A simple genetic method to distinguish mule deer and bighorn sheep fecal pellets and its application to detecting bighorn sheep colonization events in California

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FULL RESEARCH ARTICLE

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Abstract

Bighorn sheep (*Ovis canadensis*) habitat frequently is geographically discontinuous and the metapopulation model fits this species well. Consequently, extinction-colonization dynamics are important and need to be monitored. Much of the conservation history of bighorn sheep, however, was based on a theory that natural colonization is not part of the biology of this species. That theory is not supported by a growing body of evidence that natural colonization of vacant habitat does occur in this species. Here I present a simple PCR test that distinguishes bighorn sheep fecal pellets from those of mule deer (*Odocoileus hemionus*) as a useful tool in documenting bighorn sheep occupancy where mule deer are present. I include examples from California of applications of this method to investigate potential colonization events.

Key words: colonization, desert bighorn sheep, DNA extraction, extinction, fecal DNA, metapopulation, mule deer, *Odocoileus hemionus*, *Ovis canadensis*, PCR, Sierra Nevada bighorn sheep

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Introduction

Bighorn sheep have specialized habitat requirements, favoring areas with close proximity to steep rocky terrain and visual openness (Krausman et al. 1999). Suitable habitat for this species is consequently mostly discontinuous, frequently consisting of relatively small habitat patches, many of which support small populations. For instance, recent compilations of the status of desert bighorn sheep (*Ovis canadensis nelsoni*) populations in California have found that 78–82% of the populations contained 100 or fewer sheep (Epps et al. 2003; Abella et al. 2011; Prentice et al. 2019). Schwartz et al. (1986) and Bleich et al. (1990, 1996) proposed that bighorn sheep should be viewed as metapopulations, making population extinction-colonization dynamics an important consideration for conservation. Extinction of populations long has been a central conservation issue for this species (Wehausen and Epps 2021) because many populations vanished following the appearance of Europeans and their domestic livestock, notably domestic sheep, that spread fatal diseases to bighorn sheep (Buechner 1960; Goodson 1982; Wehausen et al. 2011; Besser et al. 2013).

A fundamental concept of metapopulation biology is that the rate of colonization of vacant habitat patches must exceed the rate of extinctions for the metapopulation to persist (Sjögren-Gulve and Ray 1996). This colonization/extinction rate relationship clearly was reversed following the appearance of Europeans and their livestock, but historically this was further complicated by a theory that dispersal and colonization of vacant habitat by bighorn sheep was at best a rare event (Geist 1967, 1971). A result of widespread acceptance of that theory was that restoration of bighorn sheep largely has relied on an active management approach that has used translocations to re-establish populations in vacant habitat (Bleich et al. 2021). The absence of an adequate colonization rate implied by Geist's (1967, 1971) theory makes it inconsistent with the application of a metapopulation model to this species. Consequently, it is important to document natural colonizations of vacant habitat as a test to distinguish these competing concepts of this species.

In some ranges this can be accomplished by searching on foot for bighorn sheep fecal pellets, especially near water sources in desert bighorn habitat. However, in some mountains this approach is complicated by the presence of mule deer, whose fecal pellets visually are not reliably distinguished from those of bighorn sheep.

de Ropp et al. (1998) attempted to use nuclear magnetic resonance signatures of bile in fecal extracts of a variety of herbivorous mammals, including bighorn sheep and mule deer, as a method to distinguish the source of fecal samples, but they concluded that this method was not sufficiently reliable.

In the White Mountains of California, I used the presence of protostrongylid lungworm larvae in feces to reliably distinguish deer and bighorn sheep fecal pellet—a parasite present in all 264 known bighorn

sheep samples and absent from all 27 known mule deer samples tested (Wehausen 1983). However, that approach is limited to populations of bighorn sheep with high enough infection levels of that lungworm, thus is not available for most desert bighorn sheep (Clark et al. 1985).

In the White Mountains, I also used hairs left in beds to help delineate the range of bighorn sheep (Wehausen 1983). Most deer hairs are straight and taper to a point with a change in color near that tip. In contrast, bighorn sheep have hairs shaped like a corkscrew that do not change color nor come to a point. However, mule deer also have some underbelly hairs similar to those of bighorn sheep; so, this method may not be entirely reliable, and it requires finding beds that are considerably less abundant than fecal samples.

Here I provide a laboratory method to reliably distinguish bighorn sheep and mule deer fecal samples. In presenting this method, I focus on its application to investigations of potential natural range expansions by two different subspecies of bighorn sheep in California.

Methods

Study Area

I developed this method for distinguishing bighorn sheep fecal pellets from those of mule deer for a specific investigation of Sierra Nevada bighorn sheep (*O. c. sierrae*), a federal and state endangered species (USFWS 2007). That investigation was triggered by a report in 2009 from a member of the public of having seen bighorn sheep in the southern end of the Convict Creek herd unit – historic range of Sierra Nevada bighorn sheep then considered to have been vacant for more than half a century (Wehausen and Jones 2014). In a short summer search of the region by a team of experienced biologists looking for evidence of bighorn sheep, I found the only potential evidence in a timberline meadow on the east side of Pioneer Basin between Stanford Peak and Mount Huntington, consisting of very recent tracks and three similarly fresh piles of fecal pellets, both of which could have been left by bighorn sheep or mule deer bucks. What was needed at that time was a reliable method of distinguishing fecal pellets of those two species.

After I developed a genetic method to distinguish feces from these two species and successfully applied it to the Pioneer Basin samples, fecal samples from a variety of locations were brought to me relative to occupancy questions to determine if they were from bighorn sheep, and I also collected additional samples for the same purpose. In addition to providing the details of that method here, I also summarize the results of its applications. Most samples tested (29) came from the Sierra Nevada relative to occupancy questions for Sierra Nevada bighorn sheep related to distribution goals in the recovery plan for this endangered species (USFWS 2007). In addition to the Pioneer Basin location, there were samples from Cardinal Mountain, Birch Mountain, The Thumb, Coyote Ridge, Blue Lake, Tyee Lakes, Mount Emerson, Esha Peak, Shepherd Crest, Crater Crest, and Monument Ridge. Eleven samples also have been run for desert bighorn sheep: three from the Little Maria Mountains about 12 km NW of the town of Blythe in the southeastern Sonoran Desert region of California, and two samples from each of four nearby water sources that I collected in 2018–2022 on the west side of the Inyo Mountains of California at French Spring, Union Wash, and the next two drainages north of Union Wash, about 10 km NE of the town of Lone Pine (**Fig. 1**). These desert ranges are inhabited by mule deer, but each has a different subspecies than those in the Sierra Nevada (Cowan 1936).

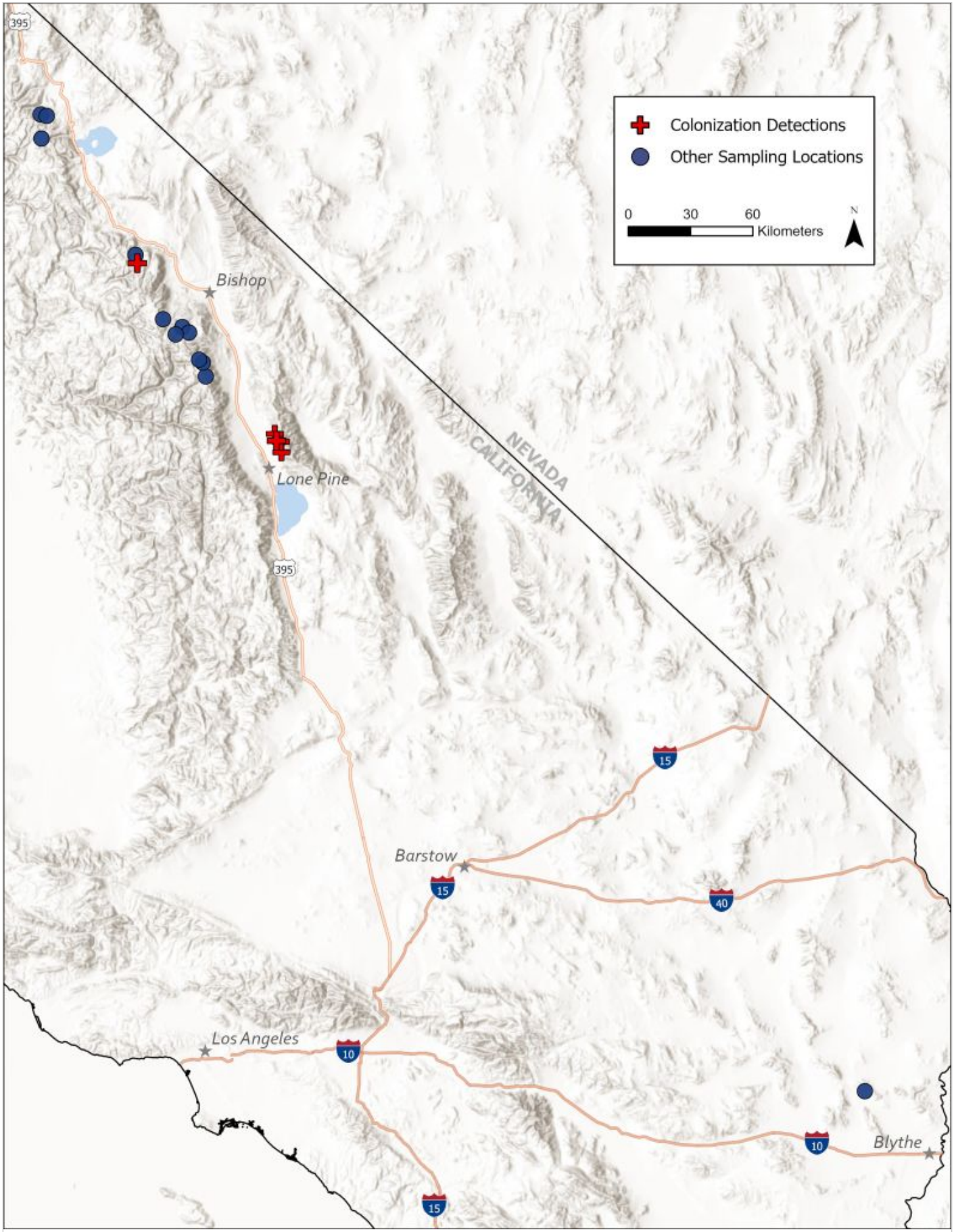

Colonization Detections
Other Sampling Locations
0
30
60
Kilometers
N
Bishop
Lone Pine
Barstow
Los Angeles
Blythe
NEVADA
CALIFORNIA
395
15
40
10

Figure 1. Map of sample locations in California with red cross symbols denoting colonization detections and blue circles indicating other sampling locations.

Genetic Analyses

See **Supplemental Information** for fecal DNA extraction method. I downloaded from GenBank (**http://www.ncbi.nlm.nih.gov**) 132 mule deer mitochondrial DNA (mtDNA) control region sequences from the study of Latch et al. (2009) from the range of this species where it overlaps bighorn sheep distribution (see **Supplemental Information** for accession numbers). Similarly, I downloaded 68 control region sequences for bighorn sheep (35 from the desert region including the Sierra Nevada, and 33 from the Rocky Mountains and Oregon; see **Supplemental Information** for accession numbers). I found all mule deer sequences to have a 19 bp region that was completely conserved (invariant) beginning at position 65 for the many mule deer sequences from Latch et al. (2009). In contrast, I found bighorn sheep sequences at that location to be somewhat variable, but all differed considerably from the conserved sequence for mule deer. Consequently, the sequence in the region conserved across mule deer was used for a deer-specific reverse primer (DeerR1). At its 3' end I added another 6 nucleotides to the primer that were largely conserved in mule deer where their distribution overlaps bighorn sheep. The only exceptions were 1 of 10 haplotypes from Montana and 3 of 14 haplotypes from Alberta for which there was a mutational change at the next-to-last primer base. That change probably will have only a small effect on the ability of the primer to anneal; but, for that northern region that primer simply could be shortened by eliminating the final two bases. For this 25-base primer sequence, bighorn sheep shared only nine nucleotides but also included three insertions near the middle of the sequence; thus, the primer was unlikely to anneal to bighorn sheep DNA.

A second reverse primer (L15712) identified by Epps et al. (2005) for sequencing bighorn sheep was used because I found that region also to be conserved in mule deer. I used a conserved region in both species near the other end of the gene for a forward primer (Beth2) that shortened the bighorn sheep PCR products by 90 bp relative to the primer (Beth) used for sequencing (Epps et al. 2005). These primer sequences (5'–3') are: Beth2: GGT TGT TGG TTT CAC GCG GCA TGG T; DeerR1: AAT AAT TTA ATA CAG TTT TGC ACT C; and L15712: AAC CTC CCT AAG ACT CAA G.

If run as a 3-primer multiplex, mule deer would give two PCR products, while bighorn sheep would give one, and both could be distinguished from failed reactions. After some experimentation I found that the Qiagen Multiplex PCR Kit gave ideal results for PCR products run out on an agarose gel when the annealing temperature was raised to 63C to eliminate potential extraneous bands. I use the following PCR conditions: 6.5 uL Qiagen Multiplex PCR Solution, 0.26 uL each primer (10uM), 5.42 uL water, 1.3 uL fecal DNA or 0.2 uL DNA from blood (plus 1.1 uL extra water). Cycling is 15 min @ 95C followed by 40 cycles of 30 sec @ 94C, 90 sec @ 63C, 60 sec @ 72C. I load the entire PCR product in a 0.7% agarose and 1.0% synergel gel run at 150 V. In addition to a negative control, I include positive controls for bighorn sheep and mule deer.

Based on the sequences used to develop these primers, expected PCR product sizes were 399 and 588 bp for mule deer and 597 bp for bighorn sheep. The shorter mule deer product was sufficiently smaller than the other products to give clearly distinguishable bands when run out on an agarose gel.

Results

PCR products consistently have exhibited the expected banding pattern (Fig. 2). Of the 40 samples that have been run, 22 have been from mule deer, 18 have been from bighorn sheep, and all results have identified the species unambiguously. Of 23 samples collected in the Sierra Nevada from habitat not known to be occupied by bighorn sheep, only the three samples from above the Pioneer Basin were from bighorn sheep. Of six additional samples collected in the Sierra Nevada from habitat already known to be occupied by bighorn sheep, all were from bighorn sheep. One of the samples collected in the Little Maria Mountains was from a bighorn sheep, and the other two were from mule deer. The eight samples from the west side of the Inyo Mountains all were from bighorn sheep (Fig. 2; Table 1).

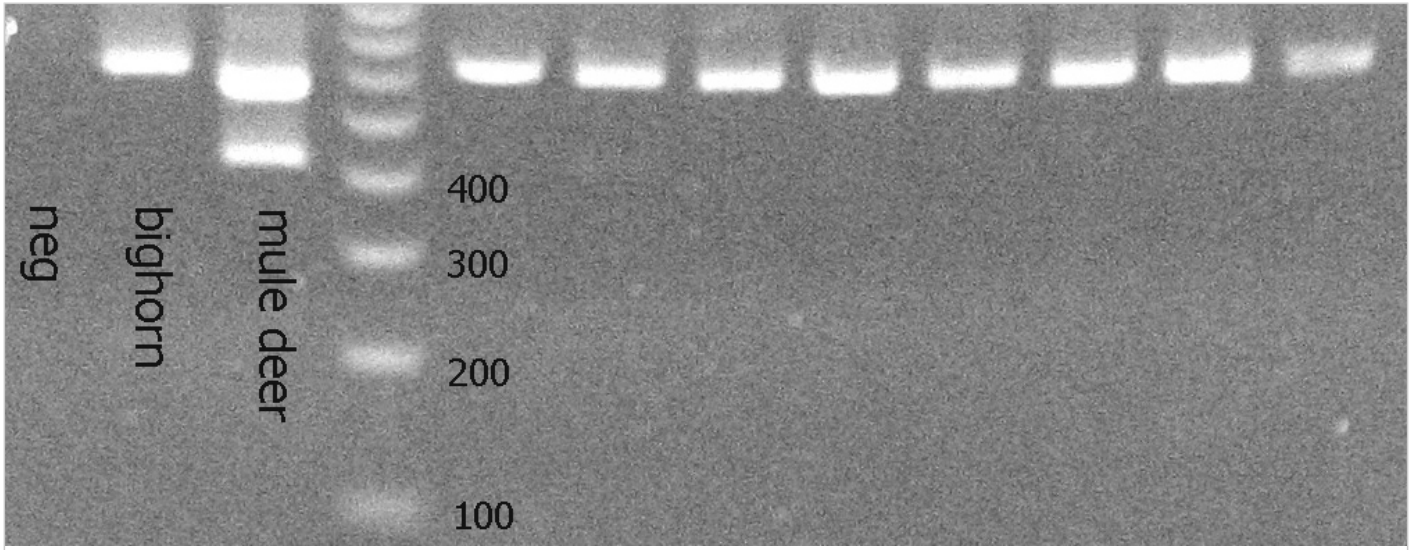


Figure 2. Results of the test of eight fecal samples from the west side of the Inyo Mountains to determine if they were from mule deer or bighorn sheep. Those eight samples are to the right of the 100 bp DNA ladder. To the left of the ladder are positive and negative controls.

Table 1. Applications in California of the method reported here to distinguish bighorn sheep and mule deer fecal pellets to investigate bighorn sheep occupancy, 2009–2022.

Bighorn Type	Location	Known Occupancy	Total	Bighorn (n)	Deer (n)
Sierra Nevada	Various	No	23	3	20
Sierra Nevada	Various	Yes	6	6	0
Desert	Little Maria Mountains	No	3	1	2
Desert	Inyo Mountains (west side)	No	8	8	0

Discussion

I have found the method provided here to reliably distinguish bighorn sheep and mule deer fecal pellets in a variety of ecosystems. However, this method should be viewed as an early step in investigations of occupancy. Male bighorn sheep range widely, so occupancy minimally should be defined by the existence of females that are the more philopatric sex (Bleich et al. 1990). For the positive bighorn sheep results reported here, I first added a PCR of the amelogenin gene to determine the sex of samples using the SE47/48 primers (Enis and Gallagher 1994). The single positive sample from the Little Maria Mountains (**Table 1**) was from a male; thus, that sampling did not document a range expansion. In the Pioneer Basin example, all three samples were from females and subsequent microsatellite analysis determined that they were three different females. In the case of the Inyo Mountains, the samples were more weathered and produced poorer DNA samples which failed to yield results on the sex for two samples. Genotyping for 18 microsatellite loci similarly produced unreliable results for 7 of those loci. Nevertheless, results of that genotyping suggested that the eight samples represented four different individuals: two females, one male, and one sex unknown. In contrast, the results of the PCR test based on mtDNA for distinguishing bighorn sheep from mule deer for those Inyo Mountains samples provided reliable results for all eight samples (**Fig. 2**). The difference between those results likely reflects the high number of copies of mtDNA in cells compared with nuclear DNA (nDNA); thus, the method presented here for distinguishing bighorn sheep and mule deer fecal pellets has an advantage over alternative genetic methods based on nDNA for samples that are more weathered. Also, the method presented here requires only a basic PCR lab because it does not use a DNA sequencer and is consequently less expensive than approaches requiring genotyping or sequencing. Nevertheless, for a lab set up to process many samples for genotyping via multiplex PCR, that extra cost may not be an issue and data development may not require more personnel time; all methods share the initial cost of DNA extraction in personnel time and reagents. The method presented here may be most useful for small, focused investigations where high throughput genotyping is not available.

The documentation of the three different female Sierra Nevada bighorn sheep east above Pioneer Basin led to more field effort in that region and eventually the ability to develop reliable visual counts of that new population. The early count data suggested that the three ewes discovered from fecal DNA in 2009 probably were the entire reproductive base of the population at that time.

Around the time when I collected the last samples on the west side of the Inyo Mountains, a resident of Lone Pine contacted me with GPS coordinates for a recently-dead bighorn sheep they had encountered close to where my samples were collected. On investigation it proved to be a yearling male, which strongly suggests a reproducing population in that area. Similar to what followed the results from the Pioneer Basin, expanded future field efforts are warranted in this part of the Inyo Mountains.

For the 40 samples I have run looking for evidence of colonization of vacant habitat by bighorn sheep in California, the results support two such range expansions: one into the Convict Creek herd unit in the Sierra Nevada, and one on the west side of the Inyo Mountains. The Convict Creek herd was one of two native populations in the Sierra Nevada that survived to about 1950 before going extinct (Wehausen and Jones 2014). In the Inyo Mountains bighorn sheep survived only on the east side of that range in the Saline Valley region. The only evidence that they once occupied the west side of that range is a detailed 1938 report by California Department of Fish and Game biologist Donald McLean.

California was late entering the era of translocating bighorn sheep to vacant habitat patches and has applied that management tool in a limited way (Bleich et al. 2021). An important benefit has been that the extensive bighorn sheep habitat in the southern desert region of California has proven to be an important natural laboratory for studying bighorn sheep metapopulation questions about gene flow (Epps et al. 2005, 2007) and extinction-colonization dynamics (Epps et al. 2004; Wehausen and Epps 2021) because much of it has not been altered by past translocation actions (Bleich et al. 2021). By its nature, the study of colonization requires a long-term perspective which has been ongoing since it was recognized that bighorn sheep should be viewed from a metapopulation perspective (Schwartz et al. 1986; Bleich et al. 1990, 1996). The first natural colonizations by this species in California were documented in the 1990s. However, those were not classified separately as natural range expansions until a 2011 compilation of population status in which five desert bighorn and two Sierra Nevada bighorn sheep populations (including the Convict Creek herd unit) were listed as natural colonizations (Abella et al. 2011). Some of those for desert bighorn sheep also were detected genetically from fecal DNA using DNA sequence data (Epps et al. 2010) prior to the development of the method reported here. Eight years later those numbers had increased to six and three, respectively (Prentice et al. 2019) and the Inyo Mountains colonization documented here increases this to seven for desert bighorn sheep. To those can be added colonizations documented in the Rocky Mountains (Singer et al. 2000) and Nevada (Jahner et al. 2019). Dispersal and colonization of vacant habitat is clearly part of the basic biology of this species. The detection method reported here adds another tool that can be used to help further expand this growing database.

Acknowledgments

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