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Identifying the Species of Bats Roosting in Redwood Basal Hollows using Genetic Methods

Abstract

Bats frequently use basal hollows in trees to gain access to interior roost sites but it has been challenging to verify which species do so because it is difficult to capture bats or identify bats using acoustic methods at these locations. We employed noninvasive genetic sampling of guano to identify the species of bats that use basal hollows in redwood (*Sequoia sempervirens*) trees in coastal northern California. Guano was collected using screen traps suspended within the hollows of trees in the northern and central range of the redwood, in Del Norte and Mendocino County, California, respectively. A representative sample of 231 guano pellets from 104 trees was selected for analysis; 149 pellets from 63 trees amplified sufficiently for genetic sequencing. Species identification is possible for 8 of the 11 species that were assumed to occur in the study area, based on previous studies using two 190 bp regions of the 16S ribosomal subunit gene. Seven distinct species, subspecies or species groups were identified; all 7 were represented from samples in the northern study area whereas only 5 of these occurred within the central study area. The long-legged bat (*Myotis volans*) was the most frequent taxa identified. Genetic sampling to identify the species using roosts will be an important contribution to the conservation of bats. This method is noninvasive and appears more efficient than other methods, such as following radio-marked bats to basal hollows or attempting to capture bats as they enter or leave a hollow. New laboratory developments in this field, such as microarrays, when combined with sequencing, will open up domains of research on individual species and species composition at various temporal and geographic scales.

Introduction

Understanding the roosting ecology of bats is fundamental to understanding the ecology of bats in forested ecosystems (Hayes 2003). Bats use cavities in trees as roosting locations and often gain access to the interior chambers in trees via basal openings (often referred to as basal hollows). The hollows form over decades or centuries from the effects of repeated fires which burn the bark and cambium and stimulate a growth response that results in a hollow base of the tree (Finney 1996). The use by bats of hollows in redwood (*Sequoia sempervirens*) in coastal California is well documented (Rainey et al. 1992, Gellman and Zielinski 1996, Zielinski and Gellman 1999, Purdy 2002, Mazurek and Zielinski 2004). Most of this work has indexed the use of particular trees using the weight of guano collected on mesh traps suspended within a hollow. Several of the studies attempted to distinguish guano 'morphotypes,' as-

suming that individual guano pellets that differed in size, texture or color were from different species or species groups. However, species that used the hollows could not be verified on this basis.

Gooding and Langford (2004) successfully trapped and identified bats as they exited hollow trees in bottomland forests in the southeastern United States. Purdy (2002) used a similar approach to capturing bats as they entered or exited redwood hollows in California with limited success. Mist nets suspended across hollow openings captured only three individuals of two species after 37 nights of effort. Hollow openings in redwoods are large, making their occlusion with a net difficult. Bats frequently detected the net and slipped past it or circled in front of the hollow, rarely proceeding far enough to be captured (Purdy 2002). Purdy's efforts demonstrated the difficulty of attempting to identify, via capture, the species of bats that use cavities in redwood trees. However, this work highlighted the value of augmenting a mist-net survey at streams with a roost survey because one of the species captured at basal hollows had not been recorded after 36 nights of mist-net effort at nearby streams; the

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other species comprised only 1 of 192 individuals captured at nearby streams (Zielinski and Gellman 1999, Purdy 2002).

The best method for identifying bat species that use redwood hollows has not been established. Using nets to capture bats at hollows is associated with significant logistical difficulties and is not a recommended method for redwood trees (Purdy 2002). It is not clear why an approach that was successful elsewhere (Gooding and Langford 2004) did not work in redwoods, but it may be related to the larger and often irregular size of the openings and the fact that, unlike the work of Gooding and Langford (2004), roosting bats could rarely be observed with flashlights, Consequently, trees could not be pre-selected as capture sites based on this information. Acoustic methods to identify the species that use redwood hollows are also unsatisfactory because few of the many *Myotis* species can be distinguished (Hayes 2000) and because when detectors are oriented toward a hollow it is uncertain whether the bats that are detected were roosting in the hollow. Furthermore, capturing bats to identify them incurs risks to the bats and the handlers, which can be avoided if a noninvasive indirect method can be developed.

Genetic identification of species using fecal material is a logical direction to pursue for identifying bats because the method has been used to verify the occurrence of many other species (e.g., Kohn et al. 1999, Eggert et al. 2003, Schwartz and Monfort, in press). Fortunately, genetic methods have been advanced to identify, from guano, a number of the species of bats that occur in the Pacific Northwest. Zinck et al. (2004) developed species-specific primers that resolved 10 of the 14 species in their study. On the basis of geographic range maps, 11 of the same 14 species are also assumed to occur in coastal northwestern California (exceptions: Keen's myotis, *Myotis keenii,* western small-footed myotis*, Myotis ciliolabrum,* and western pipistrelle, *Pipistrellus hesperus*).

Our objective was to explore the use of noninvasive genetic methods to identify the species of bats that roost in redwood basal hollows. We sampled from three pre-existing guano collections, acquired over a 10-yr period, and report the identity of species that used the hollows. We also describe the value of this method to help answer questions about bat conservation and management in redwood forests.

Methods

We attempted to assign species identification to guano that was collected during the course of 3 independent research projects conducted in either Del Norte (Gellman and Zielinski 1996, Zielinski and Gellman 1999) or Mendocino County, California (Mazurek and Zielinski 2004) (Figure 1). These regions correspond to the northern and central sections of the natural distribution of redwood (Sawyer et al. 2000). The collection locations in Del Norte County were within extensive old-growth redwood-dominated stands in federal and state parks as well as nearby remnant old-growth stands on commercial forest land. These sites included many hollow-bearing old-growth trees from which to choose when installing guano traps. Trees included in this study were selected randomly from survey units that were either 7- 18 ha (Zielinski and Gellman 1999) or 0.75 ha (Gellman and Zielinski 1996). The collection locations in Mendocino County were on commercial forest land that had been harvested, such that suitable old trees with basal hollows were much less common. Thus, we sampled almost all suitable trees that were discovered in this study area.

Guano was collected from 1992 - 1994 in Del Norte County and from 2001 - 2002 in Mendocino County. Samples were collected every two or four weeks throughout the year from guano traps (water permeable screen; 3M Weedblock, 3M Company, St. Paul, Minnesota) suspended within basal hollows of living redwood trees. Individual guano samples (hereafter, "pellets") were collected from the guano traps, oven dried (20°C for 2.5h) and stored in glass vials in the dark. Pellets for analysis were selected to achieve the greatest diversity of pellet morphology (i.e., size, shape and color) and from the greatest number of trees. Thus, despite the fact that we may have had collected from dozens to hundreds of pellets from each tree, usually no more than 2-3 pellets [maximum = 6] were selected for analysis from any one tree hollow. These were selected from the entire annual sample available from each tree. Relatively few pellets were collected per tree, and from the greatest number of trees, because we assumed that this would give us the best chance of identifying the greatest number of species. Insofar as our assumption about pellet morphological diversity mirrors species diversity, we viewed this method as the best at selecting the most diverse sample possible. Because we did not, however, sample in proportion to the number of
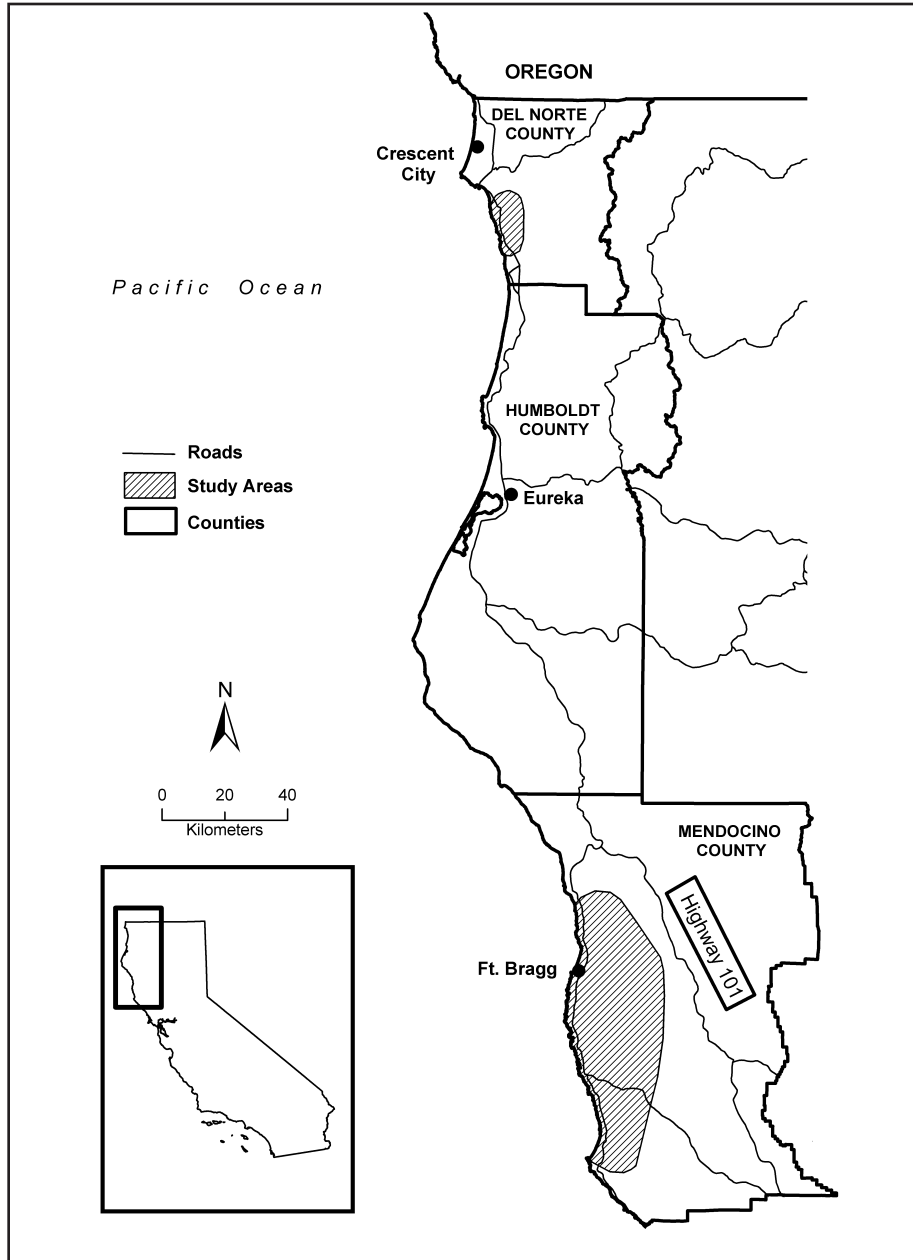
Figure 1. Location of Del Norte and Mendocino County study areas, in northwestern California.

pellets available per tree, the sample may not be ideal for addressing frequency of occurrence or relative abundance.

Two 190 bp regions of the 16S ribosomal subunit gene were amplified using two primer pairs designed for use with bat fecal samples (Mysp1/ Mysp2 and Mysp3/Mysp4; Zinck et al. 2004). Samples were amplified and sequenced following methods described in Zinck et al. (2004). Because we were interested in sequencing a set quantity of samples, and were not interested in any one pellet in particular, we first attempted to amplify the

Mysp1/Mysp2 fragment. If this was successful, we attempted to amplify the Mysp3/Mysp4 fragment. In the interest of sequencing the greatest number of samples in a cost effective manner, we did not optimize PCR conditions or re-try any individual sample. Eight of the 11 species that are presumed to occur in the study areas can be consistently and unambiguously identified from these sequences (i.e., pallid bat *Antrozous pallidus,*, Townsend's big-eared bat, *Corynorhinus townsendii,* big brown bat, *Eptesicus fuscus,* silver-haired bat, *Lasionycteris noctivagans,* hoary bat, *Lasiurus cinereus,* little brown myotis, *Myotis lucifugus alascensis,* Yuma myotis, *Myotis yumanensis,* long-legged myotis, *Myotis volans*). One group of three taxa (long-eared myotis, *Myotis evotis,* little brown myotis *M. lucifugus carissima,* and fringed myotis, *M. thysanodes*) can be distinguished from others but not from each other *(*Zinck et al. 2004). We refer to these as the *Myev/Myluca/Myth* group. There are six recognized subspecies within *Myotis lucifugus* (Fenton and Barclay 1980), two of which potentially occur in our region of study: *M. l. carissima* and *M. l. alascensis*. Although *Myotis lucifugus carissima* cannot be distinguished from *Myotis evotis* and *Myotis thysanodes* using these markers, *M. l. alascensis* is sufficiently distinct for this subspecies to be identifiable. The California myotis (*Myotis californicus)* and small-footed myotis (*Myotis ciliolabrum)* are also genetically indistinguishable (Zinck et al. 2004). Although we refer to samples that are from one of these two species as *Myca/Myci*, *Myotis ciliolabrum* is not expected to occur within either of our study areas (Holloway and Barclay 2001), so these samples are most likely *Myotis californicus*. It is also possible that *Myotis californicus* and *Myotis ciliolabrum* are a single, phenotypically variable species (Rodriguez and Ammerman 2004).

Results

A total of 231 guano pellets, derived from 104 different hollows or fire scars, were selected for analysis. DNA from 149 of the pellets (64.5%) amplified sufficiently for sequencing; 56 and 93 from the Del Norte and Mendocino County study areas, respectively. These pellets were derived from 63 of the 104 trees (43 from Del Norte and 20 from Mendocino) yielding ratios of 1.3 and 4.6 pellets successfully analyzed per tree, respectively. Seven distinct species, subspecies, or species groups were identified from these samples (Table 1). All seven were represented in the northern samples (Del Norte County), but only five were represented in the southern samples (Mendocino County; Table 1). *Myotis volans* was the most frequent taxa identified overall, and was also the most frequent taxa identified from samples collected in Mendocino County. This species was ranked second in frequency in Del Norte County, however, behind the *Myev/Myluca/Myth* group. The latter group was conspicuous by its prominence among samples in Del Norte County (32.1% of pellets) compared to Mendocino County (4.4% of pellets).

Discussion

Roosts are hypothesized to be the primary factor limiting the distribution and abundance of bats in

TABLE 1. The number of guano pellets attributable to each bat species from the sample of 149 pellets (from 63 redwood trees) in Del Norte and Mendocino Counties. *Myev/Myluca/Mythu* refers to a species/subspecies group that can be distinguished from others but not each other (*Myotis evotis*, *M. lucifugus carissima*, *M. thysanodes*). *Myca/Myci* refers to 2 species (*Myotis californicus* and *Myotis ciliolabrum*) that can be distinguished from others but not from each other.

Species	Del Norte County, CA		Mendocino County, CA		Total	
	N pellets (%)	N different trees	N pellets (%)	N different trees	N pellets (%)	N different trees
Corynorhinus townsendii	2 (3.6)	1	0	0	2 (1.3)	1
Eptesicus fuscus	3 (5.3)	3	14 (15.0)	12	17 (11.4)	15
Myev/Myluca/Myth	18 (32.1)	17	4 (4.4)	2	22 (14.8)	19
Myotis volans	14 (25.0)	13	39 (41.9)	15	53 (35.6)	28
Myotis yumanensis	2 (3.6)	2	0	0	2 (1.3)	2
Myca/Myci	13 (23.2)	12	29 (31.2)	21	42 (28.2)	33
Myotis lucifugus alascensis	4 (7.2)	4	7 (7.5)	4	11 (7.4)	8
Total	56		93		149	

many areas (Hayes 2003). A considerable amount of previous research has focused on the use of basal hollows in old-growth redwood trees by bats, but the identity of the species that used the trees was unknown. Inference from earlier studies was focused on use by bats, generically, and was based on either the weight of guano collected at a tree (e.g., Gellman and Zielinski 1996) or counts of ultrasonic vocalizations in the vicinity of the trees (e.g., Mazurek and Zielinski 2004). Until now, our knowledge of the species that used the trees had been limited to a few fortuitous sightings or captures at these unique habitat features, largely due to the difficulty of capturing bats as they enter or leave the hollows. The molecular genetic method identified seven species or species groups in our study. This is a considerable advance over attempting to capture bats in basal hollows and identify them in hand (Purdy 2002). However, it is noteworthy that the two species that Purdy (2002) captured at basal hollows (*M. thysanodes* and *M. evotis*) are members of a group of species that that cannot currently be distinguished genetically. Until this can be accomplished, we may want to view capture methods at hollows as a method that complements guano-based genetic methods. We emphasize the generally noninvasive nature of the approach promoted here, because it eliminates the need to handle bats and the incumbent risks to bats and humans (Kunz and Kurta 1988, Kunz et al. 1996, Rudran and Kunz 1996). We acknowledge, however, that the installation of guano capture nets may risk disturbing roosts of unknown size and location within the tree. Furthermore, we view the primary value of the work as a demonstration of the merit of genetic survey methods. Our sample was not adequate for a comprehensive assessment of the relative frequency of use of basal hollows by various species. Additional work will be necessary to fully understand the dynamics of how the bat community uses basal hollows in the redwood forest.

Hayes (2003) reviewed the habitat ecology of bats in the forests of western North America and segregated species into 3 groups based on their affiliation with forested habitats. Among the species identified in this study only *Myotis volans* was considered a primary associate of forests. All the species we identified have previously been described as roosting in crevice or cavities in trees or snags (Hayes 2003). Two species (*Corynorhinus townsendii* and *Myotis*

ciliolabrum), however, are not characterized as using trees as primary roost structures (Hayes 2003). Although the guano from *M. ciliolabrum* cannot currently be distinguished from the guano of *M. californicus,* these samples were probably all from *Myotis californicus* because the known range of *Myotis ciliolabrum* evidently does not include our study areas (Holloway and Barclay 2001). In addition, *M. ciliolabrum* has not been identified from the collection of bats captured in mist nets over streams in the vicinity of our Del Norte County study area, but *M. californicus* has frequently been captured (Zielinski and Gellman 1999, Purdy 2002). Thus, we assume that the *Myca/Myci* samples were *Myotis californicus*, a species that is expected to use tree cavities (Hayes 2003).

The samples verified to be *Corynorhinus townsendii* indicate that this species, which typically roosts in caves, mines and buildings elsewhere (Humphrey and Kunz 1976, Fellers and Pierson 2002, Sherwin et al. 2003) also uses tree cavities. This result, combined with previous reports of *Corynorhinus townsendii* roosting in tree hollows (Fellers and Pierson 2002, Mazurek 2004), indicates that this species may use trees as roosts in forest habitats more often than has been reported previously. Its congener in the southeastern United States, *C. rafinesquii*, was also viewed as a species that predominately roosted in caves and anthropogenic structures until research demonstrated that it used of hollows in trees (Gooding and Langford 2004). *Corynorhinus townsendii* is a species of concern throughout its range, and thus its use of trees as roosts should be considered in forest management plans.

The seven species or species-groups that were identified in this study demonstrate that hollows in old-growth redwoods are used by many of the species whose ranges include the coastal redwood zone. However, the species represented by the samples from basal hollows differ somewhat from the species captured in mist nets along streams in redwood forest. Zielinski and Gellman (1999) and Purdy (2002) trapped streams in the immediate vicinity of the location where the Del Norte County guano samples were collected. *Myotis yumanensis* was the most common species captured in both studies, followed by *Lasionycteris noctivagans* and *M. californicus. M. yumanensis* comprised over 50% of the captures in both the Del Norte County studies but < 4% of guano

samples in Del Norte County. This difference may be because *M. yumanensis* rarely roosts in basal hollows, despite the other uses they make of redwood forests, or it may be result of sampling biases. Rainey et al. (1992) reported a maternity roost of *M. yumanensis* in a basal hollow in the southern range of redwood, so it seems unlikely that the species does not use redwood hollows as roosts. The two types of samples, capture and guano, differed in the seasons of their collection. Furthermore, the guano samples in this study were selected to maximize the diversity of different guano morphotypes and representation among different trees. Thus, the guano samples may not have been particularly useful for the purpose of characterizing relative species composition.

Conspicuous by their absence from the guano sample were the lasiurines, the silver-haired (*Lasionycteris noctivagans*) and the hoary bat (*Lasiurus cinereus*), which have been captured in mist nets over streams in redwood forests (Zielinski and Gellman 1999). Hoary bats roost in foliage (Shump and Shump 1982) so evidence of their absence from hollows is not surprising. Silver-haired bats are reported to roost most frequently within crevices and cavities in decaying trees (Campbell et al. 1996, Vonhof and Barclay 1996) and, thus, would be expected to use hollows in redwoods. Their absence from the guano samples is surprising, given their prevalence in the sample of bats captured at nearby streams (Zielinski and Gellman 1999, Purdy 2002). It is possible that they do not roost in hollows despite their abundance in redwood forests, but this seems unlikely and should be the focus of additional research. Conversely, by chance alone, guano from silver-haired bats may not have been selected during the course of choosing pellets for analysis. Approximately 250 km south of our southernmost study area, Rainey et al (1992) observed 2 species using redwood hollows that we also verified via guano in our study areas: *Eptesicus fuscus* and *Myotis yumanensis*. And, *Myotis volans*, which was the most prevalent species identified from guano in this study was also verified to use a basal hollow in western redcedar (*Thuja plicata*) in Oregon (Ormsbee 1996).

Interestingly, there were fewer taxa identified from the samples in Mendocino County, in the southern portion of the California Coast Range, than in Del Norte County. This occurred even though there were more pellets in the final sample from Mendocino (n = 93) than Del Norte County (n = 56) (although the pellets were from fewer trees in Mendocino [n=20] than Del Norte [n = 43]). Particularly noteworthy is the few samples (n=4) attributable to the *Myev/Myluca/Myth* group in the Mendocino County sample compared to the Del Norte County sample, where it was the most commonly identified group. Although both study areas occurred in the redwood forest type, they are in different recognized subsections of the distribution of redwood: the northern and the central (Sawyer et al. 2000). The latitudinal variation in ecological communities associated with redwood may help explain the differences in bat species occurrence.

This work represents the first use of noninvasive genetic methods to verify the species of bats that use basal hollows in trees. Moreover, the method appears to be an improvement, in terms of efficiency and costs, over previous attempts using capture or acoustic methods to identify bats that use redwood hollows. A significant proportion of the samples were collected 10 years earlier, demonstrating the value of this method for contemporary as well as for older fecal samples. Sample storage method is important, however, because samples become degraded by repeated freezing-thawing or exposure to light. Oven-dried guano, placed in sealed glass containers out of direct light, is the best way to store guano to assure that it is suitable for analysis in the near or distant future. We encourage the collection of guano for the purpose of species identification, even in the absence of funds to conduct the analysis or the access to a laboratory for analysis. Maintaining a collection of historical samples allows for future research with broad temporal and geographical scope. Determining identity of bat species from guano collected at roosts should expand greatly the tools available for surveying bats in forests and elsewhere. The method has obvious advantages in terms of cost and efficiency. Moreover, new innovations such as microarrays will permit the identification of all species of bats within a potentially mixed-species guano sample (Zinck and Vonhof 2005). This will allow researchers to investigate species composition at natural and artificial roosts. Combining sequencing and microarray techniques will allow scientists to design studies that incorporate information about individuals and entire roosts in a cost effective manner. We also expect that innovations developed for other

mammal taxa, such as distinguishing gender (e.g., Pilgrim et al. 2005) and distinguishing individuals (e.g., Palsboll et al. 1997) will expand further the applications of molecular genetics for the conservation of bats in forests.

Acknowledgements

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