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Pliocene–Early Pleistocene Geological Events Structure Pacific Martens (*Martes caurina*)

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Abstract

The complex topography, climate, and geological history of Western North America have shaped contemporary patterns of biodiversity and species distributions in the region. Pacific martens (*Martes caurina*) are distributed along the northern Pacific Coast of North America with disjunct populations found throughout the Northwestern Forested Mountains and Marine West Coast Forest ecoregions of the West Coast. *Martes* in this region have been classified into subspecies; however, the subspecific designation has been extensively debated. In this study, we use genomic data to delineate conservation units of Pacific marten in the Sierra-Cascade-Coastal montane belt in the western United States. We analyzed the mitochondrial genome for 94 individuals to evaluate the spatial distribution and divergence times of major lineages. We further genotyped 401 individuals at 13 microsatellite loci to investigate major patterns of population structure. Both nuclear and mitochondrial DNA suggest substantial genetic substructure concordant with historical subspecies designations. Our results revealed that the region contains 2 distinct mitochondrial lineages: a Cascades/Sierra lineage that diverged from the Cascades/coastal lineage 2.23 (1.48–3.14 mya), consistent with orogeny of the Cascade Mountain chain. Interestingly, Pacific *Martes* share phylogeographic patterns similar with other sympatric taxa, suggesting that the complex geological history has shaped the biota of this region. The information is critical for conservation and management efforts, and further investigation of adaptive diversity is warranted following appropriate revision of conservation management designations.

Keywords: conservation genetics, Marten, subspecies, mitogenomics, phylogeography, ecoregion
Subject areas: Conservation genetics and biodiversity, Population structure and phylogeography

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Contemporary patterns of biodiversity and species distributions are shaped by geology, climate, and the interactions of organisms with both. Geological evolution sets the topographic template through multiple processes including folding of the earth's crust through lateral compression to form mountain ranges (orogeny), volcanic activity forming new land masses (volcanism), and the earth's crust forming on or breaking off a tectonic plate (terranes). Ancient and modern climates and their dynamics dictate environmental patterns at global, regional, and local scales (Soltis et al. 1997; Taberlet et al. 1998; Soltis et al. 2006). Although these forces operate broadly, the responses of single taxa are individualistic and contingent on variation in the demography, mobility, and ecological generality of an organism, which creates or limits ecological opportunities to exploit novel habitat (Stace 1989; Zink et al. 2000; Adamowicz et al. 2010, Mullen et al. 2010).

The interplay between topography, climate, and biodiversity has been a subject of intensive study and review in the western continental United States (Shafer et al. 2010). Mammals endemic to the northern coniferous forests display a phylogeographic split between the west coast and northern Rockies, which can be attributed to the Cascade Range uplift between approximately 5 and 2 million years ago (Graham 1999; Brunsfeld et al. 2001). Other phylogeographic splits associated with mountain chains are a result of allopatric diversification during historical glacial advances, which left multiple well-established forested refugia along the Pacific coast (Johnson and Cicero 2004; Weir and Schluter 2004). Additionally, a north–south phylogeographic split in the Coast/Cascade region, divided by what has been called the Soltis line (Soltis et al. 1997; Jaramillo-Correa et al. 2009), is apparent despite the lack of an obvious physiographic divide. This pattern has been documented within multiple taxa, including invertebrates, vertebrates, and plants (Wilke and Duncan 2004; Miller et al. 2006; Liston et al. 2007; Rankin et al. 2019).

In this region, the complex patterns of orographic lift, subsequent changes in global circulation patterns, and history of glaciation also create complex habitat patterns that are exploited by mammalian taxa. Pacific martens (*Martes caurina*) are distributed along the northern Pacific Coast of North America with disjunct populations found throughout the Northwestern Forested Mountains and Marine West Coast Forest ecoregions of the West Coast (Figure 1). A second marten species, *Martes americana*, evolved in an eastern North American refugium and later expanded across the northern forests following the retreat of the Laurentide ice sheet (Anderson 1970; Hughes 2009). Anderson (1970) provides fossil evidence suggesting that *M. americana* migrated to eastern North America across Beringia between 65 000 and 122 000 years ago with *M. caurina* arriving during a second Beringia colonization. After the last glacial maximum (LGM), *M. americana* expanded its range westward (Anderson 1970) where it ultimately contacted *M. caurina*. An alternative view is derived from molecular genetic data; mitochondrial (441–1140 bp) and nuclear (241 bp) sequence data support 2 distinct clades, suggesting a single colonization event (Stone et al. 2002; Stone and Cook 2002), which later evolved into 2 distinct species.

Species designations of North American martens have been called into question because the 2 species are known to hybridize in Montana and British Columbia (Wright 1953; Hagmeier 1961; Small et al. 2003; Colella et al. 2018). However, molecular genetic analyses support traditional morphological designation of martens (Merriam 1890; Clark et al. 1987) into the 2 distinct groups, *americana* and *caurina* (Dawson and Cook 2012; Colella et al. 2018). In fact, most molecular investigations demonstrate that mitochondrial DNA (mtDNA) divergence levels between *americana* and *caurina* are similar to those existing among the other 4 Palearctic *Martes* species with substantial nuclear allelic sorting between the 2 types, suggesting recognition as 2 distinct species (Carr and Hicks 1997; Stone and Cook 2002; Dawson and Cook 2012; Colella et al. 2018, 2019).

The subspecific status of the genus *Martes* in North America has also been extensively debated. As many as 14 subspecies of *M. americana* have been described (Hall 1981). In the Northwest Forested Mountains and Marine West Coast Forest ecoregions of the west coast, Hall (1981) classified *Martes* into *M. a. caurina* (British Columbia, Washington State, and Oregon), *M. a. vancouverensis* (Vancouver Island, BC), *M. a. sierrae* (Sierra Nevada in California, Nevada, and Oregon), and *M. a. humboldtensis* (coastal California and potentially into Oregon; Figure 2).

The subspecific status of several American marten populations from Oregon and California has been investigated using molecular genetic data (reviewed in Schwartz et al. 2012). Slauson et al. (2009) evaluated the subspecific identity of a rediscovered population of American martens within the range of a presumed extinct subspecies (*M. a. humboldtensis* or *M. c. humboldtensis*). The comparison of contemporary and historical mtDNA (1428 bp) sequence diversity within the described range of *M. a. humboldtensis* suggests that the

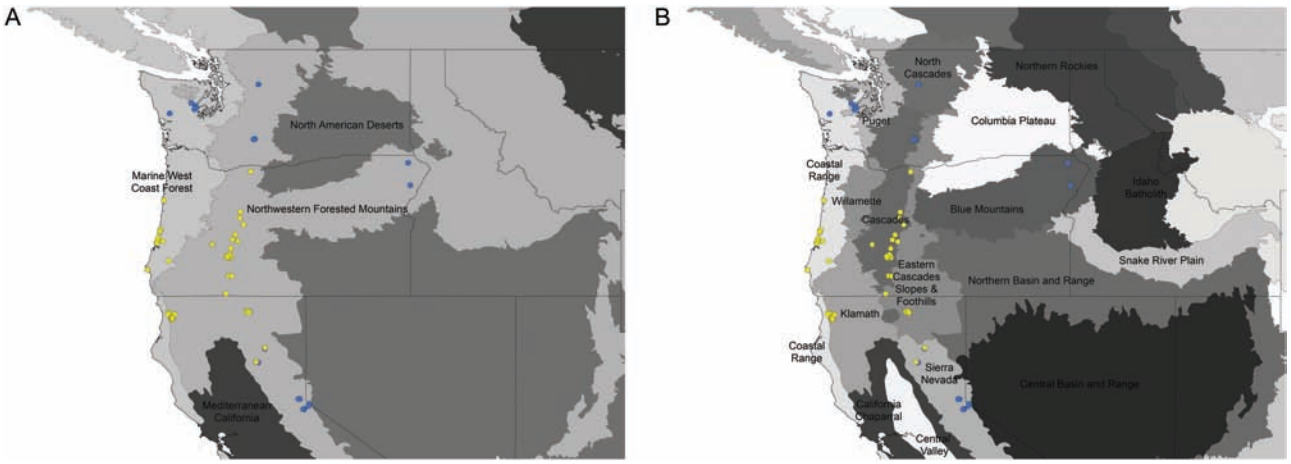

Figure 1. Map showing ecoregions defined by Omernik (1987) of the conterminous United States—representing Level 1 (a) and Level 3 (b). The blue circles represent clade 1, and the yellow circles represent clade 2 based on all mitogenome phylogenies.

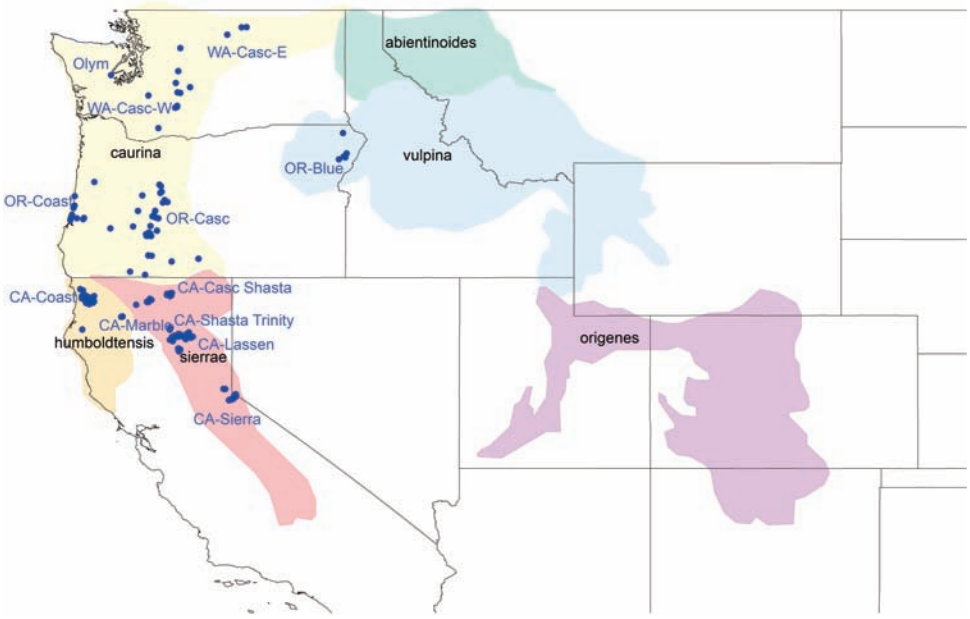

Figure 2. Map showing marten sample locations used in genetic analysis overlaid on historical subspecies designations for marten (shaded colors). Abbreviations are as follows: Sierra Nevada in California (CA-Sierra), Lassen National Forest (CA-Lassen), Trinity Alps/Marble Mountains of California (CA-Marble), Shasta Trinity National Forest (CA-Shasta Trinity), California Cascades near Mount Shasta (CA-Casc Shasta), California Coastal Range (CA-Coast), Oregon Coastal Mountains (OR-Coast), Oregon Cascades (OR-Casc), Blue Mountains in Oregon (OR Blue Mtns), Washington Cascades west of the divide (WA Casc-W), Washington Cascades east of the divide (WA Casc-E), and Olympic Peninsula (Olym).

rediscovered population represents descendants of a relictual population that previously inhabited coastal California. Additionally, the sharing of haplotypes among coastal Oregon and coastal California populations and lack of historical biogeographical barriers suggests that the subspecific boundary between *M. a. humboldtensis* and *M. a caurina* may not be valid (Slauson et al. 2009). Furthermore, Slauson et al. (2009) reported *M. a. sierrae* differed substantially in genetic variation from both *M. a. humboldtensis* and *M. a. caurina*, suggesting a separate glacial refugia.

Understanding how historical biogeography influences the contemporary distribution of genetic diversity within natural populations is a critical first step toward effective management of biodiversity. Conservation of biodiversity relies on the accurate identification and description of conservation units (Taylor et al. 2017), and national legislation (the U.S. Endangered Species Act (ESA), the Canadian Species At Risk Act) is targeted at these entities, whether they are designated as species, subspecies, or otherwise recognized as unique and significant evolutionary lineages (Haig et al. 2006; Waples et al. 2013). Taxonomic uncertainty not only clouds our appreciation of biodiversity, but also has real-world consequences for the strategic applications of always-limited conservation resources (Zink 2004; Schwartz and Boness 2017; Andrews et al. 2018). Thus, its imperative conservation units are accurately delineated using clear operational definitions.

Our research focuses on identifying conservation units of *Martes* populations in the western United States. Using both historical and contemporary samples, our goal was to address the question: how has the complex biogeography of the west coast region of the contiguous United States influenced patterns of biodiversity? Our objectives were to 1) evaluate the putative morphological subspecies designations; 2) estimate temporal splits within genetic groups; and 3) determine possible spatial genetic structure of *Martes* throughout the Pacific Northwest. Our analyses were focused on the entire mitochondrial genome because previous research on a sympatric mustelid in the region showed that partial mtDNA analysis missed important geographic patterns (Knaus et al. 2011). The implications of this research are significant, as newly discovered marten populations in coastal northwestern California and Oregon have been proposed for listing under the ESA. We use genomic data to delineate conservation units of *Martes* in the Sierra-Cascade-Coastal montane belt in the western United States.

Methods

Study Area

The far western mountain ranges of the contiguous United States are divided into 3 "level I" ecoregions: Marine West Coast Forest, Northwestern Forested Mountains, and Mediterranean California, and these are subdivided into 6 level III regions, which broadly represent similar geology, vegetation, climate, soils, and hydrology (Omernik 1987; Omernik and Griffith, 2014). The Marine West Coast Forest comprised 2 lobes bisected by a narrow band of the Northwestern Forested Mountains (Figure 1). The Marine West Coast Forest is characterized by late successional dominance of western red cedar (*Thuja plicata*) and western hemlock (*Tsuga heterophylla*) in the Pacific Northwest (Carstens et al. 2005), and coastal redwood (*Sequoia sempervirens*) forests further south. The Northwestern Forested Mountains ecoregion is a transition zone from a maritime climate to a drier continental climate. It can be divided into ecoregions that include the North Cascades, Cascades, Klamath, Blue Mountains, Eastern Cascades Slopes and Foothills, and Sierra Nevada. Each of these regions has a unique pattern of land surface forms, natural vegetation, and soils, often leading to unique flora and fauna. Coniferous forests dominate the subalpine and midelevation communities, whereas shrublands characterize

lower interior valleys. The Mediterranean California ecoregion is characterized by a warm and mild climate, dominated by chaparral vegetation and patchy oak woodlands, except on the upper slopes where coniferous forests persist (Figure 1).

Marten Samples and DNA Extraction

We analyzed 423 samples of martens collected throughout California, Oregon, and Washington (Figures 1 and 2; Supplementary Table S1). Samples of tissue, blood, hair, and turbinate bones collected from 1894 through 2014. We extracted DNA from tissue, blood, and hair samples using the QIAGEN DNeasy Blood and Tissue kit according to manufacturer's instructions, with an added 10 min 70 °C incubation after the addition of buffer AL. Hair samples were processed in a laboratory dedicated to the extraction of DNA from noninvasive samples using protocols for tissues, with overnight incubation in lysis buffer and Proteinase-K on a rocker or rotator at 60 °C and elution in 90 µl AE buffer. DNA from turbinate bones was extracted in a separate laboratory used exclusively for processing DNA from historical specimens using protocols detailed in Wisely et al. (2004) and Schwartz et al. (2007b).

Whole Mitogenome Amplification—Tissue and Blood

We obtained whole mitogenome sequences from 55 marten blood and tissue samples collected in California, Washington, and Oregon (Supplementary Table S1). Sample fragments were sequenced on an Illumina HiSeq 2000 (Oregon State University). We obtained mitogenome sequences from 27 samples using primers and protocols outlined in Knaus et al. (2011). Each polymerase chain reaction (PCR) was conducted in a total reaction volume of 20 µl with ~10 ng of total genomic DNA amplified with Phusion Flash polymerase (New England Biolabs). Cycling conditions included a 30-s activation at 98 °C, followed by 30 cycles at 98 °C for 8 s, 59 °C for 30 s, and 72 °C for 2 min. In addition, we obtained whole mitogenome sequences from 28 samples using a shotgun sequencing approach with a reference-guided assembly using the CLC-BIO assembler.

Whole Mitogenome Amplification—Bone and Hair

Bait Preparation

We attempted to obtained mitogenome sequences from 24 bone samples collected between 1894 and 1978, and 15 contemporary hair samples using an in-solution, hybridization-based DNA capture approach. To ensure consistency in laboratory methods, we re-analyzed 17 tissues with the same protocol. Marten mitochondrial baits were prepared using the protocol published by Maricic et al. (2010) with the following modifications: We used primer pairs mtI-F 5′-CAAGAGGAGAYAAGTCGTAACAAG-3′; mtI-R 5′-TCTCACCTATAATTTGACTTTGACA-3′; mtII-F 5′-AAGAAAGGAAGGAATCGAACC-3′; mtII-R 5′-TTGGAGTTGCACCAATTTTTTG-3′; mtIII-F 5′-CATGGCTTTCTCAACTTTT-3′; mtIII-R 5′-CTTTGRTTTATCCAAGCACAC-3′ to obtain mitogenome fragments in 3 separate PCR reactions from individual OR-Cascade_s48. Final concentrations of each PCR component were as follows: 0.5 µM each forward and reverse primer (desalted, Eurofins), 200 µM dNTPs (Thermo Scientific), 1× Phusion HF Buffer (New England Biolabs, hereafter written NEB), 0.006% DMSO (NEB), 1 Unit Phusion High-Fidelity DNA Polymerase (NEB), and 5-µl genomic DNA in a total reaction volume of 50 µl. Thermal cycling included a 98 °C initial denaturation for 1 min, 40 cycles at 98 °C for 10 s, annealing at 59 °C for 30 s, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. Amplicons were extracted from a 0.7% agarose gel using a QIAGEN MinElute Gel Extraction kit with 2 final 10-µl elutions. Purified amplicons were combined in equimolar amounts to a total of 1.7 µg in 132 µl. We sheared the purified amplicons to 300 bp using a Covaris E220 (Covaris Part Number 400103 Rev F). Blunt-ending and biotinylated adapter ligation steps were performed as outlined in Maricic et al. (2010), but we replaced all purification steps with a 2× SeraPure (Faircloth and Glenn 2014) bead clean and eluted in 15 µl of elution buffer.

Library Preparation

Sample DNA was quantified using a Qubit 2.0 and diluted to 5.17 ng/µl in 38.65 µl for a maximum of 200 ng per sample. DNA degradation was assessed using TapeStation Genomic DNA ScreenTapes and Reagents and D1000 Screen Tapes and Reagents. No shearing was performed in order to assess cytosine deamination at the ends of DNA fragments and because most DNA samples were already degraded to approximately 200 bp. Libraries were prepared using methods from Shapiro and Hofreiter (2012). To excise uracil bases and blunt-end DNA fragments, 50-µl reactions containing 1× NEBuffer 2, 175 ng/µl BSA, 1 mM ATP, 300 µM each dNTP, ≤ 200 ng aDNA, 20 U T4 PNK, and 3 U USER were incubated at 37 °C for 3 h in an Eppendorf Mastercycler Pro. Immediately following incubation, 6 U T4 DNA Polymerase were added and the reactions were incubated at 25 °C for 30 min in a thermal cycler. Reactions were purified using Qiagen MinElute spin columns as outlined in Shapiro and Hofreiter (2012).

To confirm error correction by USER post-sequencing, 12 samples were also blunt-ended without USER as follows: each sample (200 ng) was blunt-ended in 50-µl reactions containing 1× NEBuffer 2, 100 ng/µl BSA, 10 µM ATP, 300 µM each dNTP, 20 U T4 PNK, and 6 U T4 DNA Polymerase. Reactions were incubated at 25 °C for 30 min and then purified using MinElute spin columns as mentioned above.

Adapters were ligated to all samples in 40-µl reactions with final concentrations of 1× Quick Ligase Buffer (NEB), 62.5 nM adapter mix from Meyer and Kircher (2010), and 1-µl Quick Ligase (NEB). Reactions were incubated at 25 °C for 15 min in a thermal cycler, then purified using 2× SeraPure beads (Faircloth and Glenn 2014), and eluted in 15-µl EB. Adapter fill-in was performed in 50-µl reactions containing 1× ThermoPol Buffer, 300 µM each dNTP, and the full volume of adapter-ligated DNA from the previous step.

To determine indexing PCR cycle numbers, 2 µl of the post adapter fill-in product was diluted 1:100 in EB and amplified in a DyNAmo Flash SYBR Green qPCR assay as follows: 20-µl reactions containing 1× DyNAmo Maser Mix with ROX reference dye added, 500 nM IS4 (Meyer and Kircher 2010), 500 nM P7 indexing primer (Meyer and Kircher 2010), and 2-µl diluted DNA. Reactions were denatured initially at 95 °C for 7 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 30 s. The melt curve was generated by heating to 95 °C for 1 min, cooling to 55 °C for 30 s, and reheating to 95 °C for 30 s.

Indexing PCR was performed in 50-µl reactions containing 1× ThermoPol Buffer, 250 µM each dNTP, 5 U AmpliTaq Gold DNA Polymerase, 500 nM P5 indexed adapter, 500 nM P7 indexed adapter, and the remaining volume of DNA from the adapter fill-in step. Thermal cycling began with a 95 °C initial denaturation for 12 min followed by 7–15 cycles as determined by qPCR of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 3 min. Indexed

libraries were bead-cleaned using 2× Serapure beads and quantified using a Qubit 2.0. Libraries were pooled in equimolar concentration to a total of 2 µg.

Capture, Library Amplification, and Sequencing

The pooled library was combined with 2 nmol P5 blocking oligos, 2 nmol P7 blocking oligos, 10 µg Mouse COT-1 DNA (Life Technologies), and final concentrations of 1× Agilent blocking agent and 1× Agilent hybridization buffer in a total volume of 200 µl. The capture was performed as outlined in Maricic et al. (2010). The final mixture was purified using a QIAquick PCR Purification Kit and eluted in 50-µl EBT warmed to 60 °C. This eluate was then concentrated using a 1.8× AMPure XP bead clean and eluted in 15-µl EBT. Because the concentration of the postcapture library was low, we performed qPCR to determine cycle number for library amplification. Duplicate qPCRs were carried out in 20-µl volumes with 1× DyNAmo Flash SYBR Green qPCR Master Mix with ROX reference dye, 0.5 µM Primer IS5, 0.5 µM Primer IS6, and 2-µl captured library or water for a negative control (see Meyer and Kircher 2010 for primer sequences). The thermal profile was the same as the profile used for qPCR in the Library Preparation section. Enrichment PCR was carried out in four 50-µl final volumes to reduce potential biases produced by PCR duplicates. Each reaction contained 1× Phusion HF Buffer, 200 µM each dNTP, 0.5 µM Primer IS5, 0.5 µM Primer IS6, 1 Unit Phusion Hot Start High Fidelity DNA Polymerase, and 2-µl captured library. PCR products were purified using 1.8× AMPure XP and then pooled in equimolar concentration. The library was then sequenced on a MiSeq using paired-end, 150-bp reads, and MiSeq Reagent Micro Kit v2 (University of Montana Genomics Core).

Sequence Processing

We used a local Galaxy server (Galaxy version 1.9.0.0) to process and assemble sequence reads: we prepared reads using *FastQ Groomer*, retained only high-quality reads (quality cutoff value = 30; percent of bases in read that must have a quality cutoff ≥ 90%) using *Filter by quality* (Galaxy version 1.0.0), removed adapter sequences and reads < 15 bp using *Clip* (Galaxy version 1.0.1), and assembled reads de novo using ABySS (Galaxy version 1.9.0.0; Simpson et al. 2009) on a local Galaxy server (Blankenberg et al. 2010). Scaffolds from ABySS were then aligned in Sequencher (Genecodes).

Microsatellite DNA Amplification

We analyzed 401 samples (Supplementary Table S1) throughout California, Oregon, and Washington at 13 microsatellite loci used in previous studies of mustelids including *Ma1, Ma2*, *Ma3, Ma8, Ma18, Ma19, Gg3,Gg7* (Davis and Strobeck 1998), *Ggu216* and *Ggu234* (Duffy et al. 1998), *Mer041* (Fleming et al. 1999), *MP0197* (Jordan et al. 2007), and *Lut604* (Dallas and Piertney 1998). Microsatellites were amplified in a PCR volume (10 µl) containing 1.0 µL DNA, 1× reaction buffer (*Applied Biosystems*), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM reverse primer, 1 µM dye-labeled forward primer, 1.5 mg/ml BSA, and 1U *Taq* polymerase (Applied Biosystems). The PCR profile was 94 °C for 5 min, ([94 °C for1 min, 55 °C for1 min, 72 °C for 30 s] × 36 cycles). The resultant products were visualized on a LI-COR DNA analyzer (LI-COR Biotechnology). Museum specimens and hair samples were considered error-free only if the microsatellites produced consistent scores using a multitube approach (Eggert et al. 2003; Schwartz et al. 2004). Data were checked for genotyping errors using the software Dropout (McKelvey and Schwartz 2005).

Statistical Analyses

Phylogenetic Analyses

Haplotype networks were constructed using the whole mitogenome data set using TCS without a parsimony threshold as implemented in PopART v1.7 (Leigh and Bryant 2015). Sequences with ambiguous nucleotides were excluded from this analysis to avoid misleading networks (Joly et al. 2007).

Mitochondrial DNA sequences (i.e., protein-coding genes, rRNAs, tRNAs, and noncoding regions) were identified using the MITOS webserver (Bernt et al. 2013) under the vertebrate mitochondrial code. The obtained sequences were also aligned with known mitochondrial protein-coding genes, rRNAs, and tRNAs from *Martes* mitogenomes available from GenBank (Yonezawa et al. 2007; Yu et al. 2011; Xu et al. 2012; Li et al. 2014a,b) to define gene boundaries. Additionally, the Nucleotide Open Reading Frame BLAST was used to detect gene positions in each mitogenome. The locations of tRNAs were confirmed with tRNAscan-SE v1.21 (Schattner et al. 2005), following the generalized vertebrate mitochondrial tRNA settings and protein coding genes were converted to proteins to ensure that no stop codons were evident. The best partitioning schemes and nucleotide substitution models were determined with PartitionFinder v2.1.1 (Lanfear et al. 2017) using the corrected Akaike's information criterion (AIC_C) and constrained to the suite of evolutionary models considered by RAxML (Stamatakis 2014). Additional mitogenomes of *Martes* species (*M. zibellina*, *M. melampus*, *M. foina*, *M. flavigula*, *M. martes*, and *M. americana*) were obtained from GenBank and included in the phylogenetic analyses; *M. flavigula* was designated as the outgroup (Supplementary Table S2). Because RAxML considers a single model of rate heterogeneity in partitioned analyses, we compared AIC_C scores among maximum likelihood (ML) models using GTR, GTR+G, and GTR+G+I evolutionary models. ML analysis was performed in RAxML v8.1.21 implemented through RAxMLGUI (Silvestro and Michalak 2012) with rapid bootstrap (1000 replicates) and a thorough ML search for a single run and 1000 replicates under the GTR+I+G model.

We estimated divergence times for major clades using contemporary samples implemented in BEAST2 (Bouckaert et al. 2014). A Bayesian Model Averaging method was implemented in BEAST2 with the bModelTest package (Bouckaert and Drummond 2017) to estimate a phylogeny averaged over site models, rather than relying on likelihood-based methods to determine the site model. During the Bayesian analysis, Markov chain Monte Carlo (MCMC) proposals switch between substitution models and estimates the posterior support for gamma-distributed rate heterogeneity, proportion of invariable sites, and unequal base frequencies. The molecular clock was calibrated using 3 fossil records: 7.1 ± 0.5 million years, the age of the earliest fossil record of the genus *Pekania* (Samuels et al. 2013); 4.8 ± 0.5 million years, the divergence time between *M. flavigula* and subgenus *Martes* (Koepfli et al. 2008); 3.3 ± 0.5 million years, minimum age of the subgenus *Martes* based on the discovery of the fossil marten *M. wenzensis* (Anderson 1970). Similar calibrations have been used for clock calibration in mustelids (Li et al. 2014a; Malyarchuk et al. 2015). We used a Yule tree and a lognormal relaxed molecular clock; the MCMC was run for 200 million generations and sampled every 10 000 generations. We used Tracer v1.5 (Rambaut and Drummond 2009) to assess burn-in by plotting the log likelihood scores for each sampling generation. We discarded the first 10% of the total saved trees as burn-in, after which likelihood values reached a plateau and effective sample sizes for each parameter exceeded 200), that is, we considered the Markov chains stationary. The median

divergence times and 95% credible intervals were calculated using the 50% majority rule.

Population-Based Analyses

For microsatellite loci, we used GENALEX v6.5 (Peakall and Smouse 2006) to estimate allelic richness (numbers of alleles per locus; N_A), observed and expected heterozygosities (H_O and H_E), and number of private alleles for each population (the mean over all loci; N_P). We used rarefaction to a standardized sample size of 6 individuals to correct mean allelic richness (rarefied number of alleles per locus; A_R) in FSTAT v2.9.3 (Goudet 2000).

To assess patterns of genetic structure along the west coast of the United States, we estimated pairwise genetic differences among a priori locations. Sites with fewer than 2 individuals were removed from subsequent analyses (i.e., Siskiyou, Washington Olympic Peninsula, and Modoc). Genetic divergence among sites was assessed using pairwise Weir and Cockerham's (1984) estimate of F_{ST} in the software FSTAT. To assess the signature of isolation-by-distance, we examined the correlation of pairwise genetic distances (F_{ST}) and geographic distances between sites using Mantel tests (Mantel 1967) with 9999 permutations in GenAlEx.

To visualize population genetic structure, we conducted a principal coordinates analysis (PCoA) in the program GenAlEx using a covariance matrix from microsatellite genotypes. Additionally, we divided the samples into 3 temporal bins: historical (1894–1922), modern (1950–1990), and present (1990–2014). We conducted an additional PCoA and F_{ST} analysis with samples allocated to region and time period to look for evidence of genetic drift.

To estimate the likely number of genetic clusters (K), we used an individual-based Bayesian algorithm implemented in the program STRUCTURE v2.3.2 (Pritchard et al. 2000). This program assigns individuals to clusters by minimizing Hardy–Weinberg (HWE) proportions and linkage disequilibrium between loci within each cluster. Population genetic structure was assessed with an admixture model allowing for correlated allele frequencies to account for migration, excluding location priors, with burn-in period of 1 000 000 and 1 000 000 MCMC for 3 replicates for K values from 1 to 8. The best K value was estimated by calculating the ML value (ln[Pr(X|k)], Supplemental Material).

Results

Phylogenetic Analyses

We obtained whole mitogenome sequences from 94 martens (Supplementary Table S1), which yielded an aligned data set of 16 012 bp, containing 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes, and control region. The gene composition and order was similar to most mammals.

From the 94 samples analyzed, 11 sequences contained ambiguous bases and were not included in the haplotype network. From the remaining 83 sequences, 34 unique mitogenome haplotypes were observed. Two groups were apparent in the haplotype network (Figure 3), consistent with 2 well-supported clades in phylogenetic analyses (Figures 1, 4, and 5). Each clade was geographically discrete, except that both clades were present in samples from the Lassen National Forest (Figure 1). Clade 1 consisted of individuals from the Washington Cascades, Blue Mountains of eastern Oregon, Olympic Peninsula of Washington, and Sierra Nevada of California. Clade 2 consisted of individuals from the Cascade and Coast Ranges in Oregon and California. Clade 2-Lassen males have nuclear DNA (see Results for STRUCTURE) consistent with the coastal Oregon and California group, suggesting these males recently migrated to interior California from the coastal population.

Well-supported clades were also found within each of the main clades described above. Within clade 1, 2 well-supported clades were found: the northern Cascades clade consisting of sites in the Washington Cascades, Olympic Peninsula and Blue Mountains and the Sierra Nevada clade including sites from Sierra Nevada and Lassen range (Figure 4). Clade 2 was composed of 2 divergent clades separating the coastal and Cascades region of California and Oregon (Figure 4).

The topology of the molecular clock analysis was similar to that of the mitogenome phylogeny (Figures 4 and 5) with 2 well-supported clades. The estimate of divergence time between *M. americana* and *M. caurina* was 2.56 mya (95% credible interval: 1.78–3.41 mya; Figure 5, node A). The divergence of clade 1 and clade 2 in our study region was 2.23 mya (1.48–3.14 mya; Figure 5, node B). Within clade 1, the Sierra Nevada and Lassen regions of California split from the Oregon and Washington Cascades 1.57 mya (0.88–2.44, Figure 5, node C). Within clade 2, the coastal populations diverged from inland California and Oregon 1.82 mya (1.05–2.74 mya, Figure 5, node D). The divergence times for these major separations were supported by high posterior probabilities (>0.99). Additionally, there was some overlap among the 95% credible intervals of the time estimates meaning they could have occurred around the same time.

Population-Based Analyses

Over 13 microsatellite loci, we found some deviation from HWE (4.9% of all locality-by-locus pairs) with a single locus deviating from HWE in 2 populations (*MA18*). After sequential Bonferroni correction, we found no significant deviations from HWE and all loci were included in subsequent analyses (Table 1). Average number of alleles scored per locus ranged from 1.39 at site Washington Olympic Peninsula to 4.77 at western Washington Cascades. Overall mean rarefied allelic richness ranged from 1.38 (Washington Olympic Peninsula) to 1.68 (Oregon Blue Mountains). The lowest and highest mean levels of heterozygosity (H_O = 0.39 and 0.69) were found at Washington Olympic Peninsula and Oregon Blue Mountains, respectively (Table 1).

Pacific martens showed evidence of significant range-wide population genetic structure. Pairwise F_{ST} values suggests moderate substructure among populations (Table 2). The Marble Mountains population displayed the highest genetic distance (lowest relatedness) with respect to all other populations (F_{ST} = 0.07–0.20; Table 2). The Lassen and neighboring Sierra Nevada, California populations displayed the lowest genetic differentiation (F_{ST} = 0.05), consistent with the mtDNA results. There was a significantly positive relationship between geographic distance and F_{ST} (Mantel r = 0.39, P = 0.01) suggesting an isolation-by-distance pattern, which makes individual-based clustering results more difficult to interpret (see Schwartz and McKelvey 2009).

The microsatellite PCoA based on geographic location recovered 4 distinct clusters suggesting close genetic relationships between 1) coastal populations of Oregon and California, 2) populations in the Washington Cascades, 3) Oregon Cascades and Blue Mountains, and 4) the California Cascades-Shasta and Lassen individuals (Figure 6). The population-level PCoA dividing samples into 3 time frames (historical, modern, and present) was consistent with geographic region (Figure 6), with historical modern and present samples located in the same geographic region clustering together.

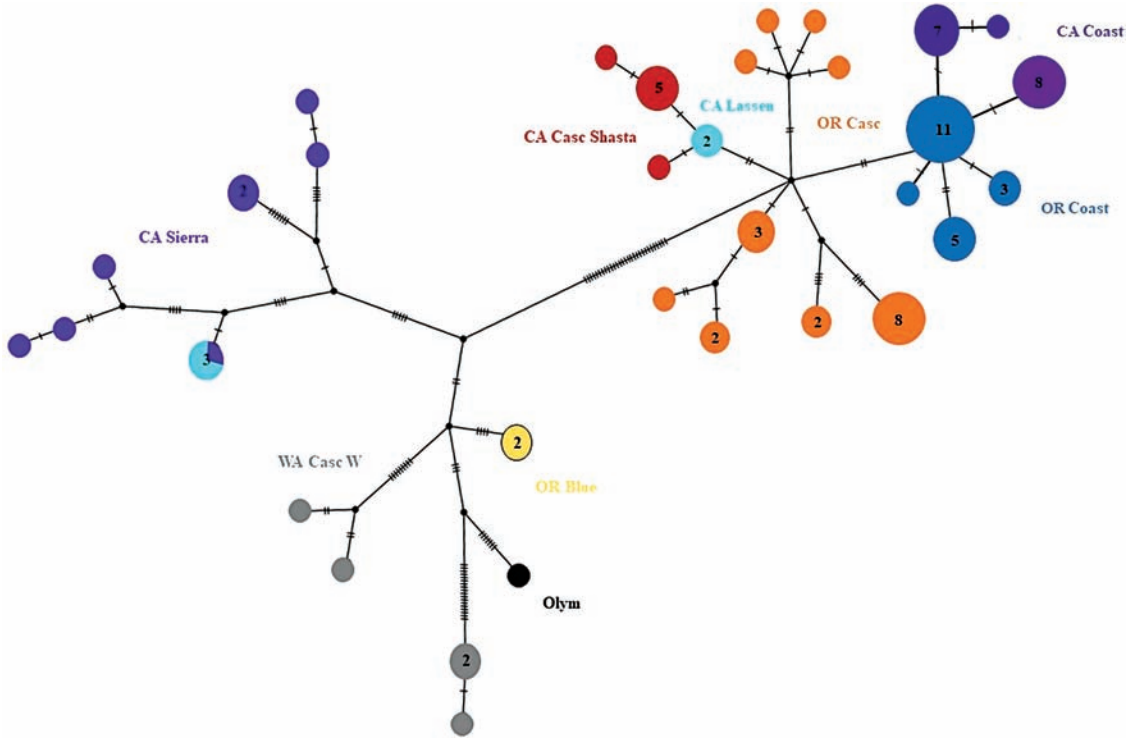

Figure 3. Haplotype spanning network for Pacific coast *Martes* based on the whole mitogenome. Circle size is proportional to the number of samples (also designated with a number in the circle) within a given haplotype and lines between haplotypes represent mutational steps between haplotypes. Color represents sampling locality.

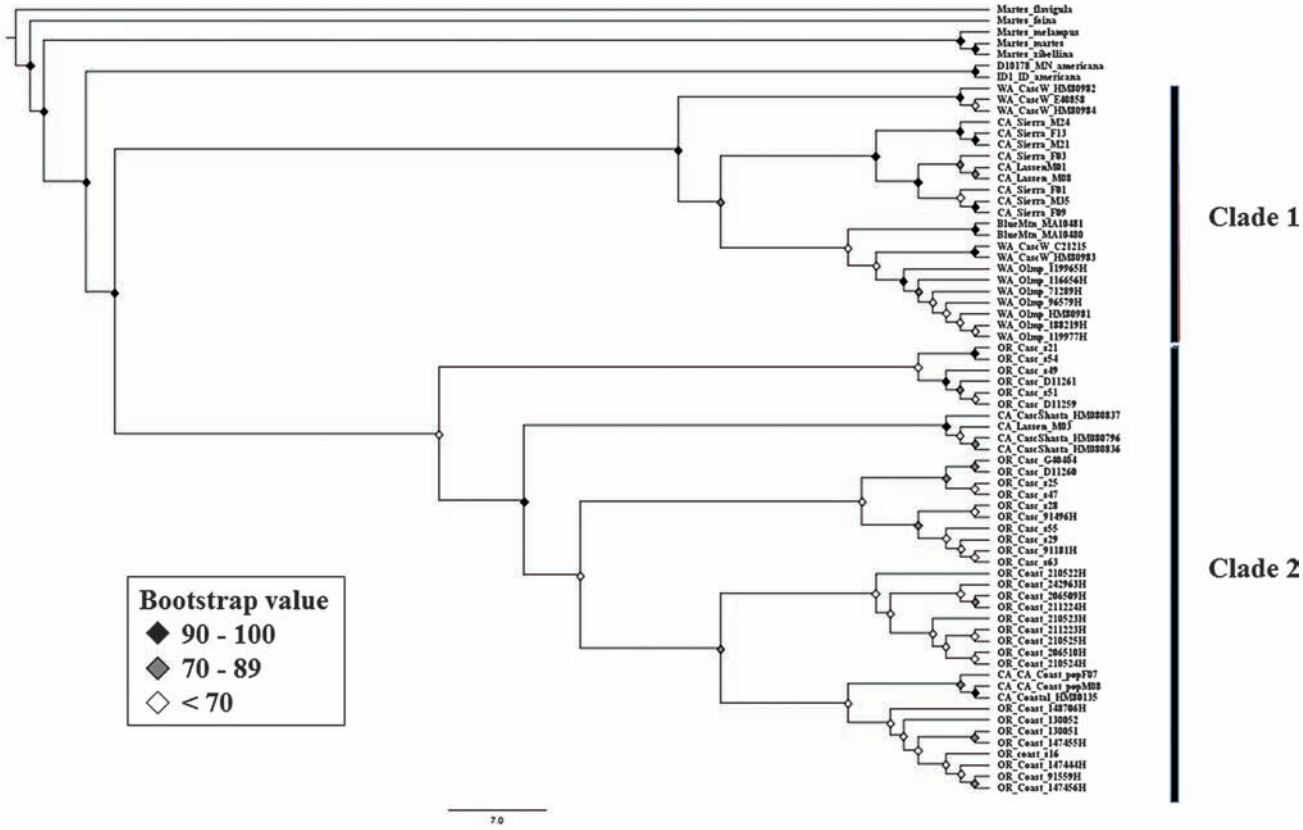

Figure 4. The best-scoring phylogenetic tree inferred from data-partitioned maximum likelihood analysis (with 1000 bootstrap replicates) based on the mitogenome.

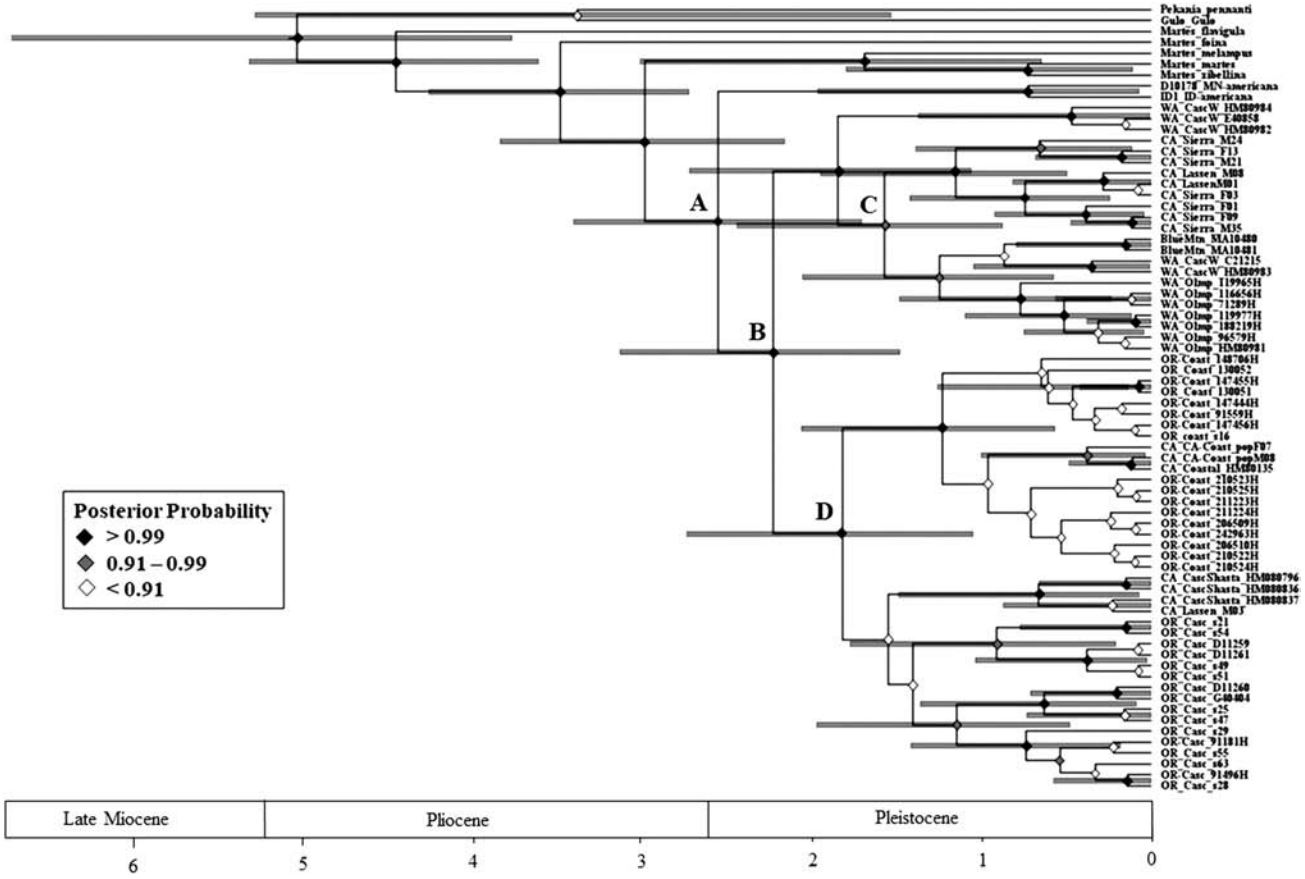

Figure 5. Maximum clade credibility tree constructed from a BEAST dating analysis of the whole mitogenome. Horizontal bars indicate 95% credible intervals of divergence times. Nodes A–D are specific divergence points.

Table 1. Descriptive statistics for 13 microsatellite loci for Pacific Coast marten

Population	N	N_A	A_R	N_P	H_O	H_E
OR Coast	14.62	2.92	1.49	0	0.43	0.48
CA Coast	61.69	3.69	1.51	0.15	0.51	0.51
Siskiyou	1.00	1.54	1.53	0.08	0.54	0.27
Marble	4.85	2.62	1.44	0	0.43	0.40
OR Blue Mtns	4.92	3.39	1.68	0.23	0.69	0.61
WA Olym	1.00	1.39	1.38	0	0.39	0.19
OR Cascades	123.92	4.69	1.61	0.31	0.59	0.61
WA Cascades E	4.39	3.62	1.64	0.08	0.57	0.54
WA Cascades W	14.23	4.77	1.53	0.23	0.61	0.62
CA Cascades-Shasta	25.85	3.78	1.60	0	0.56	0.52
CA Lassen	98.92	4.39	1.50	0	0.60	0.59
Modoc	1.92	1.92	1.57	0	0.50	0.36
Shasta-Trinity NF	5.92	3.08	1.50	0	0.60	0.52
CA Sierra Nevada	25.85	4.08	1.65	0.08	0.47	0.49

N, mean sample size; N_A, mean number of observed alleles; A_R, mean number of rarefied alleles; N_P, mean number of private alleles; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity.

The STRUCTURE analysis recovered explicit boundaries between interior California samples and the remaining martens at $K = 2$ (Figure 7). At $K = 3$, STRUCTURE further split the coastal populations of Oregon and California (Figure 7). At $K = 4$, martens from the Washington Cascades, Olympic Peninsula, and Blue Mountains segregated. For the STRUCTURE analysis, evaluating the best K value is problematic when distance is a major factor in structuring genetics (Schwartz and McKelvey 2009); however, the estimate of the likelihood of the probability of the data given the group hit an asymptote at $K = 5$, which further splits the Marble Mountains

Table 2. Pairwise estimates of F_{ST} for 13 microsatellite loci from Pacific Coast marten populations

	OR Coast	CA Coast	OR Cascade	Marble	OR Blue Mtns	WA Cascades E	WA Cascades W	CA Cascades	CA Lassen	CA Shasta-Trinity NF	CA Sierra Nevada
OR Coast	X										
CA Coast	0.06	X									
OR Cascades	0.08	0.07	X								
Marble	0.19	0.17	0.12	X							
OR Blue Mtns	0.13	0.12	0.06	0.15	X						
WA Cascades E	0.16	0.15	0.11	0.20	0.15	X					
WA Cascades W	0.12	0.13	0.07	0.14	0.09	0.05	X				
CA Cascades	0.12	0.12	0.07	0.07	0.11	0.17	0.12	X			
CA Lassen	0.08	0.09	0.06	0.09	0.09	0.14	0.09	0.06	X		
CA Shasta-Trinity NF	0.09	0.08	0.08	0.10	0.11	0.17	0.12	0.06	0.06	X	
CA Sierra Nevada	0.16	0.16	0.10	0.15	0.13	0.19	0.14	0.12	0.05	0.14	X

All values are significantly greater than zero ($P < 0.01$).

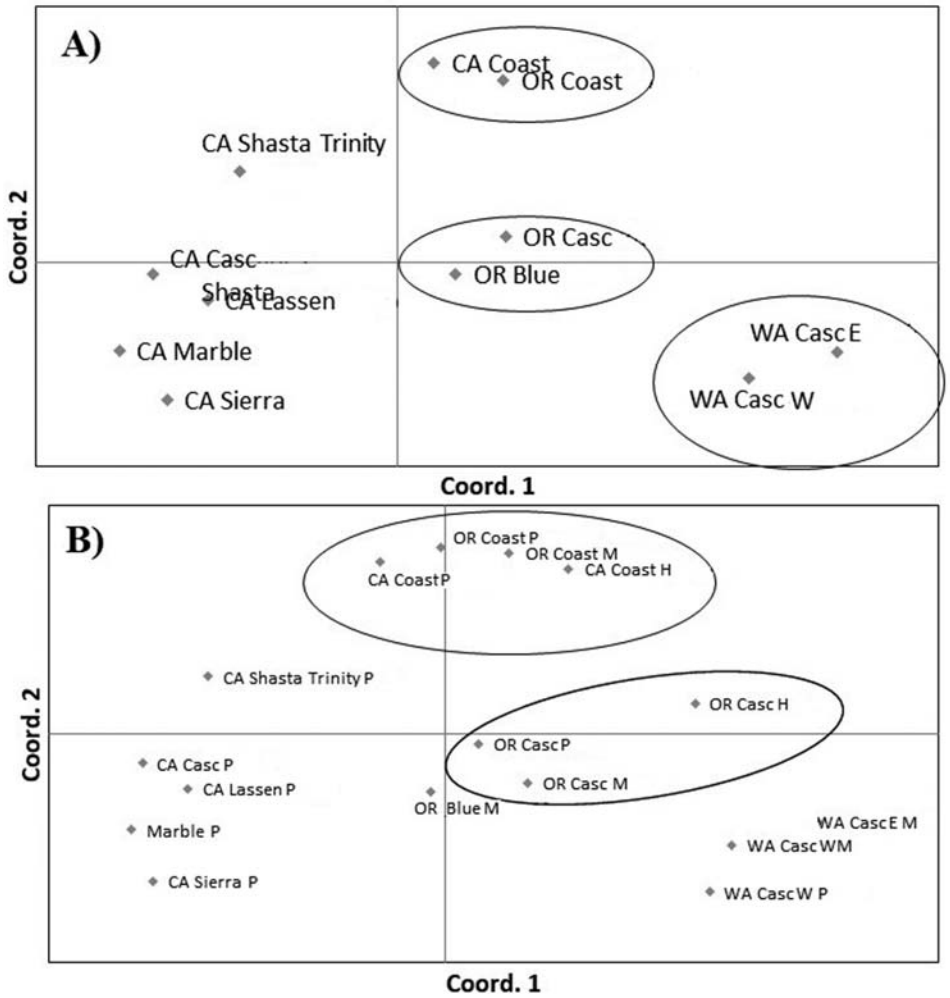

Figure 6. Population-level principal coordinate analysis of Pacific coast *Martes* using 13 microsatellites (a) general sampling location and (b) division of geographic regions into 3 time frames (historical: 1894–1922, modern: 1950–1990, and present: 1990–2014). The first principal coordinate explained 28.0% of the variance, whereas the second explained 22.9%.

group from the interior of California (Figure 7; Supplementary Figure S1).

Discussion

Our results from populations in the western continental United States confirm prior studies that show that martens are 2 distinct species in North America (Carr and Hicks 1997; Dawson and Cook 2012; Colella et al. 2018). Our molecular clock estimate, calibrated using fossil evidence estimated divergence time between *M. americana* and *M. caurina* at 2.56 mya. Pacific martens do not form one homogenous entity on the Pacific Coast in North America as both nuclear DNA (nDNA) and mtDNA sequences obtained from historical and contemporary samples suggest substantial genetic substructure throughout the range. Sequencing of the mitogenome led us to recover 2 distinct mitochondrial lineages with high bootstrap support, consistent with previous research using partial mtDNA markers on *Martes* in the region (Stone et al. 2002; Slauson et al. 2009). Divergence time estimates between these 2 *M. caurina* clades was 2.23 mya. Alternatively, Knaus et al. (2011) suggested that one synonymous mutation is expected in ~8.4 kya (5.0–17.4 kya) for fisher based on the whole mitogenome, indicating a divergence time between clade 1 and clade 2 (~47.39 bp difference; Figure 3) at 398.07 kya (236.95–824.06 kya). However, these estimates should be treated with caution because demographic factors can have a strong influence on network-based approaches for inferring rates and dates (Cox 2008; Nielsen and Beaumont 2009).

These clades form 2 primary phylogenetic breaks between the Cascades/Sierra range (clade 1) and the Cascades/coastal region (clade 2). These phylogenetic breaks correspond with previously documented phylogeographic breaks in the coastal Pacific Northwest (Brunsfeld et al. 2001), suggesting a common response to a shared physical environment and/or historic processes (Avise 2000; Arbogast and Kenagy 2001). Mesic forest species in the Pacific Northwest and Inland Northwest have diverged in allopatry after the uplift of the Cascade Mountains and formation of the dry Columbia Plateau in the Pliocene (Graham 1999; Brunsfeld et al. 2001). Our results reveal that vicariant events promoted divergence among populations/subspecies and the Plio-Pleistocene had a strong effect on the geographic structuring of genetic diversity in Pacific Coast martens (Figure 5). Because the estimated divergence time of the Pacific Coast *Martes* lineages was late Pliocene–early Pleistocene, these lineages originated with the orogeny of the Cascade mountain chain.

Based on the mitogenome, the close affinity of Sierra Nevada to the northernmost individuals, with the exclusion of coastal and Cascades region of Oregon and California, suggests a complex history of recolonization in the region. Following the LGM, Pacific coast *Martes* were thought to be isolated in one or more southern LGM refugia along the west coast, spreading northward along the Pacific Coast (early Holocene; Stone et al. 2002) or eastward to the southern Rocky Mountains (Graham and Graham 1994). Additional mitogenome samples throughout southeastern Alaska and the Rocky Mountain range are required to further examine biogeographic history and recolonization patterns in the region.

Both temporal and spatial patterns for the microsatellite data were highly concordant with 5 subclades or subpopulations (e.g., Figures 6b and 7). However, there was evidence of gene flow among neighboring populations. These 5 groups are consistent with

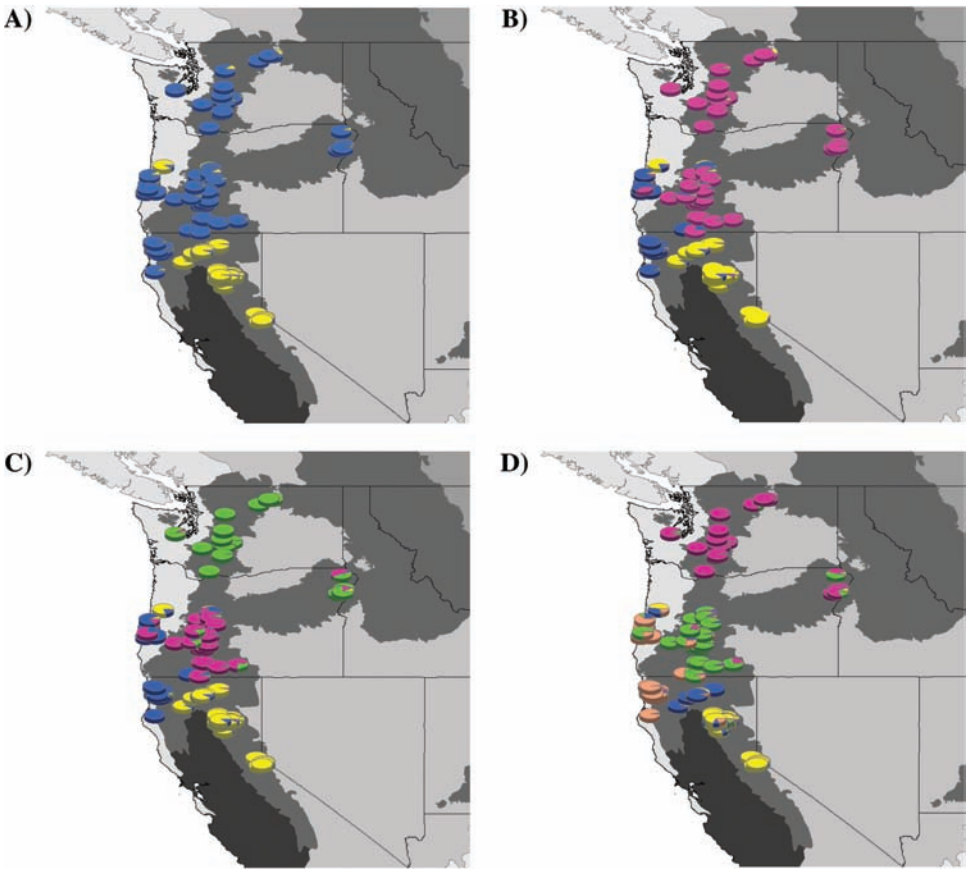

Figure 7. Microsatellite analysis results using program STRUCTURE. The maps show 4 different groupings of marten samples analyzed. Pie charts show individuals analyzed, and the colors represent the proportion of their genetics that assign to the different clusters: (a) $K = 2$, (b) $K = 3$, (c) $K = 4$, and (d) $K = 5$.

historical *M. caurina* subspecies designations. The historical subspecies designations appear correct although boundary delimitations are not. For example, *M. c. humboldtensis* was only described in coastal California with *M. c. caurina* distributed throughout Oregon. However, mitogenome and nuclear data suggest *M. c. humboldtensis* extends beyond the northern California border corroborating results of Slauson et al. (2009). This subspecies appears to be confined to the Marine West Coast Forest ecoregion, whereas a distinct group of martens appears to be in the Northwestern Forested Mountains. Additionally, within the Northwest Forested Mountains the Sierra Nevada and Lassen populations are genetically distinct, supporting the historical designation of *M. c. sierrae*, but in a more limited geographic range than originally described. Similar patterns of genetic distinctiveness of Sierra Nevada has been observed in sympatric mustelids (Schwartz et al. 2007a; Knaus et al. 2011; Tucker et al. 2012).

Subspecies are difficult to delineate, and there have been numerous definitions proposed (Mallet 1995; Mayden 1997; Hey 2001; Schwartz and Boness 2017). However, they are consistently diagnosed as entities that are taxonomically lower than a species and are progressively diverging into species in contemporary times (Wilson and Brown 1953; De Queiroz 2007). We use the general operation definition that subspecies are a population, or collection of populations, that appears to be on an independent evolutionary trajectory with discontinuities resulting from forces that restrict gene flow to the point that the group is diagnosably distinct (De Queiroz 2007; Taylor et al. 2017). Historically, *M. caurina* on the West Coast has been subdivided into 3 subspecies based on morphological characteristics; our genetic data confirmed the existence of some of these divisions. The 4 monophyletic lineages identified using microsatellite and mtDNA analyses and their genetic divergence from each other, indicate their long-term separation and evolutionary divergence. Although we could not sequence the mitogenome from Marble Mountain scat samples, microsatellite results support an additional group in the region. Whether this is due to the group being small and thus subject to strong genetic drift, sampling bias, or adaptive differences will require additional sampling and research. Overall, the 5 groups we identified are consistent with *M. c. humboldtensis* (with an extended geographic range into coastal Oregon), *M. c. sierrae* (with a more restricted zone from Lassen south), the Marble Mountains (a small isolated group, with limited samples examined), and *M. c. caurina* subdivided into a north and south group within Washington and Oregon.

There is some correspondence between subspecies designations and distinct ecoregions throughout the west. *M. c. sierrae* corresponds with the Sierra Nevada Level 3 ecoregion, the 2 *M. c. caurina* groups correspond with 2 isolated fragments of the Cascades ecoregion, and *M. c. humboldtensis* aligns with the Coast Range Klamath Mountains/California High North Coast ecoregion. Additional samples within the Klamath–Siskiyou ecoregion of southern Oregon and northern California would help identify origins of martens in the Marble Mountains and the Trinity Alps. Each of these ecoregions (level 3, Omernik and Griffith 2014) contains unique flora and fauna, providing unique vegetation communities and prey bases. Differences in dietary and ecological characteristics are known to affect morphological evolution in mammals (Tseng and Flynn 2018); thus, some of these neutral differences that we detected may, in fact, be adaptive. The molecular clock analysis suggests substantial time (e.g., >0.88 mya) for these populations to have significantly diverged to adapt to their different ecoregions. Recent research provides evidence of significant interspecific cranial shape differences within *M. caurina*, likely a result of dietary niche divergence (Colella et al. 2018). Therefore, future comparative studies examining potential ecological and/or adaptive differences among these intraspecific lineages would be valuable for determining conservation units within Pacific Coast *Martes*.

Future research should focus on 2 fronts. First, examination of larger nuclear genomic data sets (e.g., RADseq, Genotype-by-Sequencing, DNA capture of candidate genes, etc.) may confirm neutral differences found in this study and identify outlier loci that may be under selection in each geographic region. Second, additional sampling of *Martes* populations in the Great Basin, Utah, Idaho, Wyoming, and Montana will help interpret the similarity evident among populations in the Sierra and the northern Cascades (see Figure 4). It is possible that late Pleistocene or early Holocene climates allowed some connectivity from California to the southern Rocky Mountains. Alternatively, gene flow could have occurred in a stepwise manner through a now-extinct taxon, the Noble marten, *Martes americana nobilis*. This may be supported by Hall (1926) who suggested that the noble marten had a close resemblance to *M. caurina sierrae*, yet persisted at lower elevations of the eastern slope of the Sierra Nevada and throughout the Great Basin (Hughes 2009, 2012; Lyman 2011). Additional sampling in this part of the *M. c. caurina* range may shed light on the evolutionary history of this species and reveal historical connections between the western and Rocky Mountain populations of *M. caurina*.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

We deposited our microsatellite genotype data in Dryad (doi:10.5061/dryad.j6q573n9b). We placed our DNA sequences in Genbank (accession numbers BankIt2310151: MT028185 - MT028278).

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