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SHORT COMMUNICATION

Polyphyletic ancestry of historic gray wolves inhabiting U.S. Pacific states

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Abstract Given the recent re-colonization of gray wolves (*Canis lupus*) to the Pacific northwest, USA, and subsequent migration into northern California, understanding how well natural migration has restored historic diversity can inform management decisions. In this study, we report the mitochondrial DNA control region haplotypes of nine museum specimens that curators identified as *C. lupus* from Oregon, Nevada, and California. Among the nine samples currently available for genetic analysis of historic genetic diversity of *C. l.* spp. in the U.S. Pacific states, we found six previously described haplotypes including two domestic dog (*C. l. familiaris*) haplotypes. Notably, we present the first evidence of Mexican wolf (*C. l. baileyi*) ancestry in southern California while the northern Californian specimen, as well as one individual from Nevada, present a haplotype common to wolves from the historic American West and extant Canadian wolf populations. Finally, the three Oregon specimens shared a haplotype that is only observed in extant wolves from coastal British Columbia (the "coastal rainforest" wolf ecotype), indicating that the historical range of this haplotype reached as far south as southwestern Oregon. In conclusion, our results indicate that the genetic composition of historic wolf populations in the Pacific northwest and southwest states was polyphyletic and included wolves that share maternal ancestry with current populations from adjacent regions. These findings, in addition with future nuclear analyses, reveal more accurate historic range delimitations, which is critical information when designing appropriate management plans for wolves naturally re-colonizing the U.S. Pacific northwest and southwest regions.

Keywords *Canis lupus* · Mitochondrial DNA · Museum samples · Historical range · Western United States

Introduction

Due to habitat restoration and conservation efforts, but also climatic changes, large carnivores, such as gray wolves (*Canis lupus*), are expanding their current ranges to occupy territory they historically inhabited and, in some cases, have expanded outside their defined historical distributions (ex. Kays et al. 2010; Pitra et al. 2009). Wolf populations inhabiting the Pacific northwestern states (Oregon, Washington, and Idaho; U.S.A.) were historically distributed from west of the Cascade Range to the Pacific coast (Goldman 1944). The occurrence of wolves in the southwestern states (California and Nevada; U.S.A.) prior to their extirpation in the 1920's was largely based on observational data, which remains unreliable as wolves may have been confused with coyotes or dogs. Wolves historically present in these regions likely derived from distinct subspecies and ecotypes distributed throughout

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coastal British Columbia (*C. l. fuscus* encompassed in *C. l. nubilus* by Nowak 1995; Muñoz-Fuentes et al. 2009; Weckworth et al. 2010), the American West (*C. l. nubilus*; Leonard et al. 2005), and the Southwest (*C. l. baileyi*; Leonard et al. 2005; see Chambers et al. 2012 for a recent discussion of taxonomy). Therefore, gray wolves historically inhabiting mainland Pacific northwest and southwest regions were likely the representatives of diverse ancestries, and consequently constituted heterogeneous populations. During the last few decades, wolves have naturally re-established residency in Oregon and Washington (ODFW 2013; Wiles et al. 2011). More recently, a radiometry survey has reported the dispersion of a lone wolf ("OR7") from northeastern Oregon to northeastern California before returning to the southern Oregon Cascades (ODFW 2013). Application of genetics facilitates the verification of the genetic relationship between the historic populations and wolves now re-colonizing areas from which they were extirpated in the first half of the 20th century. Determining the occurrence of common ancestry between historic and extant populations of wolves in these regions may have implications for their protection status at the Federal and State level (U.S. Fish and Wildlife Service 2014, CDFW 2014).

To better understand the genetic identities of historic wolves and, subsequently, assess the genetic continuity that might exist with the newly established populations, we sequenced partial mitochondrial DNA control regions from museum specimens collected in California, Oregon and Nevada. Most of these specimens were sampled shortly before the time of the species extirpation in the 1940s (Mech and Boitani 2003). Among the nine pacific coast specimens available for genetic investigation, four were collected in disparate parts of the state of California. Our study characterizes the maternal genetic ancestry of historic wolves from the Pacific northwest and southwest, which may impact management plans for newly established wolves. Specifically, if the current population has genetic continuity with historic wolves this may imply natural migration is an appropriate source for genetic restoration.

Materials and methods

Maxillo-turbinate bone samples from nine specimens labeled as wild *C. lupus spp.* were obtained from the University of California, Berkeley Museum of Vertebrate Zoology's mammal collection (MVZ:MAMM). These specimens originated from California (n = 4), Oregon (n = 4) and Nevada (n = 1) (Table 1 and Fig. 1). DNA extractions were performed in a lab exclusively dedicated to ancient samples at University of California, Los Angeles, using methods described in Rohland and Hofreiter (2007). DNA extractions and subsequent molecular analyses were processed in parallel with extraction and PCR negative controls to monitor for potential DNA contamination. To determine species status and examine relationships within these historic wolves, a 318-bp portion of mitochondrial control region (MT-CR) was amplified using two sets of overlapping primer pairs: (i) Thr-L (Vilà et al. 1999) and ddl5R (Leonard et al. 2002); (ii) ddl1F and ddl2R (Leonard et al. 2002) following the protocol in Leonard et al. (2002). After DNA amplification, successful PCR products were sequenced in both directions using BigDye (Applied Biosystems, Inc.) on an ABI3730*XL* capillary sequencer (Applied Biosystems, Inc). All 36 fragments were replicated twice to verify the validity of the sequencing. Each of the haplotypes found in our historic samples had been previously observed suggesting that polymerase error and deamination was not an issue. Sequences were visualized and aligned in Geneious 6.0.5 (Biomatters). A local BLAST search (Altschul et al. 1997) was performed on individual consensus sequences against Genbank partial MT-CR haplotypes (n = 75 globally distributed gray wolves (*C. lupus*); n = 125 coyotes (*C. latrans*); n = 1 red wolf (*C. rufus*); n = 30 domestic dogs (*C. l. familiaris*)). Domestic dog, red wolf, and coyote haplotype sequences were excluded in subsequent analyses.

To infer the North American wolf phylogeny from the mtDNA haplotypes, we used two statistical approaches. First, a consensus median joining network (MJN) of all shortest trees was constructed using Network 4.6.1.0 (Bandelt et al. 1999). Second, we implemented BAPS 5.2 (Corander and Tang 2007) to investigate the assignment of haplotypes to specific genetic clusters using the 'clustering of linked loci' model. Prior upper bound values were specified as $K_{max} = 2–10$, with three independent replications for each K value. The average number of nucleotide differences between clusters (Tajima 1983) and the fixed differences between clusters (Hey 1991) were calculated in DnaSP v5.0 (Librado and Rozas 2009).

Results and Discussion

To infer the maternal ancestry of historic wolves from Pacific northwest and southwest regions, we amplified a 318-bp fragment of MT-CR from nine museum samples. We recovered six previously described haplotypes: lu28, lu33, lu61, lu68, D13 and D26B (Table 1). Haplotypes D13 and D26B are specific to the domestic dog (*C. l. familaris*; Vilà et al. 1997, 1999) and were found in specimens MVZ:MAMM:171944 and MVZ:MAMM:86910, respectively (Table 1). Apart from the information that these

Table 1 Mitochondrial control region (MT-CR) haplotype assignment for nine historic canid specimens from the UC Berkeley Museum of Vertebrate Zoology (MVZ) mammal collection

	MVZ: MAMM ID	Collection year	Sampling location	Canid MT-CR haplotype	GenBank accession #
#1	29771	1918	Curry County, OR	lu68	FN298179
#2	86910	1929	Douglas County, OR	D26B	AF008149
#3	86874	1931	Douglas County, OR	lu68	FN298179
#4	59682	1933	Douglas County, OR	lu68	FN298179
#5	33424	1922	Elko County, NV	lu28	FM201759
#6	33389	1922	San Bernardino County, CA	lu33	AF005313
#7	34228	1924	Lassen County, CA	lu28	FM201759
#8	129254	1962	Tulare County, CA	lu61	AY812741
#9	171944	1982	Tehama County, CA	D13	AF115714

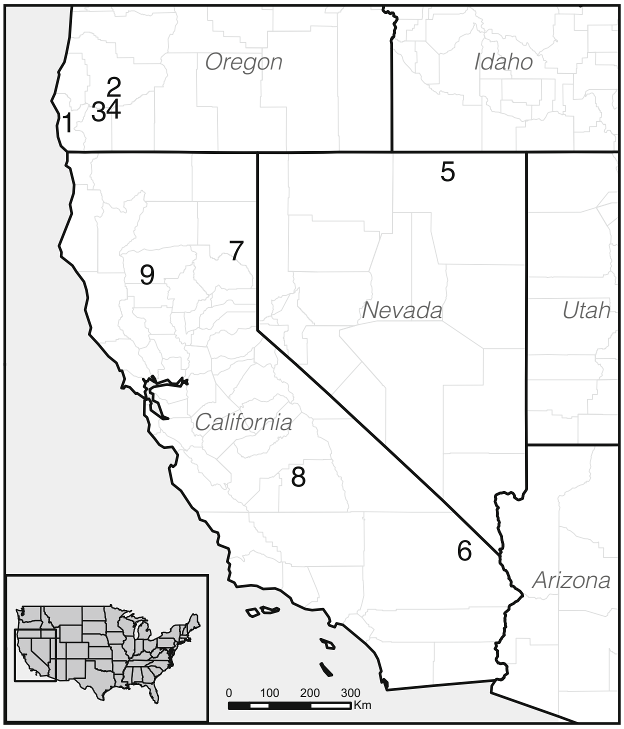

Fig. 1 Distribution of the *Canis lupus* spp. museum samples successfully amplified for the 318-bp MT-CR fragment. Each number (1–9) corresponds to a unique specimen. Individuals #1, #3–8 displayed gray wolf mtDNA haplotypes, while #2 and #9 carried domestic dog haplotypes. Further information is available in Table 1

specimens were wild caught, it was not reported whether these individuals were hunted among wolves or were thought to belong to a wolf pack. However, these results confirmed the California Department of Fish and Wildlife collection note reporting MVZ:MAMM:171944 to be incorrectly labeled. These two historical samples were not included in the phylogenetic analyses due to shared ancestry with dogs.

To assess the phylogenetic relationship of the seven wolf mtDNA sequences, we selected a 230-bp fragment that overlapped with previously published sequences. We obtained an alignment of 22 haplotypes containing 15 informative sites (Fig. 2). The MJN displayed a partitioning of these haplotypes into four haplogroups also supported by BAPS (posterior probability PP = 0.99; Fig. 2). Our clustering analysis recovered the monophyletic grouping of the previously described southern clade (Leonard et al. 2005; cluster A: lu33, lu47, lu50, lu51). The three additional BAPS clusters consisted of the following haplotypes: cluster B (lu29, lu30, lu31, lu37, lu61, G, N); cluster C (lu11, lu32, lu38, lu48, lu49, lu53, lu54, lu68); cluster D (lu28, lu52, lu67) (Fig. 2). The distribution of historical sequences in the network (Fig. 2), in addition to the average number of nucleotide differences between clusters (k = 4.8–9.7; Table S2) and the previously published Bayesian tree (Fig. 2 in Weckworth et al. 2010), suggest a polyphyletic ancestry of wolves collected in the Pacific states in the early 20th century.

We retrieved a total of four haplotypes among the seven specimens of established gray wolf maternal ancestry. Each of the three California wild *C. lupus* specimens carried a distinct gray wolf haplotype (lu28, lu33, lu61; Table 1). Individual MVZ:MAMM:33389 (San Bernardino County; Fig. 1) displayed the haplotype lu33 (Table 1), which has only been found in Mexican wolves (*C. l. baileyi;* Leonard et al. 2005). This specimen was identified as a Southern Rocky Mountain wolf (*C. l. youngi*) based on phenotypic and cranial morphometrics (Grinnell et al. 1937). Goldman (1944) proposed that this specimen represented a lone wolf that had wandered into southern California from an adjacent population in southern Nevada. However, our genetic results supported a Mexican wolf maternal ancestry and provided the first evidence that the southern clade (Leonard et al. 2005) occurred in California. Additional genetic analysis using autosomal markers would help to determine

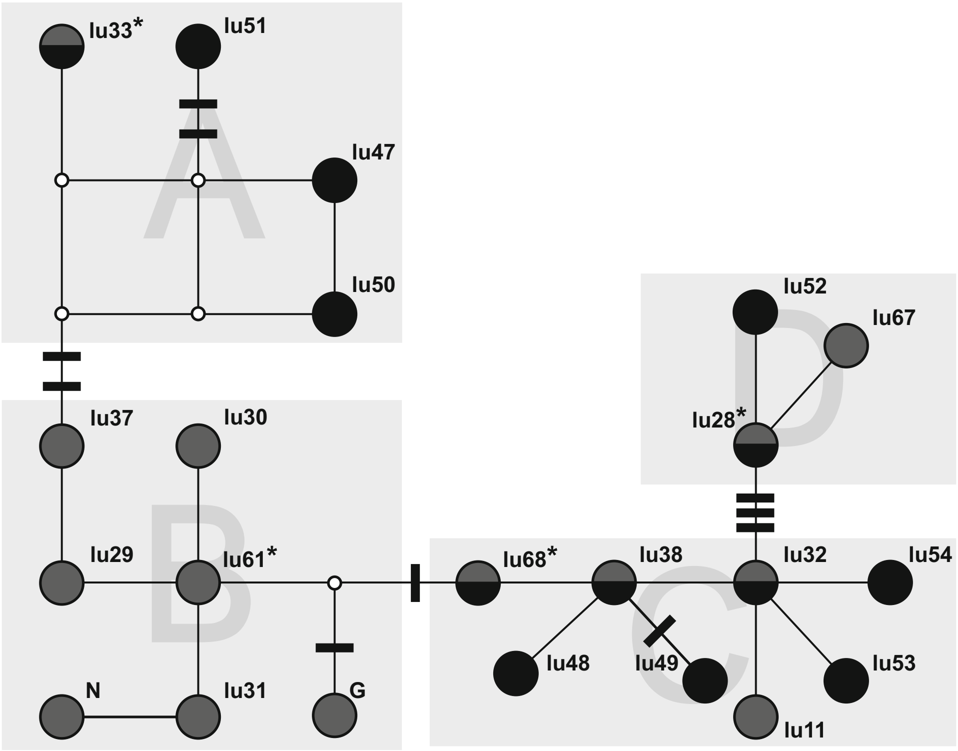

Fig. 2 Median-joining network (MJN) consensus tree and Bayesian analysis of population structure (BAPS) of North American wolf haplotypes. The consensus networks of all the shortest trees based on the 230-bp MT-CR fragment is shown. *Black coloring* of the large circles (haplotypes) indicate the occurrence of the named haplotype in the historic specimen. *Small open circles* (median vectors) depict missing intermediates (extinct or missing haplotypes). *Each vertical line* represents an additional nucleotide substitution to the one indicated by the line connecting two haplotype circles or median vectors. Haplotypes prefaced by ''lu'' were reviewed in Chambers et al. (2012). Haplotypes "N" and "G" were previously published haplotypes from Weckworth et al. (2010). Haplotypes are assigned to four distinct Bayesian clusters (A–D; posterior probability of PP = 0.99). Genetic distances between clusters are referred to in Table S2

whether MVZ:MAMM:33389 was of Mexican wolf ancestry or a hybrid between wolves from genetically distinct populations. Higher resolution of the genetic affinities would have important implications for the extension of the western historical range limits of the Mexican wolf subspecies. The U.S. Fish and Wildlife Service currently defines the historical range of the Mexican wolf to include parts of central and northern Mexico, western Texas, southern New Mexico, and southeastern and central Arizona (Parsons 1996). Redefining historical range limits to include southern California would allow for an assessment of additional habitats for the re-introduction program (Hendricks et al., *in prep*.).

The second Californian specimen (MVZ:MAMM:129254; Table 1), collected in Tulare County (Fig. 1), carried haplotype lu61, which has only been found in Alaskan and Canadian wolves (Leonard et al. 2005; Muñoz-Fuentes et al. 2009; Weckworth et al. 2010; Stronen et al. 2010). Considerable debate has surrounded the origin and taxonomy of this specimen. Shot in 1962, it was the first wolf documented in California in nearly four decades (Ingles 1963; McCullough 1967). While Ingles (1963) suggested that this individual belonged to a remnant population of wolves from the Sierra Nevada, McCullough (1967) found the specimen to be phenotypically similar to the central Asian subspecies *C. l. chanco*. Therefore, McCullough concluded that the wolf likely represented an anthropogenic introduction. Our genetic analysis supported the notion that MVZ:MAMM:129254 resulted from an introduction as the lu61 haplotype was historically absent from wolves in the conterminous U.S. (Leonard et al. 2005).

The taxonomy of the northern Californian specimen collected in 1924 (MVZ:MAMM:34228; Table 1; Fig. 1) has been debated. Although Goldman (1944) found similarity with the Cascade Mountain subspecies (*C. l. fuscus*), Grinnell et al. (1937) suggested that this individual might have migrated from western Nevada and therefore been a Plains wolf (*C. l. nubilus*). This taxonomic designation was congruent with Nowak's revision of the North American wolf subspecies classification (1995). Our results revealed that MVZ:MAMM:34228 (Lassen County, California), as well as MVZ:MAMM:33424 (*C. l. youngi*) collected in Elko County, Nevada, presented a mtDNA sequence matching lu28 (Table 1; Fig. 1). This haplotype has been found in wolves from a wide geographic distribution and in diverse ecotypes. Historically present in the western U.S. through the Central Plains (Leonard et al. 2005), lu28 currently occurs from Alaska to eastern Ontario (reviewed in Chambers et al. 2012). Although the haplotype inference provided little information about the potential ancestry of these two specimens, our analysis illustrated the ubiquity of lu28 across the North American gray wolf range (Leonard et al. 2005; Chambers et al. 2012) and suggests the western boundary of this haplotype extended to California. Lastly, the occurrence of both haplotypes lu28 and lu33 in California specimens indicate that this state had historically hosted individuals sharing diverse genetic ancestry with wolves likely originating from Oregon, Nevada or Arizona. Although there are not current plans for re-introduction programs in these regions, our results support the recent decision by the state of California to protect naturally re-colonizing wolves (CDFW 2014).

Finally, the three specimens collected in southwestern Oregon (MVZ:MAMM:29771, 59602, and 86874; Table 1 and Fig. 1) and identified as Cascade Mountain wolves (*C. l. fuscus*), shared a single haplotype, lu68. Genetic investigation of modern and historic British Columbian (B.C.) and southeastern Alaskan populations revealed that this haplotype was unique to coastal B.C. wolves (Muñoz-Fuentes et al. 2009, 2010; Weckworth et al. 2010). These wolves, described as the “coastal rainforest” ecotype, are differentiated from individuals in adjacent inland populations by morphology, habitat preference, and feeding behavior (Muñoz-Fuentes et al. 2009). An additional genome-wide study has confirmed the divergence between coastal rainforest and inland wolf ecotypes (vonHoldt et al. 2011). The genetic characterization of our three historical individuals is therefore the first evidence for the occurrence of lu68 in the conterminous U.S. The congruence of the taxonomic identification (*C. l. fuscus*) and the common maternal ancestry between our samples and the coastal B.C. population supports Goldman’s (1944) proposed historic distribution of the coastal rainforest ecotype from B.C. to Oregon. Additionally, the three museum specimens were collected in the vicinity of the northern Californian border. Given the absence of physical barriers between Oregon and California and the long distance dispersion ability of the species (Mech and Boitani 2003), our results suggests that this haplotype, currently present only in the coastal rainforest ecotype, had a distribution across the Pacific Northwest coast and California. This first genetic assignment of historic wolves in Oregon may lead to a re-evaluation of the conservation status of the recently established wolves into the Pacific Northwest, U.S.A. Further documentation and genetic characterization of the current populations in Oregon and Washington will be necessary to determine whether newly established wolves represent continuity with this historical population and hold genetic influence from extant coastal rainforest wolves.

Our analyses provide important insight into the evolutionary history of wolves that formerly inhabited California, Oregon, and Nevada. Research using nuclear analyses and additional specimens will further elucidate the distributions of the historical wolf populations/subspecies. Our genetic investigation suggests a polyphyletic ancestry of the wolf populations inhabiting these areas. These findings need to be considered when designing accurate conservation and management plans for wolves naturally re-colonizing the Pacific northwest and southwest regions.

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