

Sampling and DNA Extraction - We conducted fieldwork in the Clarno basin of central Oregon, U.S.A (44.94°N lat., 120.38°W long.). The habitat and vegetation of the study area was described in Arnold and Wilkinson (2011). Bats were captured at six sites (Fig. 2) using either mist nets extended over a water source (Site 4, CCT), mist nets placed outside the entrance to night roosts (CSH, WCB), or a 0.75 m triangular shaped net attached to a 3 m extension pole to capture bats as they exited day roosting crevices (CCS, CCN). Each bat was weighed and marked with a numbered band (National Band and Tag, Newport, KY, U.S.A.) so that individuals could be identified if recaptured. Tissue samples were obtained from wing membranes using 3 mm biopsy punches (Worthington-Wilmer and Barratt 1996) and stored in 95% ethanol until DNA was extracted from each wing-punch using a Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, U.S.A.) following the manufacturer's instructions. All field protocols conformed to American Society of Mammalogists guidelines for research on live animals (Sikes et al. 2011) and were approved by the University of Maryland Institutional Animal Care and Use Committee (Protocol R-08-39).

Mitochondrial Control Region Sequencing - We sequenced approximately 450 bp of mtDNA control region for 113 bats using primers P and E (Wilkinson and Chapman 1991). In addition, DNA extractions of wing punches taken from 18 recaptured bats were independently sequenced and compared to sequences obtained from their previous extractions to verify the repeatability of the sequence data. Polymerase Chain Reaction (PCR) amplifications were carried out in 25 μl reaction volumes consisting of 10-20 ng of template DNA, 10x PCR Buffer, 2.5mM $MgCl_2$, 0.48μM of each primer, 200 μM dNTPs and approximately 1.4 units of Taq polymerase. PCR thermocycling conditions consisted of an initial incubation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 30 min. Each sample then went through an initial purification process by treatment with ExoSap (USB Corp.) to digest unincorporated primers and dNTPs. Each sample was sequenced in both the forward and reverse directions using a BigDye Terminator V.3.1 cycle sequencing kit

(Applied Biosystems). For cycle sequencing, we used 9.5 μl PCR reaction volumes consisting of 1 μl of cleaned product, 1 μl of Big Dye, 1.5 μl of Big Dye buffer, and 0.35 μM forward or reverse primer in a PCR cycle consisting of 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequenced products were then precipitated with isopropanol and re-suspended in 10 μl of Hi-Di Formamide (Amresco) prior to fragment sizing in a 3730 capillary sequencer (Applied Biosystems).

Control region sequences obtained from bats in the family Vespertilionidae often contain variable numbers of an 81 bp repeat resulting in sequences of differing length within species (Wilkinson et al. 1997). However, all pallid bats sequenced in this study contained three repeats and yielded sequences that were approximately equal in size. Forward and reverse sequences for each bat were aligned using Sequencher 4.0 (Genescan) and checked for ambiguities. All sequences were trimmed to a common length of 425 bp and a representative of each haplotype was entered into Genbank (Genbank accession numbers XXXXX – XXXXXX).

Microsatellite Genotyping - We obtained genotypes from captured bats at 12 microsatellite loci (see Table S1 in supplemental online materials). We used the M13 tagging method developed by Schuelke (2000) to label the forward primer for loci EF4, EF5, EF21, G07, E07, H10, and G02 so that fragments could be sized by a DNA fragment analyzer. For this procedure, the PCR thermocycler conditions for loci EF4, EF5, and EF21 were 3 minutes at 95°C to denature and a touchdown protocol consisting of 20 cycles of 30 seconds at 95°C, 30 seconds at 10°C above the primer specific annealing temperature indicated in Table S1 dropping ½°C per cycle, and 30 seconds at 72°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at the primer specific annealing temperature, and 30 seconds at 72°C with a final extension of 20 minutes at 72°C. For loci G07, E10, H10, and G02 we used the M13 tagging method along with the thermocycling protocol outlined below.

For the remaining microsatellite loci (EF6, EF15, EF20, B02, and G25) we used fluorescently labeled forward primers and a PCR thermocycling profile consisting of an initial denature period of 3 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C, 30 seconds at the primer specific annealing temperature (Table S1), and 30 seconds at 72°C, followed by an extension period of 20 minutes at 72°C. Amplifications were carried out in 12 μl reaction volumes consisting of 10-20 ng of template DNA, 0.5 μM of each primer, and 2X PCR Master Mix (Lucigen Corp) containing 0.6 units of Taq DNA polymerase, 200 μM each dNTP, and 1.5 mM MgCl2 along with PCR reaction buffer (ph 9.0). For microsatellite loci EF15 and EF20, 0.5 μg/μl of Bovine Serum Albumin (BSA) and 5% Dimethyl Sulfoxide (DMSO) were added to enhance amplification of the PCR products.

Following amplification, the PCR products were run on a 3730 DNA analyzer (Applied Biosystems) and fragment sizes scored using Genemapper v.3.7 (Applied

Biosystems). In addition, extractions taken from 18 recaptured individuals were independently amplified and scored to ensure that allele calls were consistent across replicate samples. We used the program GENEPOP (Raymond 1995) to test loci for conformity to Hardy-Weinberg Equilibrium using exact tests, to evaluate for the presence of null alleles, and to examine all possible pairs of loci for linkage disequilibrium using a log-likelihood ratio test. A sequential Bonferroni correction was applied to account for multiple comparisons when considering significance for these tests (Rice 1989).

Genetic Diversity Within and Among Day Roosting Areas - Bats were assigned to one of two day roosting areas (CCN or CCS) if they were either captured exiting a day roost in that area or tracked to the roosting area using radio-telemetry. Radiotransmitters were built (Wilkinson and Bradbury 1988) and placed in the interscapular region of each bat using Skinbond adhesive (Torbot Group, Inc., Cranston, RI, U.S.A.). To minimize disruption to normal flight behavior, transmitters weighed less than 5-8% of total body weight (Aldridge and Brigham 1988). In addition, we also included bats captured at two night roosts (CSH and WCB) to examine the genetic structure of pallid bat colonies. While CSH and WCB appear to be used strictly as night roosts in that we never observed bats present in the buildings during the day, Lewis (1994) captured females at these sites and tracked them to day roosts within 2 km. Thus, we assumed bats captured at these night roosts used day roosts nearby. In addition, because only one bat was captured at the WCB site, we included this bat with those captured at the CSH night roost.

Mitochondrial sequence variability was characterized for each roosting area (CCN, CCS, and CSH) by the number of haplotypes (N_h), haplotype diversity (h, defined as the probability that two randomly chosen haplotypes from one area are different), and nucleotide diversity (π, calculated as the probability that two randomly chosen homologous sites are different). For the microsatellite data, we calculated the average number of alleles per locus (N_a) and the average observed (H_O) and expected (H_E) heterozygosities. All calculations were conducted using Arlequin v. 3.5 (Excofier and Lischer 2010).

Genetic subdivision among the roosting areas was calculated using pairwise estimates of Weir and Cockerham's (1984) Φ_{ST} (mitochondrial data) and F_{ST} (microsatellites) in Arlequin v. 3.5. In addition, we tested for any relationship between geographic distance and genetic differentiation of the mitochondrial data set by grouping sites into a western "population" (CSH and WCB) and an eastern "population" (CCS, CCN, CCT, and Site 4) to calculate the proportion of the genetic variance explained by differences among populations and differences among sites within populations using an Analysis of Molecular Variance (AMOVA) implemented in Arlequin. We used a maximum likelihood approach to identify the best fitting model for nucleotide substitutions using estimates of the Akaike information criterion (AIC) calculated in the program JModeltest v. 1.0 (Posada 2008). The value of the gamma shape parameter from

the best fitting model was utilized in the calculations of pairwise differences among populations for the AMOVA.

Phylogeographic Patterns - To visualize haplotype relationships among pallid bats in the study area, we constructed haplotype networks with the program TCS (Clement 2000) incorporating all of the sequences obtained from all capture sites. In this procedure, haplotype networks are generated under statistical parsimony where haplotypes are split into separate groups if the number of mutational steps required to join them exceeds the number of steps allowed using a 95% parsimony criterion. In addition, to place the Oregon pallid bat samples into a broader phylogeographic context, we aligned the Oregon mtDNA sequences to 80 homologous sequences from pallid bat specimens collected throughout western North America (British Columbia Canada, N=5; California, N=6; Utah, N=6; Arizona, N=2; Nevada, N=1; Texas, N=28; Oklahoma, N=2; New Mexico, N=17 and Mexico, N=13; Genbank accession numbers AY706769–AY706887) and trimmed them to a common length using Sequencher 4.0 (Genescan). We then used JModeltest to determine the appropriate model of evolution and nucleotide substitution for the data and used these parameters to create a maximum likelihood tree in the program Phyml (Guindon 2003) with the initial tree created using the BioNJ algorithm and the tree topology search using the nearest neighbor interchange (NNI) algorithm. Node support was determined from 1000 bootstrap replicates.

RESULTS

Genetic Diversity and Roost Composition - We obtained mtDNA control region sequence data from 108 females and 5 males. These 113 sequences contained 8 unique haplotypes with 57 variable sites (5 insertions/deletions, 44 transitions, and 8 transversions; see Table S2 in online supplemental material for more information). Within roosting areas, mitochondrial sequence diversity was relatively low with 3-5 haplotypes identified per roosting area (Table 1). In addition, while there was some overlap in the distribution of haplotypes among roosting areas, we identified several haplotypes that were unique to one area (e.g. haplotypes 5 and 7 from the CSH and WCB night roosts and haplotypes 2 and 3 found only at the CCS and CCN roosting areas, Fig. 2).

Females captured when exiting the same day roost crevice at the CCN and CCS sites comprised only two matrilines (haplotypes 2 and 8) and all but 1 of the 20 captured females shared a haplotype with at least one additional bat from the same crevice roost. In contrast, a male shared a haplotype with a female roostmate on only one occasion (haplotype 8) while two males exiting a day roost at CCS belonged to different matrilines than females in the same roost (males - haplotypes 1 and 3, females - haplotypes 2 and 8). (Table S3)

All 113 bats and the 18 recaptures referenced above were genotyped at 12 microsatellite loci (Table S1). Two loci were excluded from subsequent analyses due either to lack of variation (E10) or to failure to meet Hardy Weinberg expectations (H10). The remaining 10 loci had 2-24 alleles, did not deviate from Hardy Weinberg expectations (P = 0.032 – 0.81), had relatively few null alleles (< 4% for the 8 loci with > 2 alleles), and did not exhibit significant linkage disequilibrium (P = 0.02 – 1.0) after sequential Bonferroni correction. Within roosting areas, the 10 microsatellite loci showed considerable variability with 7.6 – 9.4 alleles per locus and average heterozygosity of 0.72 ± 0.07 for all genotyped bats (Table 1).

Population Structure Among Roosting Areas - As shown in Table 2, for the microsatellite data pairwise F_{ST} values among the roosting areas were low with none significantly greater than zero after sequential Bonferroni correction. In contrast, the mitochondrial sequence data showed evidence of significant population structure between CSH and both the CCN and CCS roosting areas while there was no evidence of population subdivision between the CCN and CCS roosting areas. Excluding the three males captured in the CCS roosting area resulted in evidence for marginally significant population structure between CCS and CCN using the microsatellite data, but otherwise did not alter the results. Finally, AMOVA revealed significant genetic structuring (Φ_{ST} of 0.496, P < 0.0001, 10,000 randomizations) between the eastern and western sites. This result is due to 46.0% of the mtDNA sequence variation being attributable to differences between the east vs. west sites and only 3.6% of the mtDNA variation being due to differences among sites within groups.

Phylogeographic Patterns - Statistical parsimony grouped the eight haplotypes into two distinct clades with over 12% sequence divergence (Fig. 3). However, evidence of this mitochondrial structure is not reflected in the microsatellite markers (F_{ST} between the two clades = 0.004). Haplotype 8 in clade A was found in 65 (57%) of the sequenced bats, whereas in clade B most haplotypes were evenly distributed with the exception of haplotype 6, which was found in only one bat from the CSH night roost. Finally, geographical location was not independent of haplotype frequency in that most clade A haplotypes were found in eastern sites while clade B haplotypes were found predominantly in western sites (Fisher's Exact Test, P < 0.0001) (Fig. 2).

When the control region sequences obtained for this study were aligned with those collected by Weyandt and Van Den Bussche (2007), we found that the two clades identified in this study correspond to two of the clades they located either in northern California (clade A) or in British Columbia extending south to Mexico (clade B) (Figs. 1 and 4). Clade C (Weyandt and Van Den Bussche 2007), which included bats sampled in Texas, Oklahoma, New Mexico, and three samples from Mexico, were excluded from the tree for clarity.

DISCUSSION

Population Structure and Female Philopatry - In this study, we estimated population structure from maternally inherited mtDNA haplotypes and bi-parentally inherited nuclear microsatellites to detect evidence of female philopatry and sex-biased dispersal and mating in cliff roosting pallid bats in central Oregon. Overall, similar to the findings from other temperate bats, we found evidence of strong genetic structure from maternally inherited markers and little structure from bi-parentally inherited markers. These differences indicate that female dispersal is restricted to neighboring roosting sites while male dispersal enables inter-colony mating. While in most temperate bats it is thought that mating occurs either in a swarm outside or en route to a hibernaculum (e.g. (Kerth 2004)), the extent to which pallid bats engage in similar behavior is unknown, although pallid bats have been reported to vacate summer diurnal roosting areas in the early fall presumably to hibernate (O'Shea and Vaughan 1977; Hermanson and O'Shea 1983). Regardless, given the difference in population structure revealed by the two types of genetic markers, gene flow among nuclear markers must result from males mating with females from different colonies while females remain philopatric to their natal roost sites. This conclusion is also supported by re-capture of 20 banded female bats in more than one field season at the same site and telemetry data which has shown that bats roost in the same roosting area in multiple years (B. Arnold, unpublished data).

The finding of strong female natal philopatry in pallid bats is especially interesting given that pallid bats are often regarded as being highly social when compared to other vespertilionid bats (Hermanson and O'Shea 1983). For example, pallid bats have been reported to engage in cooperative behaviors, such as preferentially placing juveniles in the center of a roosting group (Trune and Slobodchikoff 1978), guarding juveniles (Beck and Rudd 1960), and guiding mothers to distressed offspring (Brown 1976). Pallid bats are also relatively long-lived with reports of individuals surviving over nine years in the wild (Tuttle and Stevenson 1982), which along with female natal philopatry, creates opportunities for the maintenance of cooperative behaviors through kin selection if roosting groups consist of relatives or bats have a mechanism to identify relatives. Low frequency social calls given as bats return to the roost after foraging is one potential mechanism (Vaughan and O'Shea 1976). These calls are individually specific and stable through time and playbacks show that bats respond to calls by approaching the source of the sound and calling in response (Arnold and Wilkinson 2011). Thus, calling appears to facilitate roosting group formation. To determine the degree to which calling may assist in the maintenance of multigenerational social groups during roost switching, acoustic analysis of call structure to determine whether calls encode any relatedness information in conjunction with more detailed analysis of the genetic relatedness of bats sharing a crevice roost will be an important avenue for future research.

Relative to some studies conducted on temperate vespertilionid bats, the haplotype diversity exhibited within pallid bat colonies in central Oregon appears to be low. For example, in a study on *Eptesicus fuscus*, Vonhof et al. (2008) found haplotype diversity ranging from 0.615 to 0.948 within colonies and 5-15 haplotypes per colony, which is more than we found (Table 1). However, the *E. fuscus* maternity colonies sampled for the Vonhof et al. (2008) study were in buildings that have maintained stable colonies for at least 17 years. Tree roosting bats, such as *E. fuscus* and *Myotis septentrionalis* in Canada and *M. bechsteinii* in Germany that exhibit fission-fusion behavior and high roost lability may offer a better comparison to this study. In these species, the number of unique haplotypes per roosting area are two (*M. bechsteinii*, (Kerth et al. 2000)), four (*M. septentrionalis*, (Patriquin et al. 2013)), and six (*E. fuscus*, (Metheny et al. 2008b)), which are comparable to our results. Finally, the low haplotype diversity found in this study could be explained by the relatively small sample size of bats captured or tracked to roosting areas. However, multiple bats captured at the cattle trough located adjacent to the two roosting areas also showed limited haplotype diversity comparable to the maternity sites (Fig. 2, Table 1) and did not add any additional haplotypes beyond those found at either the CCS or CCN roosting areas.

The low Φ_{ST} estimate of 0.077 between the CCS and CCN roosting areas suggests that female movement between these cliff complexes is relatively high, which is not surprising given that they are only 1 km apart. Thus, as in other bat species that exhibit high roost lability such as *M. bechsteinii* (Kerth 1999), *E. fuscus* (Willis and Brigham 2004), and *M. septentrionalis* (Garroway and Broders 2007), a colony may be defined not by a given structure that bats return to in multiple years, but by a geographic area with multiple potential roosts to which females are faithful.

The high Φ_{ST} between the night roosts and both of the maternity roosting areas along with the AMOVA results indicating significant genetic differences between the eastern and western sites, suggests the presence of a geographic or behavioral barrier to female dispersal between these areas even though they are separated by only a few kilometers. Behavioral barriers to mitochondrial gene flow have been found in *M. bechsteinii* as a result of forest fragmentation even though both males and females can regularly cross clearings between forest patches when moving to swarming sites to mate (Kerth and Petit 2005). Kerth and Petit (2005) argue that dispersal by both males and females to swarming sites resulting in the spread of autosomal genes and females crossing boundaries to colonize new roosting areas can be viewed as behaviorally distinct. Thus, colonization of new roosting areas followed by female philopatry can lead to genetic divergence in maternally inherited markers over short distances, as was found in tree roosting *E. fuscus* where females moving to new roosting areas consisted of closely related maternal kin (Metheny et al. 2008a). As with most temperate bats, we know little about the behavioral mechanisms behind maternity colony formation and where and when mating takes place in pallid bats. Additional research on these topics is

needed to understand how such deep mtDNA divergence between groups can persist over such a small geographic area.

mtDNA Phylogeography - The finding that pallid bats captured sharing the same roost are representative of two divergent mitochondrial lineages is unexpected in that the two clades differ by over 12%, which is greater than the level of divergence reported for many mammalian subspecies and some species (Baker and Bradley 2006; Mayer 2007). While deep genetic divergence in sympatric populations can be indicative of cryptic species (e.g. Miller-Butterworth et al. 2005), both weight ($\overline{X}_{(\text{Clade A})} = 22.2$ g, $\overline{X}_{(\text{Clade B})} = 22.3$ g, $t_{(114)} = 0.234$, $P = 0.82$) and forearm length ($\overline{X}_{(\text{Clade A})} = 53.0$ mm, $\overline{X}_{(\text{Clade B})} = 53.6$ mm, $t_{(114)} = 1.186$, $P = 0.23$) measured from pallid bats captured at the study area are not significantly different. In addition, the acoustic structure of their low frequency contact calls also do not differ significantly (B. Arnold, unpublished data) indicating that there are no obvious morphological or acoustic differences between the two groups. More importantly, the low Fst values indicate that the two groups freely interbreed. These results correspond to other studies of bats that have found sympatric distributions of deeply diverged mtDNA lineages without evidence of divergence at nuclear markers consistent with male-mediated gene flow (Ruedi and Castella 2003; Turmelle et al. 2011).

From a phylogeographic perspective, the pattern identified by the haplotype network (Fig. 3) suggests the expansion of bats from clade A into central Oregon is relatively recent given the predominance of haplotype 8 with only two individuals represented by haplotype 7 and few presumed haplotypes not found in this study. In contrast, the pattern exhibited by clade B with many haplotypes having similar frequency is indicative of a large population maintained over a long period of time (Avise 2009).

When the sequences obtained for this study were aligned with those collected by Weyandt and Van Den Bussche (2007), we found that the two clades identified in this study correspond to two distinct geographic clades (Figs. 1 and 4) located either in northern California (clade A) or from British Columbia extending south to Mexico (clade B). Recent phylogeographic studies on mammals in the Pacific Northwest have identified similar divergence zones resulting in clades corresponding to the geographic locations of northern California into central Oregon and British Columbia (Chavez and Kenagy 2010; Galbreath et al. 2010). These distributions are indicative of post-glacial range expansions from southern refugia northwards on either side of the Sierra Nevada Mountains with secondary contact occurring recently in central Oregon. Further sampling both west and east of the study area is needed to determine if this interpretation of range expansion of pallid bats in northwestern North America is correct.

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Table 1. Measures of genetic variation for *A. pallidus* at six sites in central Oregon. Sample size (N), number of haplotypes (N_h), haplotype diversity (h), nucleotide diversity (π), mean number of alleles per microsatellite locus (A), observed (H_O) and expected (H_E) heterozygosities

		Mitochondrial Variability			**Microsatellite Variability**		
Site Location	N ($n_f/n_{m)}$	N_h	h	π	A	H_O	H_E
CCS	25 (22/3)	5	0.597	0.061	7.7	0.68	0.702
CCN	18 (18/0)	3	0.307	0.036	7.6	0.72	0.734
Site 4	2 (2/0)	2	1	0.122	3	0.83	0.778
CCT	47 (45/2)	5	0.456	0.050	9.4	0.706	0.732
CSH	20 (20/0)	5	0.663	0.02	8.2	0.735	0.712
WCB	1 (1/0)	1	–	–	–	–	–

Table 2. Weir and Cockerham's estimates of F_{ST} (microsatellite data, lower diagonal) and Φ_{ST} (mitochondrial data, upper diagonal) between *A. pallidus* roosting sites. Significance was determined using a permutation procedure implemented in the program Arlequin with alpha set to 0.017 to correct for multiple testing. Values in parentheses were calculated excluding the three males captured at the CCS roosting area.

	CCS	**CCN**	**CSH**
CCS	–	0.077 (0.045)	0.391*** (0.438) ***
CCN	0.01 (0.017)*	–	0.678***
CSH	0.0083 (0.0086)	0.0035	–

* P=0.013

*** P<0.001

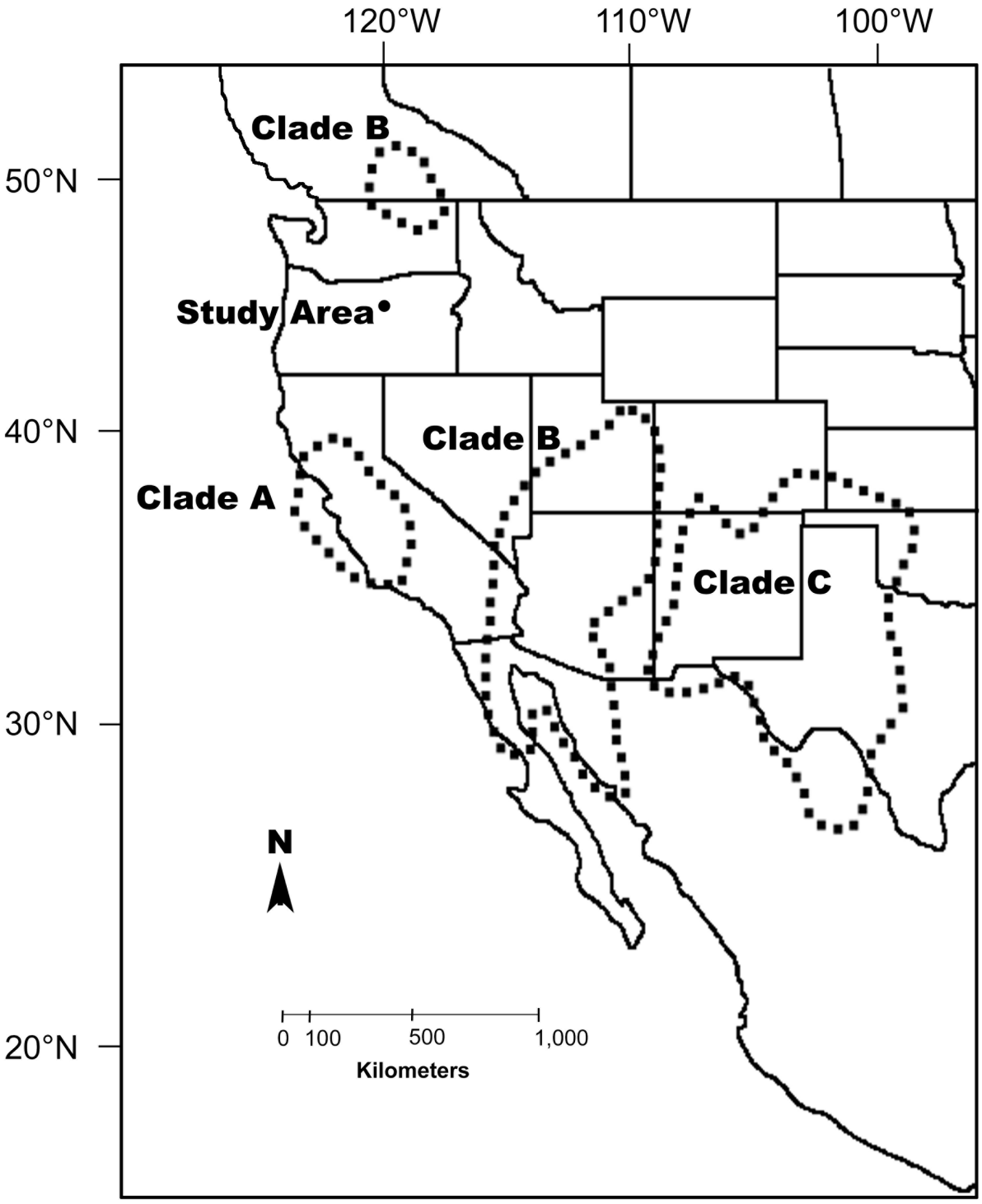
FIGURE LEGENDS

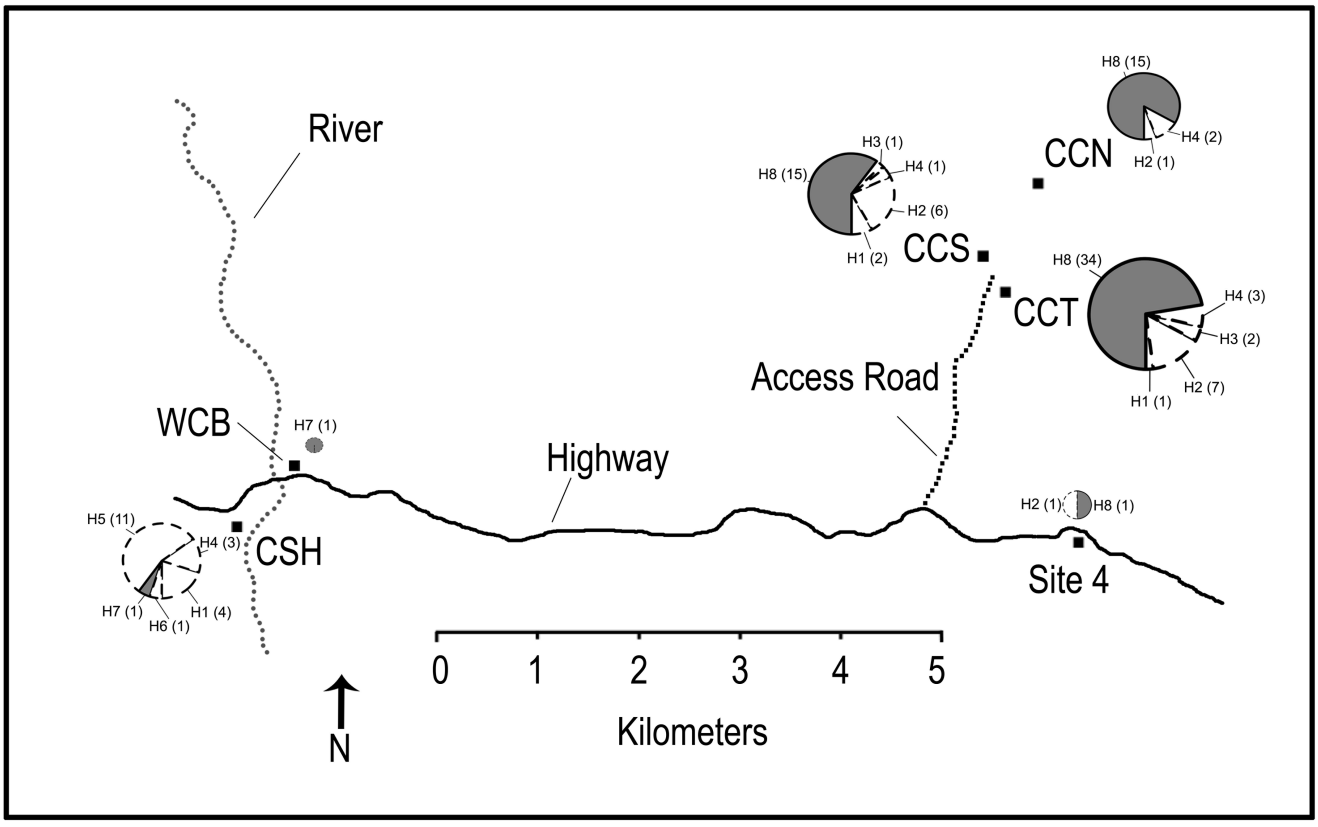
Fig. 1 – Map showing the location of the study area relative to the known occurrence of mtDNA clades of pallid bats in North America. The figure was modified from Weyandt and Van Den Bussche (2007) with dashed lines encompassing the sampling locations utilized for their study.

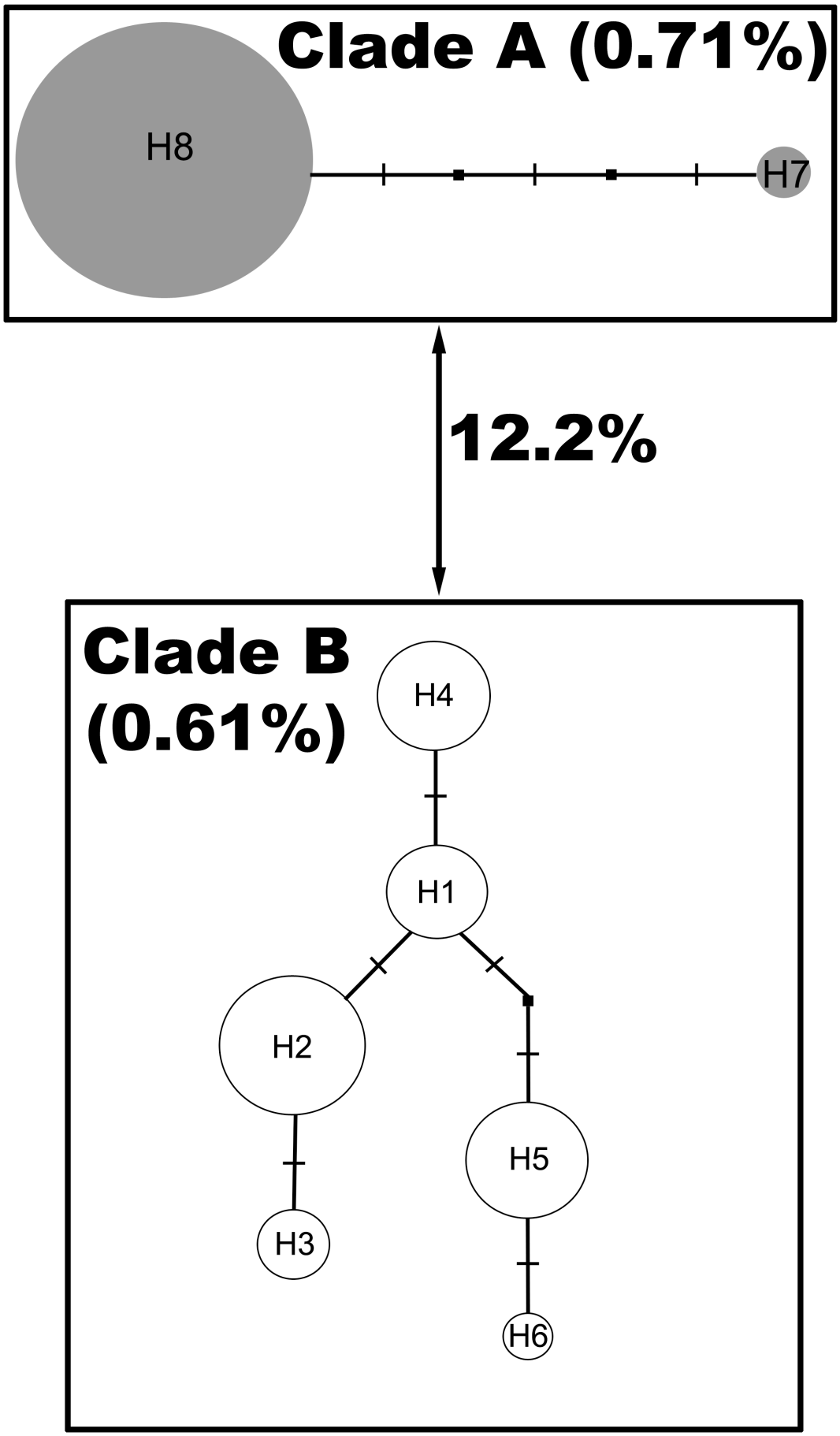
Fig. 2 – Map of the study area showing the capture sites for *Antrozous pallidus*. Pie charts indicate mtDNA control region haplotype frequencies at each site. Haplotypes shaded in grey align closely with haplotypes identified as "Clade A" in Weyandt and Van Den Bussche (2007) while haplotypes with no shading align closely with haplotypes identified as "Clade B". Capture site abbreviations are: Cove Creek North (CCN), Cove Creek South (CCS), Cove Creek Cattle Trough (CCT), Clarno School House (CSH), and Will Cole Barn (WCB).

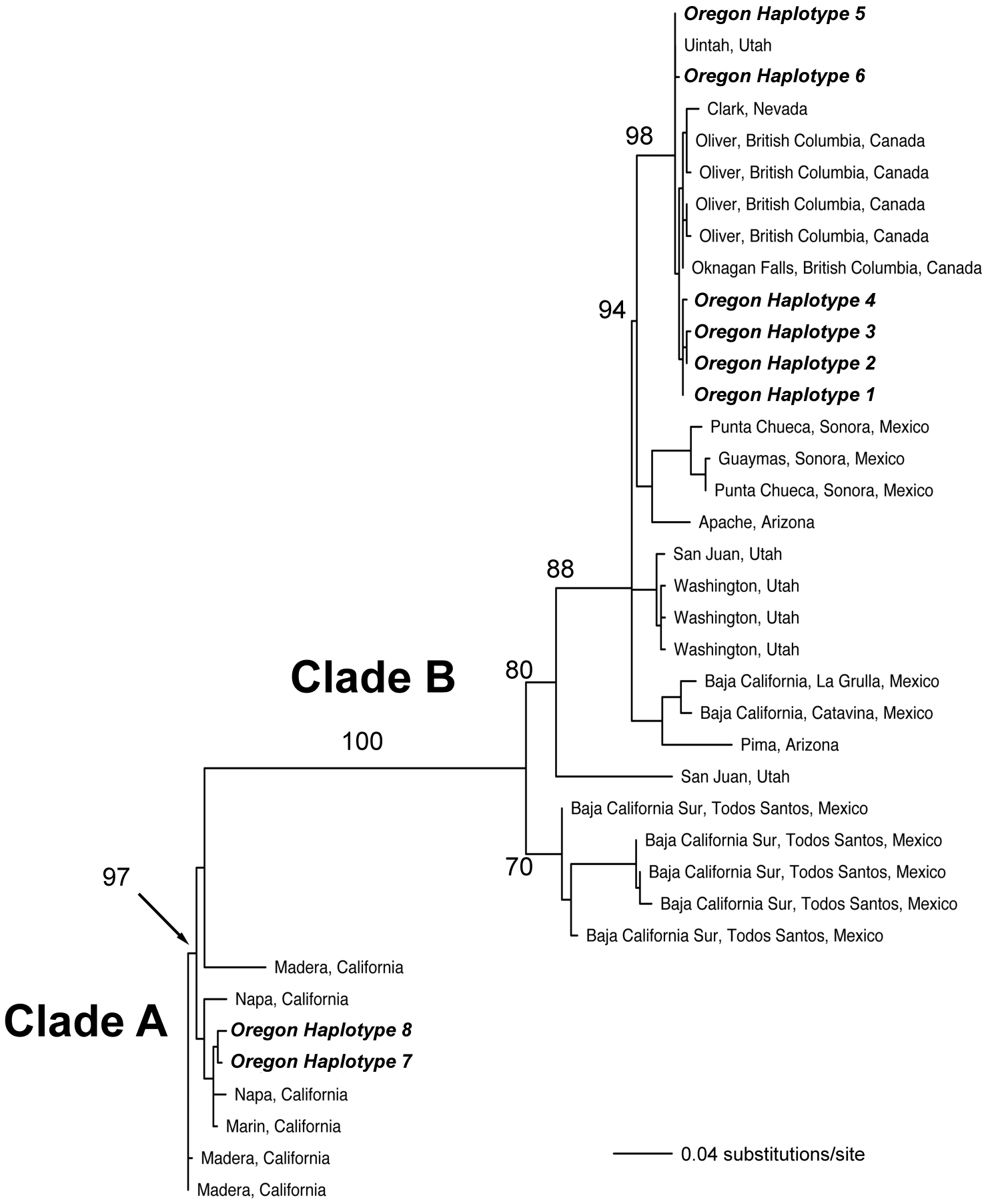
Fig. 3 – Parsimony network of mtDNA control region haplotypes showing clades identified using 95% confidence in the program TCS. A bar between haplotypes represents a single mutation and solid squares designate presumed haplotypes not identified in this study. Percent sequence divergence is shown within clades (in box) and between clades (outside box).

Fig. 4 – Unrooted maximum likelihood tree obtained for mtDNA control region sequences of bats sampled in Oregon (shown in bold italics) or obtained from Genbank and representative of clades A and B (Weyandt and Van Den Bussche 2007). The tree was constructed based on the TrN + G (gamma=0.15) model of nucleotide substitution obtained from JModeltest (see methods). Bootstrap support (percentage of 1000 replicates) is shown for the nodes leading to major groups.


120°W
110°W
100°W
50°N
40°N
30°N
20°N
Clade B
Study Area
Clade B
Clade A
Clade C
N
0 100 500 1,000
Kilometers


River
WCB
H7 (1)
Highway
Access Road
CSH
H5 (11)
H4 (3)
H7 (1)
H6 (1)
H1 (4)
H3 (1)
H8 (15)
H4 (1)
H2 (6)
H1 (2)
CCS
H8 (15)
H4 (2)
CCN
H2 (1)
H8 (34)
H4 (3)
CCT
H3 (2)
H2 (7)
H1 (1)
H2 (1)
H8 (1)
Site 4
0 1 2 3 4 5
Kilometers
N


Clade A (0.71%)
H8
H7
12.2%
Clade B
(0.61%)
H4
H1
H2
H5
H3
H6


Oregon Haplotype 5
Uintah, Utah
Oregon Haplotype 6
Clark, Nevada
98
Oliver, British Columbia, Canada
Oliver, British Columbia, Canada
Oliver, British Columbia, Canada
Oliver, British Columbia, Canada
Oknagan Falls, British Columbia, Canada
94
Oregon Haplotype 4
Oregon Haplotype 3
Oregon Haplotype 2
Oregon Haplotype 1
Punta Chueca, Sonora, Mexico
Guaymas, Sonora, Mexico
Punta Chueca, Sonora, Mexico
Apache, Arizona
88
San Juan, Utah
Washington, Utah
Washington, Utah
Washington, Utah
Clade B
80
Baja California, La Grulla, Mexico
Baja California, Catavina, Mexico
100
Pima, Arizona
San Juan, Utah
Baja California Sur, Todos Santos, Mexico
Baja California Sur, Todos Santos, Mexico
70
Baja California Sur, Todos Santos, Mexico
97
Baja California Sur, Todos Santos, Mexico
Baja California Sur, Todos Santos, Mexico
Madera, California
Napa, California
Clade A
Oregon Haplotype 8
Oregon Haplotype 7
Napa, California
Marin, California
Madera, California
Madera, California
0.04 substitutions/site

ONLINE SUPPLEMENTAL INFORMATION

Table S1. Annealing temperatures, observed size ranges, and genetic diversity (heterozygosity expected (H_E), heterozygosity observed (H_O) for the 12 microsatellite loci used in the genetic analyses of 113 *Antrozous pallidus* samples collected in central Oregon, U.S.A.

Locus	Observed Size Range (bp)	T_A (°C)	No. of Alleles	H_E	H_O	Source Species	Primer Source
EF4	232-260	50	11	0.86	0.84	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF5	157,159	50	2	0.49	0.39	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF6	174-205	47	9	0.74	0.72	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF21	217-242	50	10	0.77	0.81	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF15	77-108	55	12	0.87	0.86	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF20	90-228	47	24	0.92	0.93	*Eptesicus fuscus*	(Vonhof et al. 2002)
G25	129-151	53	6	0.59	0.54	*Myotis myotis*	(Castella andRuedi 2000)
B02	160-192	53	16	0.91	0.88	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
G07	338,340	53	2	0.26	0.27	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
G02	185-210	53	12	0.85	0.87	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
H10	255-317	53	20	0.93	0.82	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
E10	307	53	1	NA	0	*Corynorhinus rafinesquii*	(Piaggio et al. 2009a)

Table S2. Haplotypes obtained from the 425 bp mtDNA control region sequences of 113 *Antrozous pallidus* captured at six sites in central Oregon (Sample sizes for each haplotype are: H1–7, H2–15, H3–3, H4–9, H5–11, H6–1, H7–2, H8–65)

	Nucleotide Position																			
	28	**30**	**31**	**34**	**36**	**41**	**48**	**51**	**52**	**70**	**72**	**73**	**74**	**78**	**79**	**87**	**96**	**99**	**101**	**105**
H1	A	A	A	A	C	A	T	G	C	G	C	T	G	C	T	A	T	G	A	A
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
H7	–	G	C	G	T	G	C	A	T	A	T	C	A	T	A	.	C	A	G	–
H8	–	G	C	G	T	G	C	A	T	A	T	C	A	T	A	.	C	A	.	–

	Nucleotide Position																			
	111	**114**	**115**	**117**	**118**	**127**	**138**	**141**	**159**	**160**	**188**	**190**	**195**	**197**	**201**	**219**	**224**	**225**	**228**	**235**
H1	C	–	C	G	G	C	T	A	C	C	A	T	–	T	A	T	C	T	G	C
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	T	T	T	A	T	T	C	G	T	T	C	A	A	C	.	C	T	C	A	A
H8	T	T	T	A	T	T	C	G	T	T	C	A	A	C	G	C	T	C	A	A

	Nucleotide Position																
	236	**237**	**238**	**248**	**252**	**255**	**272**	**281**	**298**	**306**	**334**	**344**	**350**	**356**	**360**	**371**	**414**
H1	C	T	C	T	T	–	C	T	C	A	C	T	C	T	C	T	A
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H4	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.
H7	T	C	T	C	C	A	A	.	T	G	A	C	T	.	T	C	.
H8	T	C	T	C	C	A	A	.	T	G	A	.	T	.	T	C	.

Table S3. Haplotype composition of captured *Antrozous pallidus* roosting groups at the study site in Central Oregon (CCS = Cove Creek South, CCN = Cove Creek North)

Capture Event	**Haplotype Distribution**
CCS1	H2 – 2 females
	H8 - 8 females, 1 male
CCS2	H1 – 1 male
	H2 – 1 female
	H3 – 1 male
	H8 – 6 females
CCN	H8 – 3 females

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