

Field identification of the cryptic vespertilionid bats, *Myotis lucifugus* and *M. yumanensis*

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Recent advances in molecular techniques have provided new tools for confirming species identities, however they can be expensive and results are not immediately available. *Myotis lucifugus* and *M. yumanensis* are morphologically cryptic species of bats sympatric in western North America that can be difficult to distinguish in the field. We evaluated a set of models that used morphological and echolocation call characters obtained in the field to predict species identity as determined by DNA analysis. We constructed models using data from 98 *M. lucifugus* and 100 *M. yumanensis* captured throughout the Pacific Northwest from which we had obtained high-quality, time-expansion recordings of their echolocation calls. The best model for distinguishing the species combined forearm length and characteristic frequency of echolocation calls and was able to identify 92% of *M. lucifugus* and 91% of *M. yumanensis* individuals, with $\geq 95\%$ confidence. We evaluated the applicability of our model by testing it on additional datasets. Our model correctly classified 83% of *M. lucifugus* ($n = 30$) and 93% of *M. yumanensis* ($n = 29$) individuals captured in north-central Oregon, whose echolocation calls were recorded using a zero-crossings echolocation detection system. It also correctly classified 86% of *M. lucifugus* ($n = 22$) and 85% of *M. yumanensis* ($n = 26$) individuals, captured throughout our study area, for which only poor-quality time-expansion recordings of echolocation calls were obtained. Combining morphometrics with echolocation call characteristics may be a useful approach for distinguishing among pairs of cryptic species of bats in other areas.

Key words: cryptic species, echolocation, geographic variation, mtDNA, species identification, *Myotis lucifugus*, *M. yumanensis*

INTRODUCTION

Species identification is a fundamental prerequisite for successful ecological research and conservation yet can be difficult to achieve for morphologically cryptic species. Recent advances in molecular techniques have provided tools for investigating

relatedness between outwardly similar species and identifying morphological characteristics to discriminate amongst them (Bradley and Baker, 2001; Hebert *et al.*, 2004; Reed *et al.*, 2004; Rodriguez and Ammerman 2004; Russo *et al.*, 2006). Many new cryptic species groups have been identified and genetic confirmation of species'

identities has become increasingly routine (Bradley and Baker, 2001; Hebert *et al.*, 2004). Crypsis among genetically distinct species occurs in many genera of vespertilionid bats (Chiroptera: Vespertilionidae) including several species in the genus *Myotis* (Baker, 1984; Jones and van Parijs, 1993; Mayer and von Helverson, 2001; Jacobs *et al.*, 2006).

In western North America, *Myotis lucifugus* and *M. yumanensis* are a frequently encountered cryptic-species pair and where sympatric, as they are throughout the Pacific Northwest, can be challenging to identify in the field (Fenton and Barclay, 1980; Nagorsen and Brigham, 1993; Verts and Carraway, 1998). Although historically suspected of hybridizing based on morphological similarities (Harris, 1974; Parkinson, 1979), more recent investigations have shown considerable genetic distance between the two species and no evidence of hybridization (Herd and Fenton, 1983; Zinck *et al.*, 2004). Several morphological differences between these species have been proposed and biologists have considered them useful for field identification (Fenton and Barclay, 1980; Nagorsen and Brigham, 1993; Verts and Carraway, 1998).

The two species are similar in many of the external morphologic characters typically used to distinguish bats. They are similar in size, possess brown pelage, and lack keeled calcars (Nagorsen and Brigham, 1993; Verts and Carraway, 1998). *Myotis yumanensis* is generally the smaller species and forearm lengths of < 35 mm in Oregon (Verts and Carraway, 1998) and < 36 mm in British Columbia (Herd and Fenton, 1983; Nagorsen and Brigham, 1993) have been suggested as starting points for discriminating it from *M. lucifugus*. However, forearm length overlaps broadly between the species (Parkinson, 1979; Herd and Fenton, 1983; Scott, 2005) and additional non-quantitative characters are typically used to

distinguish the species in the field (Nagorsen and Brigham, 1993; Verts and Carraway, 1998).

Characters such as forehead slope, dorsal pelage sheen, and behavior have been suggested as means to help discriminate the species (Harris, 1974; Barclay and Fenton, 1980; Nagorsen and Brigham, 1993; Verts and Carraway, 1998). Nevertheless it can be difficult to assess such relative differences under field conditions or to standardize their assessment among observers. Additionally, recent work has found these characters to be unreliable for distinguishing the species (Luszcz *et al.*, 2003; Scott, 2005). Further, geographic variation in morphological characters (e.g., size and color) across large spatial scales could reduce their discrimination ability.

Echolocation call characteristics have been used to distinguish between other pairs of morphologically similar species of bats (Jones and van Parijs, 1993; Jacobs *et al.*, 2006). For example, recognition that the echolocation calls of European pipistrelles took two forms, one with maximum energy ca. 45 kHz and the other ca. 55 kHz, led to separate species designations for *Pipistrellus pipistrellus* and *P. pygmaeus*, respectively (Jones and van Parijs, 1993; Barratt *et al.*, 1997). Early reports on the echolocation calls of *M. lucifugus* and *M. yumanensis* suggested that they may be too similar for diagnostic use in the field (Herd and Fenton, 1983; Thomas, 1987). More recent work has reported minimum frequencies ca. 40 kHz for *M. lucifugus* and ca. 50 kHz for *M. yumanensis* (Saunders and Barclay, 1992; O'Farrell *et al.*, 1999; Murray *et al.*, 2001), a difference which should be sufficient to distinguish the species. However, intraspecific variation in echolocation call parameters is common among vespertilionids (Obrist, 1995; Betts, 1998; Murray *et al.*, 2001; Broders *et al.*, 2004) though it has not been well-described for the species

under consideration, especially for *M. yumanensis*. If intraspecific variation is large relative to inter-specific variation it may preclude the use of echolocation characters to discriminate between the species.

Herd (1983) described an antibody test for use in the field to distinguish between these species. Although effective, the antibody test required collection of a blood sample from the bat and use of 'anti-*lucifugus*' antibodies that are not commercially available. Possibly because of this complexity, that method has not been widely adopted. Fortunately, genetic divergence between *M. lucifugus* and *M. yumanensis* is high throughout western North America (Herd and Fenton, 1983; Zinck *et al.*, 2004). As a result, laboratory analysis of DNA, obtained from tissue or guano samples collected in the field is the most reliable method for establishing species identity. At present, DNA analysis of samples requires access to a genetics laboratory and additional funds to conduct analyses. Perhaps more importantly, because samples must be transported to the laboratory for testing, DNA analysis does not provide immediate species identification.

The objective of our study was to provide a means to distinguish between *M. lucifugus* and *M. yumanensis*, with specified probabilities of accuracy, using simple measures that could be obtained in the field. We created a set of predictive models from characters that could be quantitatively determined from live animals in the field (e.g., forearm length) and specifically excluded measures that tend to be subjectively determined (e.g., fur sheen). For the model to be useful it must be relevant to a wide variety of users and apply to conditions encountered in the field. We used data collected throughout the Pacific Northwest to select a model and validated it on three additional datasets. These allowed us to evaluate how use of a different echolocation

detection system or incorporation of poor quality echolocation recordings may affect model performance.

MATERIALS AND METHODS

Study Area

We captured *M. lucifugus* and *M. yumanensis* at 100 locations throughout northwest California, Oregon, and northwest Washington during 2002–2004 (Fig. 1). Capture sites were non-randomly selected to meet the needs of this and other concurrent studies. The study region encompassed over 300,000 km² and contained 11 ecological subregions (Bailey, 1995). The north-south trending Cascade Mountain Range bisects the study region (Fig. 1). The portion of the study area west of the Cascade Range is characterized by a temperate marine climate dominated by mesic evergreen forests with abundant surface water. The east side of the Cascade Range is primarily dominated by xeric coniferous forests and semi-arid shrub-steppe. Mean annual precipitation ranged from > 300 cm in some western locations to < 25 cm in eastern portions of the study area.

Capture and Morphometrics

We captured bats by placing mist nets designed specifically for bats (Avinet, Inc., Dryden, New York) over water and in presumed flyways. We typically selected net sites where water features, topography, and vegetation provided the best opportunities to catch bats. At roosts (e.g., bridges), we captured bats with standard or modified hand nets, such as a hand-held 'H' nets (Waldien and Hayes, 1999). We determined the sex of captured bats by external inspection (Racey 1988). We measured forearm length (FA) using a ruler (± 0.5 mm) or calipers (± 0.1 mm). We distinguished the *M. lucifugus*/*M. yumanensis* pair from other *Myotis* species in the region using published regional keys (Nagorsen and Brigham, 1993; Verts and Carraway, 1998) and genetic analysis confirmed that no non-target species were mistakenly sampled.

Molecular Analysis

We collected a 3 mm biopsy from the wing membrane of each individual (Worthington Wilmer and Barratt, 1996) and stored them in plastic vials containing desiccant (anhydrous calcium sulphate; W.A. Hammond DrieRite Co. Ltd., Xenia, Ohio, USA) at

ambient temperature. DNA was extracted from wing biopsies in the laboratory using DNeasy™ tissue extraction kits (QIAGEN Inc. Valencia, California, USA). Primers Myp1 and Myp2 were used to amplify an approximately 190 base pair region of the 16S ribosomal subunit gene (Zinck *et al.*, 2004). Two restriction enzymes (Ssp I and Aci I) were used for restriction fragment length polymorphism analysis (RFLP) to distinguish *M. lucifugus* from *M. yumanensis* (Scott, 2005). Differences in resulting fragment number and fragment size between the two species allowed for accurate and consistent distinction between the species. In the rare case that a sample

could not be identified to species using RFLP, DNA sequencing was utilized (Zinck *et al.*, 2004). Although three subspecies of *M. lucifugus* (*alacensis*, *carissima*, *lucifugus*) and two subspecies of *M. yumanensis* (*saturatus*, *sociabilis*) occurred within our study region we were concerned with field identification at the species level and limited identification using DNA accordingly. All bats were handled following the American Society of Mammalogists Animal Care and Use Guidelines (Animal Care and Use Committee, 1998), and procedures were approved by the University of Idaho and Portland State University Institutional Animal Care and Use Committees.

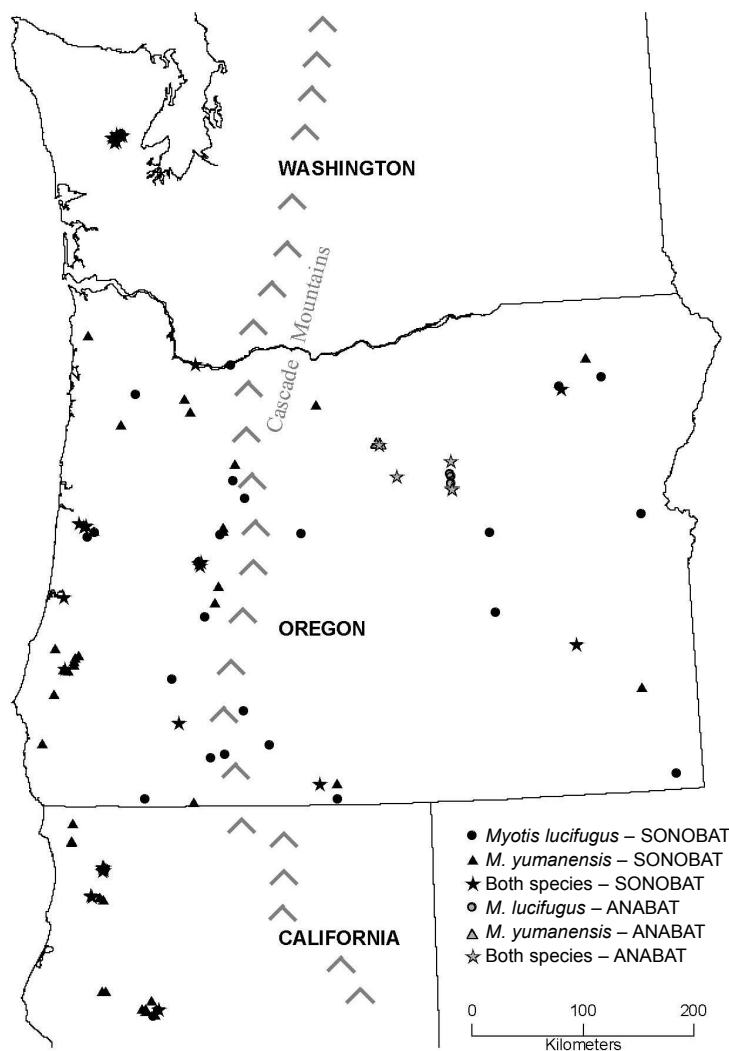


FIG. 1. Locations where *M. lucifugus* and *M. yumanensis* were captured in northwest California, Oregon, and northwest Washington, USA, 2002–2004. Echolocation call recordings from individuals were recorded using Pettersson D-240X time-expansion bat detectors and analyzed using SONOBAT software or recorded using Anabat II detectors and analyzed using ANALOOK software

Echolocation Call Recordings

After recording morphological information and taking DNA samples, we recorded echolocation calls from bats using either a time-expansion detector system (Pettersson D-240X, Pettersson Elektronik AB, Uppsala, Sweden) or zero-crossing detector system (Anabat II bat detectors with type 6 Anabat Zero-Crossings Analysis Interface Modules; Titley Electronics, Ballina, NSW, Australia). Call sequence files were stored to analog cassette players, digital recorders, or laptop computers in the field. All files were eventually transferred to personal computers for analysis. Each bat was recorded either as it flew along a tethered zip-line (Szewczak, 2004) or after it was released from an observer's hand, sometimes with a chemiluminescent light tag (Hovorka *et al.*, 1996) attached to its ventral side using temporary, non-toxic glue.

Sonobat Call Analysis

We used SONOBAT call analysis software (version 2.5; DNDesign, Arcata, CA, USA) to analyze echolocation sequences recorded using time-expansion detectors (hereafter this equipment combination is referred to as SONOBAT). We assigned a quality rating of either good or poor to each sequence. Good quality sequences contained ≥ 1 , and usually many, calls where the signal was clearly distinguishable from noise, appeared fully-formed (i.e., no missing frequency components), and in many cases displayed harmonics which indicated calls had been well-recorded (Fig. 2A; see Szewczak 2004). Poor quality sequences generally had poor signal-to-noise ratios and all calls within the sequence had ≥ 1 of the following characteristics: short duration (< 2.5 ms), reduced bandwidth, or oversimplified shapes (Fig. 2B). For individuals for which we had recorded > 1 echolocation sequence using SONOBAT, we selected the sequence that had the best signal-to-noise ratio and contained calls that spanned the broadest bandwidth.

Characteristics of echolocation calls produced by an individual, particularly those within the same sequence, are not independent therefore we used a single call per individual to avoid pseudo-replication (Hurlbert, 1984; Denzinger *et al.*, 2001; Jacobs, 2006). Because the distance and angle between bat and detector changed throughout each recording, all calls within a sequence were not recorded in equal detail. We analyzed a single echolocation call from the selected sequence that had a high signal-to-noise ratio, broad bandwidth, and, when present, one or more harmonics (Waters and Jones, 1995; Szewczak, 2004; Waters and Gannon, 2004). These characteristics

sometimes varied independently of one another and we selected the call that represented the best combination of all characteristics. We used the analysis tool in SONOBAT to manually place cursors on a time-frequency sonogram of the selected call that indicated the locations of lowest frequency (LF, kHz) and characteristic frequency (Fc, kHz — Fig. 2). LF was synonymous with the frequency at the end of the call. Fc, defined as the endpoint of the portion of the call with the least frequency change over time, was $\geq$ LF for all calls.

ANABAT Call Analysis

We analyzed echolocation sequences recorded using Anabat detectors with ANALOOK software version 4.8p (hereafter this equipment combination is referred to as ANABAT). We developed a custom filter designed to select high quality, search-phase calls ≥ 2.5 ms in duration (Corben and O'Farrell, 1999; Britzke and Murray, 2000). The filter screened pulses by frequency, duration, and several other algorithms to eliminate echoes, extraneous noise, fragmented calls, and poor quality calls within a sequence (Fig. 2C; O'Farrell *et al.*, 1999). The ANALOOK analysis function was applied to all remaining post-filter calls to calculate call duration, Fc, and LF of each call (Corben and O'Farrell 1999). No manual editing of call files was conducted. When > 1 sequence was obtained from a single bat, all calls were pooled for that individual. Calls with the longest duration typically had the broadest bandwidth.

We used the call parameters to construct two ANABAT test datasets. For the first, we used the mean values of LF and Fc as calculated by ANALOOK for all post-filtered calls recorded for an individual. This dataset was in keeping with conventional use of ANALOOK in which an entire sequence of calls, rather than an individual call, are viewed and analyzed simultaneously (O'Farrell *et al.*, 1999; Murray *et al.*, 2001; Broders *et al.*, 2004). A second dataset was created for consistency with the SONOBAT single-call approach. We selected the call with the longest duration for each individual and recorded values of LF and Fc for that call (Fig. 2C).

Statistical Analyses and Model Building

We evaluated association between FA and Fc using Pearson's correlation coefficients (PROC CORR; SAS Release 9.1; SAS Institute, Cary, North Carolina, USA). We considered results of genetic analysis of mtDNA samples to be the true species identity for each individual and used logistic regression to

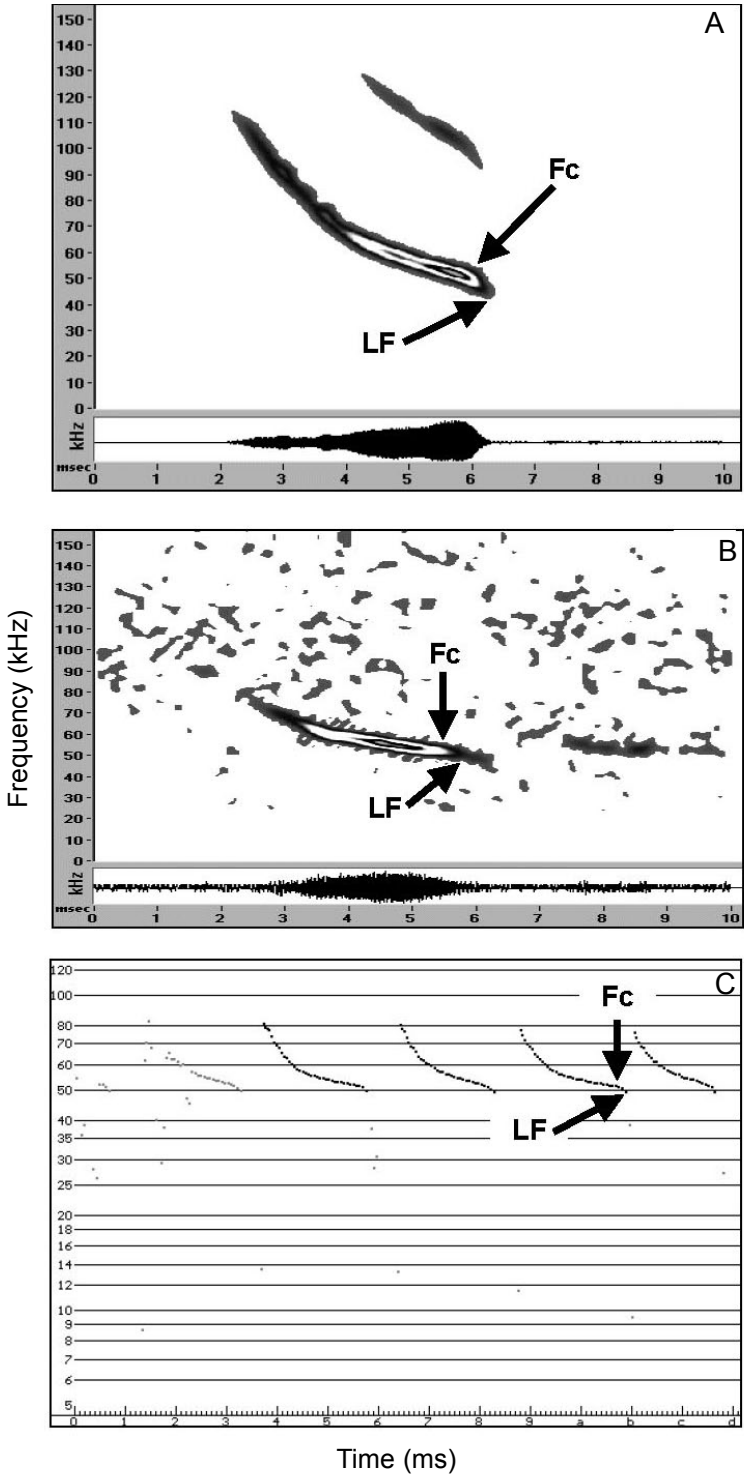


FIG. 2. Sonograms of *M. yumanensis* illustrating the location of lowest frequency (LF) and characteristic frequency (Fc) for A — a high quality SONOBAT call, B — a poor-quality SONOBAT call, and C — a filtered sequence of ANABAT calls. The interval between alpha-numeric tick marks for (C) is 2.5 ms

determine the combination of morphological and echolocation characters that best discriminated between *M. lucifugus* and *M. yumanensis* (PROC LOGISTIC; SAS Release 9.1; SAS Institute, Cary, North Carolina, USA). We selected morphological and echolocation characters that could be reliably quantified in the field and used them to construct 10 a priori models (Table 1). We did not include LF and Fc in the same model because they are alternative measures commonly used to characterize echolocation calls, and we were interested in which of them would provide better resolution for distinguishing the species. We used Akaike's Information Criterion (AIC) to rank each model and calculated its Akaike weight, i.e., the likelihood that a model was the best approximating model given the data (Burnham and Anderson, 2002).

Training Dataset

Each individual from which we recorded a good-quality SONOBAT echolocation sequence was included in the training dataset (sensu Fielding and Haworth, 1995). In all, this included 98 *M. lucifugus* and 100 *M. yumanensis* individuals captured at 78 locations throughout study area. We applied parameter estimates from the models to calculate, for each individual, the probability that it was a *M. lucifugus*. Because we used a binary model, the probability that a species was identified as *M. yumanensis* was one minus the probability it was *M. lucifugus*. We considered an individual to be correctly assigned to species if the modeled probability was > 95% and it matched results of genetic analysis.

Test Datasets

We evaluated performance of the best model by applying it to three additional test datasets, which

differed from the training dataset in terms of recording equipment, echolocation analysis methods, or quality of echolocation recordings. The three test datasets consisted of: 1) 30 *M. lucifugus* and 29 *M. yumanensis* individuals captured at 13 locations in north-central Oregon (Fig. 1) where parameters from the longest duration echolocation call for an individual recorded using ANABAT were used as model input, 2) the same group of individuals where input parameters were based on mean value for all calls > 2.5 ms recorded for an individual using ANABAT, and 3) 22 *M. lucifugus* and 26 *M. yumanensis* individuals captured at 30 locations throughout the study area from which only poor-quality SONOBAT echolocation calls were recorded. Some individuals in the poor quality SONOBAT data were captured at the same sites as individuals in the training dataset. We applied parameter estimates from the best model, identified from the training dataset, to each individual in the test datasets to calculate the probability that it was identified as *M. lucifugus* or *M. yumanensis*. We calculated correct classification rates for each test data set as the proportion of individuals for which species assignment by the model, with > 95% probability, matched the species identification from genetic analysis.

RESULTS

Species Differences

Mean values for FA were longer and Fc and LF of echolocation calls were lower for *M. lucifugus* than *M. yumanensis* in each of the datasets (Fig. 3). Nevertheless, values for all three characters overlapped broadly between the species. Overlap between the

TABLE 1. Models evaluated for field identification of *M. lucifugus* and *M. yumanensis* in the Pacific Northwest. FA — forearm length, Fc — characteristic frequency of echolocation call, LF — minimum frequency of echolocation call. Log (*L*) is the log-likelihood value, K is the number of parameters, Δ AIC is the difference in AIC values from the lowest AIC model, and w_i is the AIC model weight

Model	log (<i>L</i>)	K	AIC	Δ AIC	w_i
FA + Fc	20.65	3	26.65	0.00	0.707
FA + Fc + Sex	20.42	4	28.42	1.78	0.291
Fc	35.24	2	39.24	12.60	0.001
Fc + Sex	33.87	3	38.87	13.23	0.001
FA + LF	52.84	3	58.84	32.19	< 0.001
FA + LF + Sex	52.35	4	60.35	33.70	< 0.001
LF + Sex	77.51	3	83.51	56.87	< 0.001
LF	81.05	2	85.05	58.41	< 0.001
FA	197.54	2	201.54	174.90	< 0.001
FA + Sex	196.67	3	202.67	176.02	< 0.001

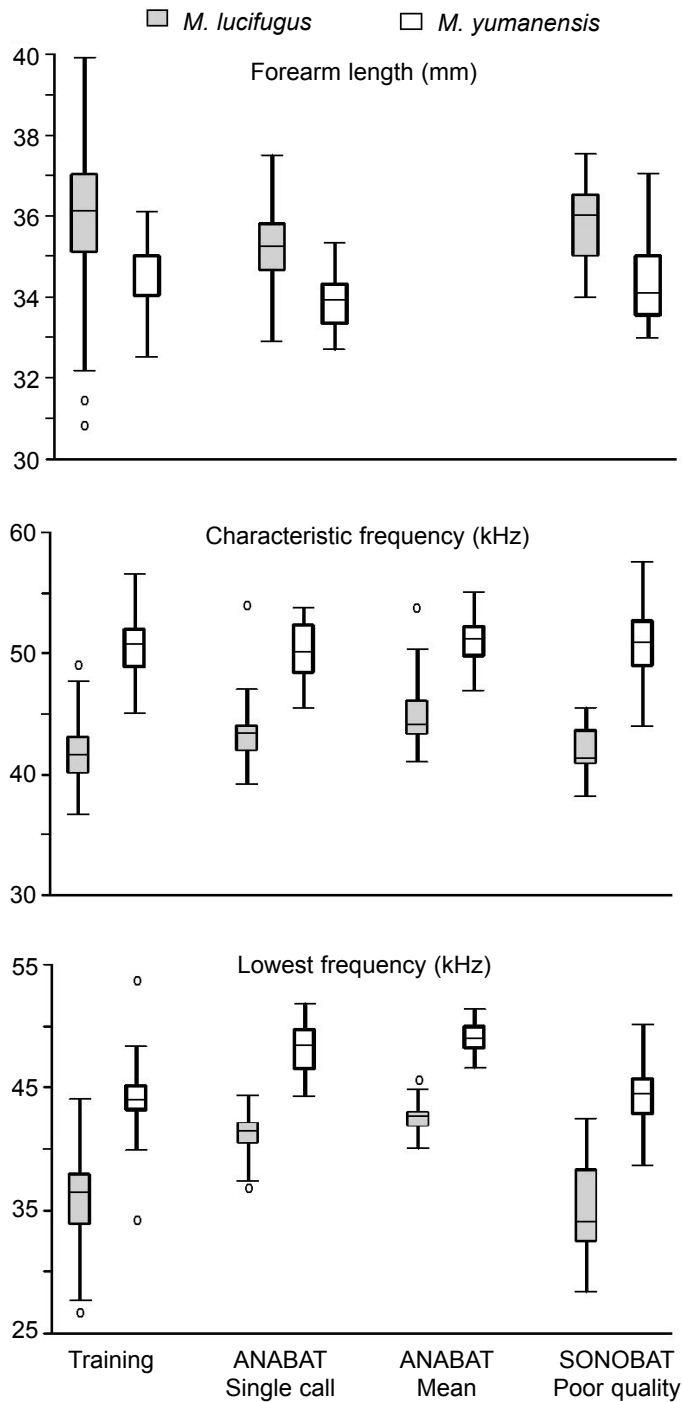


FIG. 3. Box and whisker plots for three parameters used to describe differences between *M. lucifugus* and *M. yumanensis* in the Pacific Northwest. Values for forearm length of the ANABAT mean data are not depicted because the same individuals were used in the ANABAT single call dataset. Sample sizes for *M. lucifugus* and *M. yumanensis*, respectively were 98 and 100 for the training dataset; 30 and 29 for the ANABAT datasets; and 22 and 26 for the poor-quality SONOBAT dataset

two species was 93% for FA and 56% for Fc (Fig. 4). Sixteen of 155 (10.3%) *M. yumanensis* individuals had FA ≥ 36 mm, though only one had FA > 36.5 mm. Ten of 150 (6.7%) *M. lucifugus* had FA ≤ 34 mm. Only two of 155 (1.3%) *M. yumanensis* had Fc < 45 kHz and none had Fc < 44 kHz. However, 13 of 150 (8.6%) *M. lucifugus* individuals had Fc > 45 kHz. Fc was not correlated with FA in *M. lucifugus* ($r = -0.11$, $n = 150$, $P = 0.17$) or *M. yumanensis* ($r = -0.01$, $n = 155$, $P = 0.95$).

Training Dataset Model

The top-ranked model for distinguishing *M. lucifugus* and *M. yumanensis* using the training dataset was the combination of FA and Fc (Table 1). This model was 2.4 times as likely to be the best model, based on AIC weight (w_i), as the second-ranked model which added sex of the individual to FA and

Fc. Together the top two models composed 99.8% of the Akaike weights from the 10 models we considered. Because the proportion of correctly identified individuals was identical and maximized log-likelihood values for the model with sex included were similar to those from the best model (Table 1), we did not use model-averaged values (Burnham and Anderson, 2002). We used parameter estimates from the best model to generate an equation determining the probability that an individual was *M. lucifugus* given a specific forearm length (FA) and characteristic call frequency (Fc):

$$P_{MYLU} = 1 / (1 + e^{-37.1268 - 1.6827*FA + 2.0823*Fc})$$

The probability that an individual was *M. yumanensis* was:

$$P_{MYU} = 1 - P_{MYLU}$$

The best model correctly classified, with probability $\geq 95\%$, 91.8% of *M. lucifugus* and 91.0% of *M. yumanensis* individuals

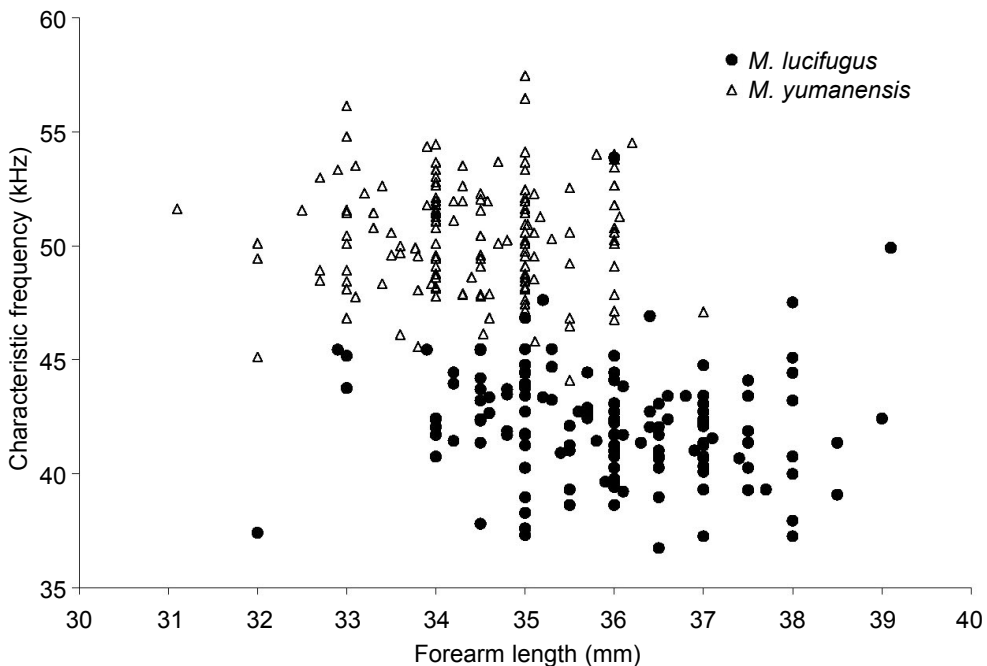


FIG. 4. Forearm length versus characteristic frequency of echolocation calls for *M. lucifugus* ($n = 150$) and *M. yumanensis* ($n = 155$) in the Pacific Northwest, 2002–2004. Values depicted are from training and test datasets. Fc was obtained from the longest duration call for individuals recorded with ANABAT

included in the training data set. If the probability for an individual to be assigned to a species was increased to 99%, the best model correctly classified 88.8% of *M. lucifugus* and 84.0% of *M. yumanensis*. We applied the species probability equations above to the commonly observed values for FA and Fc in our dataset (Table 2).

Validating Model on Test Datasets

The training dataset model correctly classified, with probability $\geq 95\%$, $> 84\%$ of *M. yumanensis* individuals in each of the test datasets (Table 3). It correctly classified $> 83\%$ of *M. lucifugus* individuals in the datasets where Fc was based on the longest duration ANABAT call or poor quality SONOBAT calls (Table 3). It correctly classified only 60% of *M. lucifugus* individuals when Fc was based on the mean values of all ANABAT calls > 2.5 ms duration for an individual.

DISCUSSION

Species Identification from Single Characters

Distinct genetic differences between *M. lucifugus* and *M. yumanensis* (Zinck *et al.*, 2004), allowed us to evaluate inter- and intra-specific variation in FA and Fc. We confirmed that, in the Pacific Northwest, *M. lucifugus* tends to have higher values of FA and echolocates at lower Fc than *M. yumanensis*. Nevertheless there is considerable overlap in these traits. By comparison, there was only a 5% overlap in frequency of maximum energy in echolocation calls between *P. pipistrellus* and *P. pygmaeus* in Europe (Jones and van Parijs, 1993). In our study area, use of threshold values for a single trait to discriminate between the species will result in a large proportion of mis- or unidentified individuals. For instance,

TABLE 2. Predicted probability (%) with which individual bats may be identified as *Myotis lucifugus* as a function of their forearm length and characteristic frequency (Fc) of their echolocation call. Values in parentheses represent the probability that an individual was *M. yumanensis*. Values in bold represent combinations of the variables that provide $> 95\%$ probability of correct assignment to either species

Fc (kHz)	Forearm length (mm)													
	32	32.5	33	33.5	34	34.5	35	35.5	36	36.5	37	37.5	38	
41	99.63	99.84	99.93	99.97	99.99	99.99	>99.99	>99.99	>99.99	>99.99	>99.99	>99.99	>99.99	
42	97.12	98.74	99.45	99.76	99.90	99.96	99.98	99.99	>99.99	>99.99	>99.99	>99.99	>99.99	
43	80.76	90.68	95.76	98.13	99.18	99.65	99.85	99.93	99.97	99.99	99.99	>99.99	>99.99	
44	34.34	54.82	73.78	86.72	93.80	97.23	98.79	99.47	99.77	99.90	99.96	99.98	99.99	
45	6.12	13.14	25.97	44.86	65.36	81.40	91.03	95.93	98.20	99.22	99.66	99.85	99.94	
46	(99.19)	(98.15)	(95.81)	9.21	19.04	35.30	55.86	74.59	87.19	94.05	97.34	98.84	99.50	
47	(99.90)	(99.77)	(99.46)	(98.75)	(97.15)	6.37	13.62	26.79	45.91	66.31	82.03	91.37	96.09	
48	(99.99)	(99.97)	(99.93)	(98.84)	(99.64)	(99.16)	(98.07)	(95.64)	9.57	19.70	36.27	56.90	75.38	
49	(>99.99)	(>99.99)	(99.99)	(99.98)	(99.95)	(99.89)	(99.76)	(99.43)	(98.70)	(97.03)	6.62	14.13	27.62	
50	(>99.99)	(>99.99)	(>99.99)	(>99.99)	(99.99)	(99.99)	(99.97)	(99.93)	(99.84)	(99.62)	(99.12)	(97.99)	(95.46)	
51	(>99.99)	(>99.99)	(>99.99)	(>99.99)	(>99.99)	(>99.99)	(>99.99)	(99.99)	(99.98)	(99.95)	(99.89)	(99.75)	(99.41)	

simply applying a threshold for FA of 36 mm (Nagorsen and Brigham, 1993) would misidentify 10% of *M. yumanensis* and 43% of *M. lucifugus* individuals in our sample. Alternately, reserving species identification for individuals which fell above or below threshold values for FA (e.g., 34 mm and 37 mm) would leave 80% of individuals unidentified (Fig. 4).

Model for Identifying Species

In spite of intra-specific variability and overlap in FA and Fc values, our top-ranked model combined both traits and assigned a large proportion of individuals to species with a high degree of confidence. Although it may appear that Fc alone would provide good discriminative power (Fig. 4), the model that only included Fc was 543 times less likely to be the best model and correctly identified 82% of individuals in the training dataset as compared to 91% for the best model. As with other *Myotis*, females of both species had slightly longer FA than males, however differences between sexes were small relative to inter-specific differences and inclusion of sex as a covariate did not improve our ability to identify species in the field. Thus, in the interest of parsimony and ease of use, we opted for a single model to discriminate between the species rather than separate models for each sex. Juveniles were included in our sample and our model correctly identified all seven *M. lucifugus* and 16 of 22 (73%) *M. yumanensis*

juveniles included among our datasets. Because of our low sample sizes, a measure of caution is warranted if applying our model to juveniles.

The model that combined Fc and FA was far superior to the one that combined LF and FA. Although previous studies have characterized echolocation calls based on LF (Herd and Fenton, 1983; Thomas *et al.*, 1987; O'Farrell *et al.*, 1999), Fc is increasingly recognized as a more consistent feature than LF by which to describe the echolocation calls of North American species (O'Farrell *et al.*, 2000; Szewczak, 2000; Broders *et al.*, 2004). Because LF is generally of lower intensity than other parts of a call it is not always well-recorded, particularly in field settings, and can be poorly depicted in time versus frequency sonograms (Fenton, 2000; Szewczak, 2000). This may have resulted in greater apparent variation in LF among calls and individuals of a species and consequently diminished its ability to help discriminate between species in this study.

Validating the Model on Test Datasets

Classification rates usually decline when models are applied to independent datasets (Fielding and Haworth, 1995). Hence, the fact that the model created from our training dataset performed comparably when applied to test datasets that used a different detection system, had lower quality echolocation calls, or were drawn from only

TABLE 3. Correct classification rate (%) for the best model for identifying *M. lucifugus* and *M. yumanensis* in the field and number of individuals (*n*) on which the model was evaluated

Dataset	<i>M. lucifugus</i>		<i>M. yumanensis</i>		Total	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Training Dataset						
SONOBAT Good quality	98	91.8	100	91.0	198	91.4
Test Datasets						
ANABAT Single-call	30	83.3	29	93.1	59	88.1
ANABAT Mean call	30	60.0	29	100.0	59	79.7
SONOBAT Poor quality	22	86.4	26	84.6	48	85.4

a subsection of our study area indicated to us that it may be broadly applicable. The only situation for which the model substantially under-performed was for *M. lucifugus* individuals recorded using ANABAT where the input value for Fc was the mean of all calls recorded from an individual. Mean Fc values for both species were consistently greater than those obtained from the longest duration call recorded from an individual (see Fig. 3). As a result, Fc values for *M. lucifugus* were closer to those expected for *M. yumanensis* and were therefore difficult for the model to classify. By contrast, mean Fc values for *M. yumanensis* individuals also tended to be higher and thus not likely to be confused with *M. lucifugus*, which helps explain why the *M. yumanensis* dataset, based on mean Fc values for an individual, achieved 100% correct classification. Evaluation of entire sequences of calls may be necessary to identify free-flying individuals (O'Farrell *et al.*, 1999) but we found a single-call approach to be more reliable, particularly when call duration is used as a selection criterion, to achieve our goal of confirming identification of captured individuals.

Choice of Bat Detection System

Encouragingly, we found that choice of bat detection system alone did not substantially change the proportion of individuals that could be correctly identified using our model. Previous work has suggested there are differences between the two detection systems used in this study in terms of their sensitivity and abilities to accurately describe some characteristics (e.g., LF) of echolocation calls (Fenton, 2000). However we found them similar in their ability to identify Fc with enough precision to discriminate these species. Further, even poor-quality SONOBAT calls, which would generally not be used to identify free-flying

bats (O'Farrell *et al.*, 1999), represent Fc well enough to help discriminate between captured individuals of the two species. Therefore, for confirming species identity of captured individuals, methods that allow for multiple attempts at recording calls (e.g., tethered zip-lines or flight tents) may be preferable to those where echolocation calls are not always recorded (e.g., hand-release — Siemers 2004; T. J. Weller *et al.*, unpublished data).

Impact of Geographic Variation

Geographic variation has been suggested as a primary contributor to variation in both size and echolocation call characteristics of bats (Findley and Traut, 1970; Harris, 1974; Burnett, 1983; Thomas *et al.*, 1987; Bogdanowicz, 1990; O'Farrell *et al.*, 2000; Barclay and Brigham, 2004). In our study it was important to consider the role of geographic variation when determining the proper geographic scale at which to fit a species discrimination model. If the high levels of variation in FA and Fc we observed were due to differences among sub-areas within our study region, it would contraindicate the use of a single, region-wide model to identify species in favor of local models where more precise parameter values could be fit to local conditions. Our study was not designed to address geographic variation, but we found value ranges of FA and Fc within several sub-areas that approximated those for the study region as a whole. For instance, at a single locality in northern California we recorded values of 32–38 mm for FA and 37–45 kHz for Fc for *M. lucifugus*. The fact that the range of values observed within sub-areas approximated that of the entire study region indicated to us that intra-specific variation was high throughout the study area and not necessarily a result of geographic variation.

CONCLUSIONS

Our model provides a means of assigning species identity in the field to ca. 90% of *M. lucifugus* and *M. yumanensis* individuals encountered in the Pacific Northwest, with 95% confidence, using quantitative measures that are easy to obtain from live animals. Importantly, neither the type of echolocation recording equipment used nor the quality of echolocation recordings substantially reduced our ability to identify the species; nevertheless use of high-quality echolocation sequences is likely to improve one's confidence. When species identity for a particular individual must be known with 100% certainty, genetic analysis is still required. We recommend that genetic samples (e.g., tissue or guano) be collected from each individual such that species identity can be confirmed via genetic analysis if necessary and because genetic material can be banked for future uses. Nevertheless, for many research and monitoring questions, investigators may be satisfied with the ability to assign species identity in the field for individuals that exceed pre-established benchmarks (e.g., 95 or 99%) for correct identification based on our model. This certainly represents an improvement over assuming correct species identification on the basis of morphologic characters observed in the field, and may allow some investigators to avoid cost and time delays associated with genetic analysis. Combining morphometrics and echolocation call characters may be an effective approach for field identification of cryptic species of bats in other areas.

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