A Blind-Test Comparison of the Reliability of Using External Morphology and Echolocation-Call Structure to Differentiate Between the Little Brown Bat (*Myotis lucifugus*) and Yuma Myotis (*Myotis yumanensis*)

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A BLIND-TEST COMPARISON OF THE RELIABILITY OF USING EXTERNAL MORPHOLOGY AND ECHOLOCATION-CALL STRUCTURE TO DIFFERENTIATE BETWEEN THE LITTLE BROWN BAT (*MYOTIS LUCIFUGUS*) AND YUMA MYOTIS (*MYOTIS YUMANENSIS*)

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ABSTRACT—Species that overlap in their morphologies are sometimes difficult to distinguish from one another, which can complicate species' conservation and management. The Little Brown Bat (*Myotis lucifugus*) and Yuma Myotis (*Myotis yumanensis*) are sympatric in parts of their range in western North America, and they overlap in morphology, making them difficult to tell apart in the hand in some areas, such as the Pacific Northwest. We compared various methods of distinguishing between *M. lucifugus* and *M. yumanensis* to genetic results, using a blind test approach to remove observer bias. Using multiple independent observers, we used external morphology and echolocation-call structure to classify bats from a maternity colony consisting of both species. Genetic analysis confirmed 13 *M. lucifugus* and 40 *M. yumanensis*. Minimum echolocation-call frequency separated 100% of *M. lucifugus* from *M. yumanensis* using a cut-off of 43 kHz. All *M. lucifugus* had a minimum echolocation-call frequency ≤42.8 kHz, whereas *M. yumanensis* had a minimum frequency ≥44.55 kHz. There was some overlap in forearm length; a cut-off of 36 mm would have correctly identified 77% of *M. lucifugus* and 100% of *M. yumanensis* to species. Criteria based on subjective assessment of fur sheen and length as well as ear color were moderately successful (90.5 and 77% success by 2 separate observers) in distinguishing between the 2 species. The use of Munsell soil color charts and multivariate statistics to classify fur and membrane color and confirm species identification was not successful. Our results suggest that mean minimum call frequency alone is sufficient for distinguishing between *M. lucifugus* and *M. yumanensis*. Use of quantitative rather than qualitative criteria eliminates observer bias and appears to be better for identifying these 2 species.

Key words: bats, blind test, Chiroptera, echolocation, Little Brown Bat, *Myotis lucifugus*, *Myotis yumanensis*, species identification, Yuma Myotis

The inability to accurately identify species that overlap wholly or partially in morphology can have scientific, management, conservation, and even economic, implications (Arlettaz and others 1997; Davidson-Watts and Jones 2006; Bickford and others 2007; Sattler and others 2007; Boston and others 2010). There are multiple examples of morphological overlap across various mammalian taxa, including rodents (Russo and others 2006; Lalis and others 2009), tenrecs (Olson and others 2004), and bats (*Pipistrellus pipistrellus* and *P. pygmaeus*: Barlow and others 1997; Häussler and others 2000; *Myotis myotis* and *M. blythii*: Arlettaz 1999; *Corynorhinus townsendii*, *C.*

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rafinesquii, and *C. mexicanus*: Piaggio and Perkins 2005; *Myotis evotis* and *M. keenii*: Nagorsen and Brigham 1993; and *Myotis lucifugus* and *M. yumanensis*: Fenton and Barclay 1980; Nagorsen and Brigham 1993). The prevalence of examples among bats is likely due to the fact that flight constrains morphology; therefore, bats of the same size tend to be similar in their morphology (Barclay and Brigham 1991).

The Little Brown Bat (*Myotis lucifugus*) and the Yuma Myotis (*Myotis yumanensis*) are sympatric in parts of their range in western North America, and they overlap in size and morphology, making them difficult to tell apart in the hand in some areas (Barbour and Davis 1969; Parkinson 1979; Herd and Fenton 1983; Nagorsen and Brigham 1993; Olson and others 2014). The 2 species were even once thought to hybridize in some areas where their ranges overlap (Barbour and Davis 1969; Parkinson 1979), although more recent research does not support this (Herd and Fenton 1983; Piaggio and others 2002). Despite their similarities in morphology, *M. yumanensis* is more maneuverable than *M. lucifugus* (Aldridge 1986). The 2 species also have different foraging preferences and diet (Herd and Fenton 1983; Saunders and Barclay 1992; Nagorsen and Brigham 1993), as well as different conservation issues. *Myotis lucifugus* was recently assessed as Endangered in Canada due to the effects and continued threat of white-nose syndrome on populations (COSEWIC 2013), and subsequently listed on Schedule 1 of the Species at Risk Act (SC 2002) in December 2014 under the emergency listing provisions of that Act. These differences in ecology and conservation status make it especially important to be able to distinguish between *M. lucifugus* and *M. yumanensis*.

External morphology has been used to distinguish between *M. lucifugus* and *M. yumanensis* in the field, with varying success (Fenton and Barclay 1980; van Zyll de Jong 1985; Nagorsen and Brigham 1993; Rodhouse and others 2008). *Myotis lucifugus* is described as having long, sleek, and glossy dorsal fur, dark basal fur on the shoulders, dark brown to blackish ears, and a forearm length usually >36 mm (Herd and Fenton 1983; van Zyll de Jong 1985; Nagorsen and Brigham 1993), although in the Okanagan Valley, British Columbia, non-reproductive females had a dull pelage (Herd and Fenton 1983). *Myotis yumanensis*, the smaller of the 2 species, is characterized as having short and dull dorsal fur, no dark basal fur on the shoulders, paler ears, and a forearm length usually ≤36 mm (Nagorsen and Brigham 1993). In 1 study, pelage sheen separated the 2 species with 96% success, whereas ear color was 82% successful (Rodhouse and others 2008). For some other species, pelage color is the key character used to distinguish between them in the field (for example, Piaggio and Perkins 2005). However, pelage color can vary with geography and reproductive status even within a species (Herd and Fenton 1983; Warner and Czaplewski 1984; Manning and Jones 1989; Solick and Barclay 2006). Likewise, in a study of 101 bats, only 18% of *M. yumanensis* and 17% of *M. lucifugus* were correctly identified to species based on forearm length due to considerable interspecies overlap in these measurements (Rodhouse and others 2008).

Differences in echolocation-call structure have been used to distinguish between species of bats with morphological overlap. For example, the cryptic species *Pipistrellus pipistrellus* (Common Pipistrelle) and *Pipistrellus pygmaeus* (Soprano Pipistrelle) are almost impossible to distinguish morphologically (Barlow and others 1997; Häussler and others 2000), but can be reliably distinguished by a 10-kHz difference in the mean frequency of maximum energy in their echolocation calls (Jones and van Parijs 1993; Barlow and Jones 1997). Herd and Fenton (1983) found significant differences in the search phase echolocation calls of *M. lucifugus* and *M. yumanensis*, but did not think that they could be reliably identified in this way due to overlap in measurements. Within species such as *M. lucifugus* that forage in a variety of habitats, echolocation-call structure varies with the amount of clutter (Broders and others 2004; Wund 2006), which further complicates the use of echolocation-call measurements to distinguish between species (Barclay and Brigham 2004). Clutter, which has been adapted from radar theory, refers to any obstacle (to a flying bat) that produces echoes that may interfere with detection of echoes from a prey target, or presents an obstacle to flight (Fenton 1990). Despite the interspecific overlap, the minimum frequency of echolocation-calls of *M. lucifugus* ranges from about 35 to 43 kHz (Saunders and Barclay 1992; O'Farrell 1999; Murray and others

2001; Broders and others 2004; Wund 2006), while that of *M. yumanensis* ranges from about 46 to 50 kHz (O'Farrell and others 1999), suggesting that these ranges may be a reliable measure to distinguish between species. Differences in characteristic frequency, the end point of the flattest (in terms of frequency) portion of an echolocation call (Corben and O'Farrell 1999), have also been used to distinguish between the 2 species (Weller and others 2007). In fact, characteristic frequency has been suggested as a better measure than minimum frequency because it is less likely to be affected by decreasing amplitude (such as when the distance between the bat and the microphone increases as the bat flies away; Chris Corben, pers. comm.; Szewczak 2000). Depending on the type of ultrasonic detector used, there may be broad overlap in minimum frequency and characteristic frequency (Weller and others 2007).

Due to observed overlap in morphological and acoustic characteristics, none of the aforementioned criteria are reported to provide 100% success in distinguishing *M. lucifugus* from *M. yumanensis* (Fenton and Barclay 1980; Nagorsen and Brigham 1993; Rodhouse and others 2008). Using a combination of characteristics has provided varying degrees of success (Herd and Fenton 1983; Weller and others 2007; Rodhouse and others 2008). For instance, Weller and others (2007) successfully identified 92% of *M. lucifugus* and 91% of *M. yumanensis* in the Pacific Northwest using a combination of forearm length and characteristic frequency of echolocation calls. Likewise, others found that a combination of forearm length and pelage sheen correctly identified 92% of *M. yumanensis* and 66% of *M. lucifugus* (Rodhouse and others 2008).

Classifying a species using qualitative characteristics such as fur length or sheen is subjective because it depends on the experience of the observer and environmental conditions (such as lighting), and, if relied on exclusively, could result in different identifications from 1 observer to the next (for example, Lobert and others 2001). The ideal way to determine the reliability of an identification technique is to use blind testing with multiple observers (for example, Scheuer 2002). Despite the difficulties in distinguishing between *M. lucifugus* and *M. yumanensis* in some areas of geographic overlap, there are no published studies that have compared external morphology, echolocation-call structure, and genetics in a blind test. The objective of our study was to determine the validity and reliability of using external morphology, echolocation-call structure, or both to distinguish between *M. lucifugus* and *M. yumanensis* using a blind-test approach and genetic confirmation of identity. A maternity colony of both species roosting in the same building provided the ideal opportunity for such a study.

METHODS

Our study site was a maternity colony for both *M. lucifugus* and *M. yumanensis* in a maintenance building at the Hozomeen Ranger Station in North Cascades National Park, USA (UTM: Zone 10, 2377506.85E, 6248600.55N, NAD83; elevation = 500 m) in the Skagit Valley of the Cascade Range. Both species roosted in the attic of this large building, although we do not know if they were spatially separated from each other. As a part of a larger study (Luszcz 2004), we conducted fieldwork for this project over 2 d (17 and 18 May 2002). All capture and handling of bats followed guidelines set by the University of Calgary Animal Care Committee, and the Canadian Council on Animal Care. At dusk each night, we captured bats exiting the building using mist nets erected along nearby flyways. We held captured bats in cloth bags until mist nets were closed and measuring could begin.

Each bat was assigned a number, which was used throughout the study to link all measurements. Each observer was responsible for 1 identification task throughout the entire measurement process, and tentative identifications were not disclosed. Only 1 observer assessed sex, age, mass, and forearm length because these measures are less subject to observer variation. Two observers independently assessed fur and membrane (ear or wing) color using Munsell® Soil Color Charts (Munsell Color Laboratory 2000) as outlined in Solick (2004). Munsell Soil Color Charts consist of color chips organized by their hue, value, and chroma, the 3 color attributes recognized by the Munsell notation system (Miller 1958). Hue describes a color's relation to red, yellow, green, blue, and purple, with higher hue numbers

being more yellow and less red. Value refers to the brightness of a color, and ranges from 0 (pure black) to 10 (pure white). Chroma denotes the degree of saturation, on a scale from 0 to 10. High chroma indicates a rich, vibrant color, whereas low chroma colors are pale (Munsell Color Laboratory 2000). Each observer classified fur and membrane color by placing the Munsell Soil Color Chart over the fur on the back below the scapula and on a membrane, using the same light fixed at a standard distance from the bat.

Two other observers, with similar bat identification experience, independently identified bats as *M. lucifugus* or *M. yumanensis* based on morphological criteria from the literature. Fur length was classified as either short or long, fur sheen as either dull or glossy, and ear color as either light to medium brown, or dark brown. A bat required a minimum of 2 out of 3 criteria (fur length, fur sheen, and ear color tone) to be classified to a species.

To assess whether wing loading or aspect ratio (2 measures of wing size and shape; Norberg and Rayner 1987) were useful in identification, 2 observers extended the right wing of each bat to its fullest and traced its outline onto paper. We later scanned the wing tracings and imported the digital images into BatWing.exe 1.0 software (Harley and Miller-Butterworth 2000). Using the software, we obtained measures of wing area and length and then calculated wing loading and aspect ratio following methods outlined in Solick and Barclay (2006). Wing loading and aspect ratio provide information about a bat's ability to exploit cluttered habitats (Norberg and Rayner 1987).

Another observer took a 3-mm diameter wing biopsy punch from each bat for genetic analysis (Worthington and Barratt 1996), conducted at Portland State University (JZ). DNA was extracted from the biopsy samples using the Qiagen^{TM} DNEasy Tissue Extraction Kit. A 190 base-pair mitochondrial DNA fragment from the 12S ribosomal subunit gene was amplified under standard PCR conditions (Zinck and others 2004). The resulting PCR product was sequenced using an ABI 3100 automatic sequencer. Sequences were aligned using Seqed (ABI), and analyzed in PAUP 4.0. Sequences were initially compared to a library of over 300 additional *Myotis* sequences. We used the genetic analysis to identify individuals to species and then used those identifications to compare to the identifications based on the characteristics measured in this study.

We released each bat in the same location along a laneway in a forested campsite less than 500 m from the roost, and we recorded echolocation calls using Anabat II ultrasonic detectors (Titley Electronics, Ballina, NSW, Australia) set to a division ratio of 16. Detectors were turned on only once the bats were free-flying, and we recorded calls until bats flew out of range. We used Analook software to purge call sequences of extraneous noise (pixels) and to measure call morphology. We adapted rules from Patriquin (2001) to clean echolocation calls. The final rules were: (a) pixels had to follow the same general trajectory of a call or they were deleted; (b) along a trajectory of points in a call, a lone pixel near the beginning or end of the call had to be separated from the rest of the call by no more than 5 kHz or it was deleted; and (c) along a trajectory of points in a call, groups of points near the beginning or end of the call had to be separated from the rest of the call by no more than 10 kHz or they were deleted. Minimum call-frequency values and characteristic-frequency values were each averaged to obtain 1 data point per bat. Number of usable calls recorded per bat ranged from 7 to 172, except in 1 case where only 1 usable call was recorded.

For all statistical analyses, we used the online statistical tool VassarStats (http://vassarstats.net/) and Microsoft Excel. We compared minimum echolocation call-frequency, characteristic call-frequency, mass, forearm length, wingspan, wing loading, and aspect ratio within and between the 2 species using 2 sample t-tests ($\alpha = 0.05$). Means and standard deviations are reported unless otherwise indicated. For the qualitative ear and wing variables, we examined agreement between observers, and between each observer and the genetic results, by calculating Cohen's Kappa statistic, a measure of inter-observer agreement whereby agreement due to chance is factored out (Cohen 1960). Kappa values can help to assess the reliability of a method of categorization. Kappa values range from 1 to –1. A Kappa value of 1 indicates perfect agreement between

TABLE 1. Descriptive statistics for measurements of echolocation-call frequency and external morphology of *Myotis lucifugus* (MYLU) and *M. yumanensis* (MYYU) from the Skagit Valley, Washington, 2002. Mean ± standard deviation, range in parentheses, and sample size are provided.

	MYLU (all female)	MYYU males and females	MYYU females	MYYU males
ECHOLOCATION				
Minimum frequency (kHz)	39.82 ± 1.84 (36.32–42.80) $n = 12$	47.48 ± 1.35 (44.55–50.29) $n = 40$	47.37 ± 1.31 (44.55–50.29) $n = 31$	47.87 ± 1.52 (46.56–50.24) $n = 9$
Characteristic frequency (kHz)	42.31 ± 3.08 (38.99–50.42) $n = 12$	51.86 ± 2.21 (45.85–57.26) $n = 38$	51.66 ± 1.95 (45.85–54.33) $n = 30$	52.60 ± 3.04 (47.85–57.26) $n = 8$
MORPHOLOGY				
Mass (g)	5.51 ± 0.49 (4.7–6.2) $n = 13$	4.90 ± 0.36 (4.0–5.4) $n = 40$	5.03 ± 0.23 (4.5–5.4) $n = 31$	4.46 ± 0.38 (4.0–4.9) $n = 9$
Forearm length (mm)	36.47 ± 1.14 (34.40–38.30) $n = 13$	34.43 ± 0.70 (32.30–35.58) $n = 40$	34.50 ± 0.67 (32.30–35.58) $n = 31$	34.18 ± 0.78 (32.95–35.50) $n = 9$
Wingspan (cm)	23.10 ± 0.81 (21.91–24.76) $n = 13$	22.00 ± 1.03 (19.16–23.77) $n = 39$	22.16 ± 0.95 (19.16–23.77) $n = 31$	21.38 ± 1.14 (19.29–23.02) $n = 8$
Wing loading (N/m^2)	6.04 ± 0.38 (5.28–6.50) $n = 13$	6.18 ± 0.55 (5.06–7.99) $n = 39$	6.19 ± 0.50 (5.06–7.11) $n = 31$	6.15 ± 0.76 (5.65–7.99) $n = 8$
Aspect ratio	5.98 ± 0.30 (5.39–6.49) $n = 13$	6.19 ± 0.36 (5.29–6.86) $n = 39$	6.15 ± 0.39 (5.29–6.86) $n = 31$	6.32 ± 0.19 (6.01–6.59) $n = 8$

observers, 0 means that any agreement is totally due to chance, and –1 means there is perfect disagreement. Common criteria for Cohen's Kappa are as follows: <0 = no agreement; 0 to 0.20 = poor agreement; 0.2 to 0.4 = fair agreement; 0.4 to 0.6 = moderate agreement; 0.6 to 0.8 = good agreement; and ≥0.8 = very good agreement (Landis and Koch 1977). We also used Cohen's Kappa to examine inter-observer agreement of the Munsell Soil Color Chart variables (hue, value, and chroma).

RESULTS

Over 2 nights of mist netting, we captured 55 bats during their dusk exit, including 1 *Myotis evotis* (Long-eared Myotis) female. We obtained complete information, including usable echolocation calls, for 52 bats (43 females and 9 males); we did not obtain echolocation calls for 1 additional bat, but we included it in the morphological analyses. Genetic analysis confirmed 13 *M. lucifugus* (all females) and 40 *M. yumanensis* (of which 31 were females). Descriptive statistics for measurements of echolocation-call frequency and external morphology are summarized in Table 1.

There was no significant difference in mean minimum echolocation-call frequency between male and female *M. yumanensis*, or in mean characteristic frequency (Table 2); thus, we combined male and female *M. yumanensis* in the interspecies analysis of call parameters. Mean minimum call frequency was significantly different between *M. lucifugus* (39.82 ± 1.84 kHz) and *M. yumanensis* (47.48 ± 1.35 kHz; $t = 15.77$, $df = 50$, $P < 0.0001$; Table 2, Fig. 1). The highest minimum frequency for *M. lucifugus* was 42.8 kHz and the lowest minimum frequency for *M. yumanensis* was 44.5 kHz. Thus, in our study, a minimum frequency cut-off of 43 kHz separated 100% of *M. lucifugus* from *M. yumanensis*. Characteristic frequency also differed significantly between *M. lucifugus* and *M. yumanensis* ($t = 11.85$, $df = 48$, $P < 0.0001$; Table 2), although there appeared to be some overlap between species (ranges: MYLU = 38.99–50.42, MYYU = 45.85–57.26; Table 1). However, we considered the highest characteristic frequency for *M. lucifugus* (50.42 kHz) as an outlier. Thus, if we consider the second-highest characteristic frequency (45.55 kHz) for *M. lucifugus* as the correct highest characteristic frequency for the species in our study, and

TABLE 2. Results of t-tests to determine statistical differences in measurements of echolocation-call frequency and external morphology between *Myotis lucifugus* (MYLU) and *M. yumanensis* (MYYU) from the Skagit Valley, Washington, 2002. We report t-value with degrees of freedom, P-value, and estimate ± 95% confidence intervals. Significant P-values are indicated in bold. Asterisk (*) indicates when t-tests for samples with unequal variances were performed.

	MYLU versus MYYU	Females only MYLU versus MYYU	MYYU females versus males
ECHOLOCATION			
Minimum frequency (kHz)	$t_{50} = 15.77$ **$P < 0.0001$** 7.66 ± 0.98	$t_{41} = 15.11$ **$P < 0.0001$** 7.54 ± 1.01	$t_{38} = 0.98$ $P = 0.33$ 0.50 ± 1.04
Characteristic frequency (kHz)	$t_{48} = 11.85$ **$P < 0.0001$** 9.55 ± 1.62	$t^*_{14.65} = 9.77$ **$P < 0.0001$** 9.36 ± 2.04	$t_{36} = 1.07$ $P = 0.29$ 0.94 ± 1.78
MORPHOLOGY			
Mass (g)	$t_{51} = 4.83$ **$P < 0.0001$** 0.61 ± 0.25	$t^*_{14.17} = 3.35$ **$P = 0.005$** 0.48 ± 0.31	$t^*_{9.7} = 4.3$ **$P = 0.002$** 0.57 ± 0.30
Forearm length (mm)	$t^*_{15.03} = 6.06$ **$P < 0.0001$** 2.04 ± 0.72	$t^*_{15.6} = 5.78$ **$P < 0.0001$** 1.96 ± 0.72	$t_{38} = 1.21$ $P = 0.23$ 0.32 ± 0.53
Wingspan (cm)	$t_{50} = 3.50$ **$P = 0.001$** 1.10 ± 0.63	$t_{42} = 3.11$ **$P = 0.003$** 0.94 ± 0.61	$t_{37} = 1.99$ $P = 0.054$ 0.78 ± 0.80
Wing loading (N/m^2)	$t_{50} = 0.86$ $P = 0.39$ 0.14 ± 0.33	$t_{42} = 0.97$ $P = 0.34$ 0.15 ± 0.31	$t_{37} = 0.17$ $P = 0.86$ 0.04 ± 0.45
Aspect ratio	$t_{50} = 1.86$ $P = 0.07$ 0.21 ± 0.22	$t_{42} = 1.43$ $P = 0.16$ 0.17 ± 0.25	$t^*_{24.33} = 1.72$ $P = 0.10$ 0.17 ± 0.20

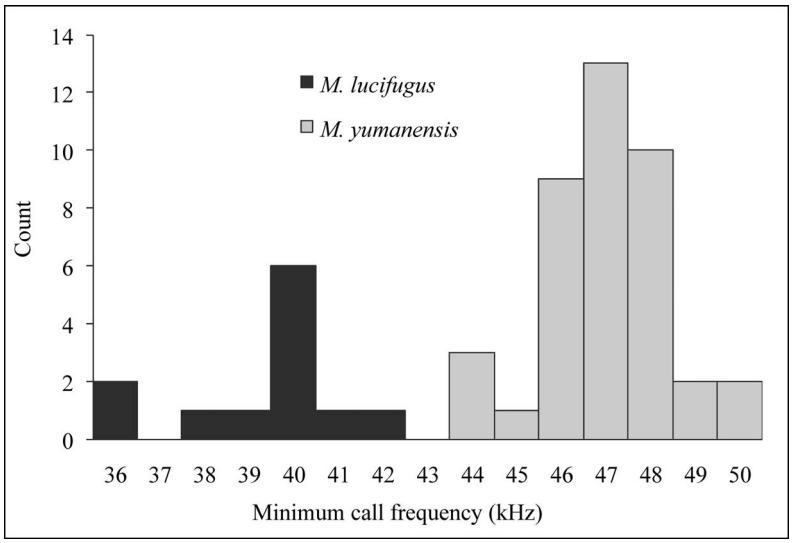

FIGURE 1. Frequency distribution of the minimum echolocation-call frequency of 12 adult *Myotis lucifugus* and 40 *Myotis yumanensis* from the Skagit Valley, Washington.

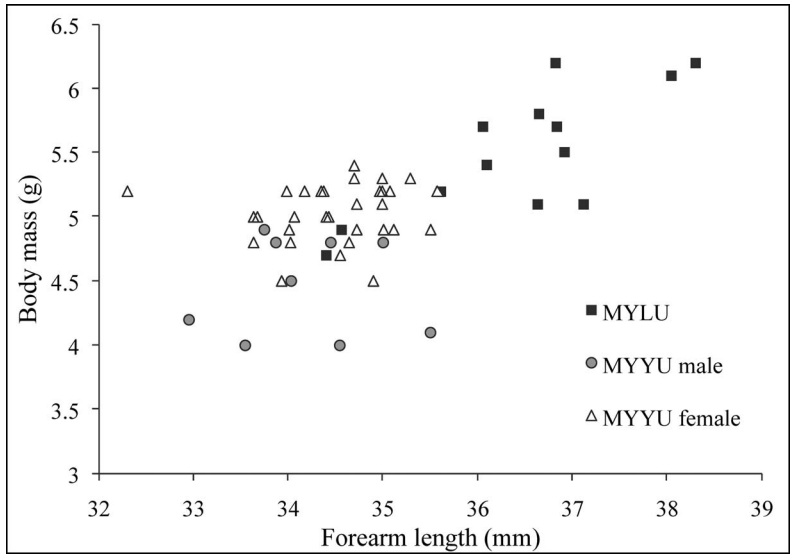

FIGURE 2. Body mass plotted against forearm length for *Myotis lucifugus* (MYLU) and *Myotis yumanesis* (MYYU), illustrating the overlap in these measurements between the 2 species. The 3 *M. lucifugus* that overlap with *M. yumanensis* were correctly identified to species by their fur length and sheen, and ear color, as well as minimum echolocation-call frequency.

compare that to the lowest characteristic frequency for *M. yumanensis* (45.85 kHz), then the characteristic frequencies for the 2 species do not overlap.

Forearm lengths and wingspans did not differ significantly between male and female *M. yumanensis,* nor did wing loading or aspect ratio (Table 2). Although female *M. yumanensis* had a slightly greater wingspan than males (Table 1), this difference was not significant (Table 2). Female *M. yumanensis*, however, were significantly heavier than males ($t = 4.3$, $df = 9.7$, $P = 0.002$; Table 2). Therefore, given that all measurements of external morphology (except mass) for *M. yumanensis* were relatively similar, we combined males and females of this species when we compared *M. lucifugus* and *M. yumanensis*. *Myotis lucifugus* were significantly heavier, had longer forearms, and greater wingspans than *M. yumanensis* (Table 2, Fig. 2). Ten of the 13 *M. lucifugus* had a forearm length >36 mm, and all *M. yumanensis* ($n = 40$) had a forearm length ≤36 mm. Therefore, 77% of *M. lucifugus* and 100% of *M. yumanensis* in our study could have been correctly identified to species on the basis of forearm length alone if a cut off of 36 mm was used. Despite the differences in wingspan, the 2 species did not differ in their wing loading or aspect ratio (Table 2). When we compared females only, the results were the same. Female *M. yumanensis* weighed significantly less and had shorter forearms and smaller wingspans than female *M. lucifugus* (Table 2). They did not, however, differ in their wing loading or aspect ratio (Table 2).

Compared to the 100% success in identifying individuals to species using echolocation-call characteristics, criteria based on subjective assessment of fur sheen and length, and ear color were less successful in distinguishing between *M. lucifugus* and *M. yumanensis*. Observer 1 correctly identified 48 of 53 bats (90.5% success) to species (Cohen's Kappa statistic $\kappa = 0.76$; good agreement), and Observer 2 correctly identified 41 of 53 bats (77% success) to species ($\kappa = 0.49$; moderate agreement). There was moderate inter-observer agreement in the species identifications made by each of the 2 observers ($\kappa = 0.56$). In the cases of misidentification: Observer 1 misidentified *M. yumanensis* as *M. lucifugus* in 4 cases, and *M. lucifugus* as *M. yumanensis* in 1 case; Observer 2 misidentified *M. yumanensis* as *M. lucifugus* in 10 cases, and *M. lucifugus* as *M. yumanensis* in 2 cases. In 8 of 14 cases of the misidentification of *M. yumanensis* as

M. lucifugus, *M. yumanensis* was classified as having all 3 *M. lucifugus* traits: long glossy fur and dark ears. In the remaining 6 cases, *M. yumanensis* was classified as having 2 out of 3 *M. lucifugus* traits. In the cases of *M. lucifugus* being misidentified as *M. yumanensis*, *M. lucifugus* was classified in 2 of 3 cases as having all 3 *M. yumanensis* traits: short dull fur and light ears. Finally, the 3 *M. lucifugus* with forearm lengths <36 mm, which placed them in the area of overlap with the range of *M. yumanensis* forearm lengths (Fig. 2), were successfully identified to species using external morphology as well as minimum echolocation-call frequency.

Attempts to identify bats to species using Munsell Soil Color Charts were unsuccessful. Hue, value, and chroma variables for fur color resulted in poor (or no) agreement between observers (κ = –0.24, 0, and 0.10, respectively). Based on membrane color, there was also poor agreement for hue (κ = 0.08) and moderate agreement for value and chroma (κ = 0.49 and 0.49, respectively) between observers. In the 2 instances of moderate agreement, there was little variation in value or chroma raw data values; thus, no further analysis was undertaken with these data.

Discussion

Our blind test approach using multiple observers to compare methods for distinguishing between *M. lucifugus* and *M. yumanensis* provided valuable insight into the reliability of each method as a species identification tool. In our study, minimum echolocation-call frequency was the best variable for species confirmation. Based on the genetic results, the separation of minimum frequency at 43 kHz was distinct, with no overlap between species. In another study that examined the same parameter between *M. lucifugus* and M. *yumanensis*, there was no overlap in mean lowest frequency from Anabat data sets (and little overlap for Anabat single calls recorded from Anabat zero-crossing detectors; Figure 3 in Weller and others 2007), lending support to our methods and results. Weller and others (2007) did, however, observe broad overlap in lowest frequency between the 2 species in data recorded using time-expansion detectors and analyzed using SonoBat call analysis software. These differences in results may relate to differences in the 2 detection systems (Fenton 2000).

Minimum frequency is less consistent than characteristic frequency, because if there is a significant droop, or tail, after the characteristic frequency point, the extent to which this is detected becomes highly dependent on the distance to the bat. This is 1 reason why characteristic frequency is considered a better measure for acoustically distinguishing between species. As a bat flies further away, less of the tail will be recorded as its amplitude decreases as the frequency drops (Chris Corben, pers. comm.). On the other hand, as clutter increases, a bat's echolocation call becomes steeper. The characteristic frequency increases and requires more magnification to see changes in the slope in order to define it (Broders and others 2004; Wund 2006), compared to minimum frequency. Using our rules for cleaning Anabat calls, we did not find minimum frequency to be misleading. However, we did find what appeared to be overlap in characteristic frequency between the 2 species, although it was due to what we consider to be an outlier frequency for 1 *M. lucifugus*.

More recently, field researchers in California and British Columbia have successfully distinguished between captured *M. lucifugus* and *M. yumanensis* in the field using a "bag test" (Dave Johnston, HT Harvey and Associates, Los Gatos, CA, pers. comm.; Cori Lausen, Birchdale Ecological Ltd., Kaslo, BC, pers. comm.). They recorded echolocation calls of bats while they were still in their cloth holding bags, differentiating the 2 species by their minimum frequency. In British Columbia, they used an Echo Meter EM3 detector (Wildlife Acoustics, Massachusetts, USA), and observed emitted pulses in the field: minimum frequencies of 30 to 37 kHz for *M. lucifugus* and >42 kHz for *M. yumanensis* (Cori Lausen, pers. comm.). Their minimum frequency cut-off was similar to that observed in our study. This "bag test" was 100% accurate when compared to genetic results (n = 13; Cori Lausen, pers. comm.). A bat echolocating in a bag is responding to a high clutter environment. Broders and others (2004) similarly found that rates of misclassification were lowest in high clutter. Determining minimum echolocation-call frequency *in situ* would make identification of *M. lucifugus* and *M. yumanensis* much easier than

our methods that measure the mean minimum frequency of all calls in a sequence after manual call cleaning, or even methods that apply custom filters in Analook or auto-identification software (Corben and O'Farrell 1999; Weller and others 2007). Application of the "bag test" in other geographic areas would be an ideal confirmation of its strength in distinguishing between *M. lucifugus* and *M. yumanensis* in the hand. Furthermore, comparing "bag test" minimum frequency to that of calls collected from a free-flying bat after it has been released would be an excellent test of the strength of both methods.

Measurements of external morphology resulted in variable and sometimes inconclusive species identifications. Although body mass and wingspan significantly differed between *M. lucifugus* and *M. yumanensis*, the interspecific overlap in these measurements reaffirmed that they alone are not adequate for species identification. Measurements of wing loading and aspect ratio were too similar to be of any value in separating the 2 species. Contrary to Rodhouse and others (2008), we found little overlap between species in measurements of forearm length; however, our sample size was relatively small and only from 1 geographic location. As has been found in other *Myotis* species, female *M. yumanensis* were significantly heavier than males (for example see Kalcounis and Brigham 1995). Had we captured male *M. lucifugus* in our study, we might have observed even more overlap in body mass and forearm length. The 3 *M. lucifugus* that overlapped with *M. yumanensis* in mass and forearm length (Fig. 2) were identified to species correctly by their fur length and sheen, and ear color, as well as minimum echolocation call frequency. It should also be noted that bats misidentified by their fur length and sheen, and ear color would have been correctly identified by their forearm length and by their minimum echolocation-call frequency, although we did not have an echolocation call recording for 1 individual.

Distinguishing between *M. lucifugus* and *M. yumanensis* based on fur characteristics and ear color (van Zyll de Jong 1985; Nagorsen and Brigham 1993) was only moderately successful and is quite subjective. Both observers had a similar amount of bat identification experience, yet showed only moderate agreement in their species identification. In our study, only 2 *M. lucifugus* were misidentified as *M. yumanensis* (due to short dull fur), whereas 8 misidentifications in the opposite direction occurred (due to long glossy fur and dark ears). The Skagit Valley, where this study occurred, is a coastal-interior transition zone in the Cascade Range. Thus, some of the *M. yumanesis* in our study may have been the coastal subspecies, *M. yumanensis saturatus* Miller 1897, which is a dark-brown to chestnut form with black ears and membranes (van Zyll de Jong 1985). Species identification using these characteristics could have been further complicated if we had conducted this study later in the season when juveniles were flying, because *M. lucifugus* juveniles have been reported to have darker fur than adults (Fenton and Barclay 1980), whereas yearlings tend to have duller fur than adults (Herd and Fenton 1983). Our attempts to use Munsell Soil Color Charts to distinguish differences in fur and membrane color between *M. lucifugus* and *M. yumanensis* were unsuccessful because the results were irreproducible, as evidenced by poor inter-observer agreement (Cohen 1960), suggesting that this method is likely not worthy of further testing.

In conclusion, our results demonstrate that mean minimum frequency can be a strong predictor of species on its own when distinguishing between *M. lucifugus* and *M. yumanensis*. However, due to variation in echolocation in different geographic locations and habitats across the range of these 2 sympatric species (for example, Weller and others 2007), a 43 kHz minimum frequency cut-off may not be applicable in all areas. Furthermore, we caution that this method should not be applied to free-flying bats across various habitat types, but should be limited to identification of captured bats released into the same conditions (for example, capture site). Further study in different geographic areas and habitats could determine whether our methods and specific cut-off value are generally applicable.

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