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Data, sample sizes and statistics affect the recognition of species of bats by their echolocation calls

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Identification of bat species based on analysis of echolocation calls can be affected by the way data are manipulated, the diversity of species, and call variability. We document the effects of sample sizes and a priori assignment of calls by species on the outcome of discriminant function analysis (DFA) and multinomial logistic regression (MLR) of features of echolocation calls, and determine which features of calls are most useful for identification. We used recorded echolocation calls of eight species readily distinguishable by call features, including molossids, emballonurids and a moormopid recorded at sites in Belize, Brazil, and Mexico. On individual calls, we measured four features: frequency with most energy, highest and lowest frequencies and call durations obtained from sequences consisting of 10 calls. Cluster analysis and multiple analyses of variance indicated significant differences between the calls of different species. Outcomes of DFA and MLR were affected by both sample sizes (numbers of calls, numbers of sequences) and the subjective approach that researchers take to their data (i.e., categorizing calls or sequences of calls by species). Levels of variation in calls of some species in our sample often precluded the use of single calls in making call-based identifications. Accurate documentation of variability in echolocation behavior of sympatric bats is a prerequisite for an effective sound-based bat survey.

Key words: echolocation calls, emballonurids, molossids, mormoopids, variation, cluster analysis, discriminant function analysis, multiple logistic regression

INTRODUCTION

Recognizing species of echolocating bats by their calls has been valuable in assessing the distribution of, and habitat use by, bats not prone to capture (e.g., Kalko, 1997; Ochoa *et al*., 2000). Many authors (e.g., Fenton and Bell, 1981; Ahlén, 1990; O'Farrell and Miller, 1997, 1999; Russo and Jones, 2002; Rydell *et al*., 2002) have demonstrated that it is possible to distinguish among species of bats by their echolocation calls, reflecting diagnostic patterns of frequency change over time and species-specific frequencies in calls. The feasibility of detecting and identifying a bat by its echolocation calls is a direct function of call intensity. Species producing low intensity echolocation calls (the 'whispering bats' of Griffin, 1958) are more difficult to sample than bats using high intensity echolocation calls. Among species producing

high intensity echolocation calls, some call features appear to be particularly useful in distinguishing species. For example, in some vespertilionids (Fenton and Bell, 1981; O'Farrell and Miller, 1999), and molossids (Fenton and Bell, 1981), lowest frequencies in calls are important, while in emballonurids, frequencies with most energy are particularly useful (Kalko, 1995). Other authors report that the frequency values measured at the ends of calls could be most useful in discriminating between species from different families (Parsons and Jones, 2000; Russo and Jones, 2002). However, the use of apparently diagnostic frequencies to identify bat species by their calls is complicated in species that vary frequencies between calls such as *Saccopteryx bilineata* and *S. leptura* (Kalko, 1995), *Molossus molossus* (Kossl *et al*., 1999), or *Barbastella barbastellus* (Denzinger *et al*., 2001).

Identification of bat species by their echolocation calls is affected by variability. Individuals can dramatically change the features of their calls as they search for, detect, and close on flying prey (Griffin *et al*., 1960), under different environmental conditions (Kalko and Schnitzler, 1993; Kalko, 1995; Obrist, 1995) and/or in the presence of conspecifics (Obrist, 1995; Ratcliffe *et al*., In press; Ulanovsky *et al*., In press). Furthermore, calls may vary within species geographically (Thomas *et al*., 1987; Parsons, 1997; Law, 2002), sexually (Neuweiler *et al*., 1987; Jones *et al*., 1992), by age (Jones and Ransome, 1993; Jones and Kokurewicz, 1994; Kazial *et al*., 2001; Russo *et al.*, 2001) and as a result of bats using different foraging tactics (Ratcliffe and Dawson, 2003). Variability in call detection can be generated by a variety of recording artifacts. Included are discrepancies in recording equipment (Obrist, 1995; Barclay, 1999), relative distances between microphones and bats (Barclay, 1999), wind, temperature, and humidity-related transfer functions of acoustic signals (Lawrence and Simmons, 1982). Also contributing to variation are movements of bats' heads relative to the microphone (O'Farrell and Miller, 1999) and vegetation (Schnitzler and Kalko, 1998; Patriquin *et al*., 2003).

In a bat fauna of few species, some people accustomed to listening to bat detector representations of bat echolocation calls may learn to distinguish between species by the audio output of bat detectors. But, quantifying call features can make recognition of bats by their echolocation calls a more repeatable procedure leading to the next step, statistical analyses, which are fundamental for quantification and objectification of the process. Among the many statistical analyses available to researchers, discriminant function analysis (DFA) has been more widely used (Obrist, 1995; Barclay, 1999; Jones *et al*., 2000; Parsons and Jones, 2000; Kazial *et al*., 2001; Russo and Jones, 2002) than other analyses such as artificial neural networks (Jones *et al*., 2000; Olden and Jackson, 2002), synergistic pattern recognition (Obrist *et al*., 2004), decision trees (Herr *et al*., 1997) and multiple logistic regression. DFA has the ability to identify which variables (call features) discriminate between groups, vital information when using echolocation calls to identify species.

Statistical procedures are performed on samples of the total populations in question. Although sample sizes fluctuate due to many factors, there are general guidelines on minimum sample sizes acceptable (Cohen, 1988). Similarly, the way in which data are obtained, organized and submitted to the statistical procedure vary across schools of thought, laboratories, and individual researchers. Thus, although statistics are used with the intent of objectifying analyses of echolocation calls, they are inherently subject to biased factors due to the researcher's data selection process, and also the variability in the calls themselves.

Our paper has two goals: 1) to test the utility of DFA and MLR in the face of call variability, data manipulation, and sample size manipulation; and 2) to determine which features of calls are most useful for identification of species.

Materials and Methods

Preliminary Procedures

We recorded bat calls in Belize (near Lamanai Outpost Lodge; 17°46'N, 88°39'W; from 16 to 23 May 2000, 7 nights), Brazil (around Alter do Chao, Para' State; 02°30'S, 54°57'W; from 1 to 23 April 2002, 15 nights of recordings) and in Mexico (Mexico City; 19°25'N, 99°10'W; and Chamela, Jalisco State; 19°31'N, 105°02'W; from 21 June to 20 July 2002, 13 nights of recordings).

We used data on the calls of eight species of bats: four emballonurids, three molossids and one mormoopid from among the species occurring in the study areas (Sanchez *et al*., 1989; Ceballos and Miranda, 2000; Chavez and Ceballos, 2001; Fenton *et al*., 2001; Bernard and Fenton, 2002). Using features such as pattern of frequency change over time as well as frequency and time parameters, we identified the species we studied from recordings (field recordings and those from hand-released bats) and the literature. We caught *Saccopteryx bilineata* (Belize and Brazil), *Peropteryx macrotis* (Brazil), *Nyctinomops macrotis* (Mexico) and *Tadarida brasiliensis* (Mexico). We weighed each captured bat, measured length of forearm, classified them as adults or subadults, and recorded sex. We released the bats when no other bats flew in the area and recorded the echolocation calls they produced. We compared the data from these recordings with those obtained from free-flying bats that we watched while recording. We recorded *Saccopteryx leptura* in Brazil, *Pteronotus davyi* in Mexico, a *Molossus* sp. in Brazil and an unknown emballonurid in Brazil. Identification of *S. leptura* was based on previous captures in the area (Bernard, 2001) and on published data about its echolocation calls (Kalko, 1995). Call features suggested the presence of *Pteronotus davyi* in Mexico (O'Farrell and Miller, 1997), while identification of the *Molossus* sp. in Brazil was based on previously reported data about Neotropical molossid echolocation calls (Kossl *et al*., 1999; O'Farrell and Miller, 1999). Our recordings also suggested the presence of an unknown emballonurid in Brazil. The sonogram parameters and shape of these calls is typical of Neotropical emballonurids (Barclay, 1983; Kalko, 1995; O'Farrell and Miller, 1997; Rydell *et al*., 2002), and on our recordings of the calls of released emballonurids.

We recorded calls by connecting the high frequency output of a Pettersson D980 (Pettersson Elektronik AB, Uppsala, Sweden) bat detector via an F 2000 Control (Pettersson Elektronik AB) filter and an Ines DAQ i508 or a National Instruments DAQ 6062E high speed card to a personal computer running BatSoundPro 3.31 (2001; Pettersson Elektronik AB) software. We set the sampling frequency at 250 kHz and the sample rate at 44,100 Hz (16 bits/sample, mono). The D980 was mounted on a tripod, 1.3 m above the ground and pointed upwards at an angle of 45° from the horizontal.

We reviewed 1,169 recorded minutes of calls from free-flying bats, selecting for analysis, only a fraction of these recorded minutes, those with sequences that met our criteria. Specifically, we analyzed only calls selected from sequences of 10 successive calls emitted by one bat as it flew through the airspace sampled by the microphone. We used six criteria to select call sequences for analysis: 1) high signal-to-noise ratio, i.e., the call was at least three times stronger than background noise as displayed on the time-amplitude window, 2) calls were not saturated, 3) at least 10 sequential calls from one individual based on interpulse intervals, 4) no evidence of the presence of conspecifics; 5) search phases calls as defined by Griffin *et al*. (1960), and 6) we randomly selected sequences recorded on different nights to minimize the chances of using sequences from the same individual (pseudoreplication).

We analyzed the calls using BatSoundPro. We measured call duration (DUR) as well as inter-pulse intervals (IPI) taken from the end of one call to the beginning of the next, from the time-amplitude display (see Fig. 1A). We measured frequency components from the Fast Fourier Transform (FFT) power spectrum (Obrist, 1995; Parsons and Jones, 2000; Ibáñez *at al.*, 2002; Russo and Jones, 2002; Fig. 1B — size 512, Hanning window), taking -50dB as the criterion for isolating the highest (HF) and lowest (LF) frequencies (kHz) for each call (Fenton *et al*., 2004). We measured frequency with most energy (FME) (kHz) from the peak of the power spectrum (Obrist, 1995; Parsons and Jones, 2000; Ibáñez *et al*., 2002; Russo and Jones, 2002; Fig. 1C). These basic call features (DUR, HF, LF, and FME) have been widely used in the past (Obrist, 1995; Masters *et al*., 1991; Britton *et al*., 1997; Surlykke and Moss, 2000). We also used the FFT power spectrum to identify harmonics.

We minimized the variability in calls due to environmental conditions (Kalko and Schnitzler, 1993;

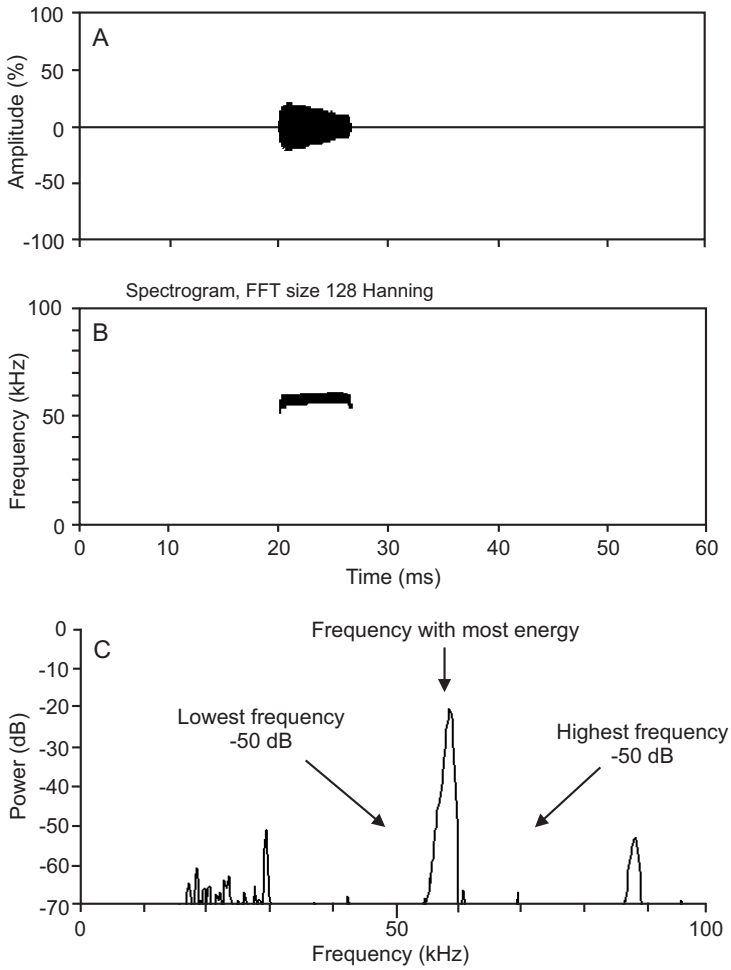

FIG. 1. Displays used for ecolochation call measurements. A) a time-amplitude, B) a spectrogram, and C) a FFT power spectrum

Kalko, 1995; Obrist, 1995) by making recordings in open spaces (bats > 10 m from nearest obstacles). In Brazil we recorded on a lake away from vegetation, in Mexico City in an 'open' city park or at Chamela, Jalisco State (Mexico) on a beach and at Lamanai in Belize from a dock or a canoe. We grouped sequences by species according to our criteria. We did not use data obtained from calls recorded from hand-released bats in the statistical analyses. We calculated descriptive statistics from 560 single calls using Statistix 7.0 for Windows, 2000 (Analytical Software, Talahassee, Florida).

We used both hierarchical and K-means clustering analysis of the call data to confirm that these taxa were readily distinguishable by call features. For hierarchical clustering we used a custom program in Matlab 6.5 for Windows, 2002 (The MathWorks, Natick, Massachusetts), for K-means clustering, SPSS 10.0 for Windows, 1999 (SPSS, Chicago, Illinois). In hierarchical clustering, we used the 'single' linkage method in the calculation and distance between the observations was calculated as the Euclidean distance. In K-means clustering, we specified eight groups.

Testing the DFA and MLR Procedures

Discriminant Function Analysis (DFA) is often used to assess the percentage of species correctly identificied, to determine which variables (DUR, HF, LF and FME) discriminate most both between and within species, and to classify species into groups or guilds. Descriptive DFA has been commonly used in analyses of bat calls (e.g., Obrist, 1995; Boughman

and Wilkinson, 1998; Jones *et al*., 2000; Parsons and Jones, 2000; Kazial *et al.,* 2001; Russo and Jones, 2002). We assessed the effects of data input and sample size on the efficacy of DFA for the identification of bat species by their echolocation calls, and also determined which variables (DUR, HF, LF and FME) discriminate most both between and within species.

We used SPSS 11.0.1 for Windows, 2001 (SPSS, Chicago, Illinois) and entered call feature data grouped by sequences. We used DUR, HF, LF, and FME as predictors, or independent variables, while 'species' served as the group membership or dependent variable. For all discriminant analyses, we calculated cross-validated classification results using the leave-one-out method to assess the generalizability of the model to calls outside our sample (Olden and Jackson, 2002). However, our objective was to study the accuracy of DFA given the information available, rather than to assess its classification of unknown calls, so we have reported original results. Prior probabilities were set to equal (all species were equally considered in the classifications) for the purposes of these exercises (Wollerman and Wiley, 2002). We also used Multinomial Logistic Regression (MLR) as an alternative to DFA (Press and Wilson, 1978; Affi and Clark, 1990) to classify individuals into groups on the basis of multiple measures.

Prior to each DFA and MLR analysis, we performed a multivariate analysis of variance (MANOVA). Again, the call feature data were entered into the analyses grouped by sequences. We used DUR, HF, LF and FME as criterion variables, and 'species' as the factor, with 8 levels, to test for significant inter-sequence differences both within and between species, with DUR, HF, LF and FME taken together. We found that the variance of our dependent variables was not equally distributed across groups but Q-Q plots and the skew of each dependent variable indicated that the variables were adequately normally distributed. Further, DFA is relatively robust to departures from normality (Dillon and Goldstein, 1984). The results of a Box's Test suggested that the covariance of our dependent variables was not equal across groups, but Lachenbruch (1975) has asserted that DFA is relatively robust even when there are violations of these assumptions. MLR is not affected by such assumption violations (Affi and Clark, 1990).

Results

Preliminary Statistical Results

CV values and visual inspection of spectrograms indicated that while some species we recorded (e.g., the unknown emballonurid; Table 1; Fig. 2) consistently produced similar echolocation calls, the calls of others were more variable. Results of the cluster analysis confirmed our impression that the eight species were readily distinguishable by their calls (Table 2). In fact, there is little difference between the clustering by all four parameters or when only the two most significant (FME and LF) are used, probably reflecting strong correlations between call parameters (Table 3). Hierarchical clustering provided the best separation by groups, although the analysis reveals that some call variants complicate the situation. Using call data from three sequences for each of the eight species, a MANOVA showed significant differences in call features between all species used (Table 4).

Effects of Data Input and Sample Size on DFA and MLR

The output of the DFA corresponded to our subjective grouping of sequences by species 93.8% of the time (Table 5). This DFA revealed that LF and FME were the most important call features, DUR the least important (Table 6). When the 24 sequences were randomly associated with species by grouping sequences with no regard to species, the DFA classification accuracy was 29.6%.

To further explore the effect of data loading on the outcomes of DFA we used a 'blind analysis' with data from the 24 sequences (3 from each 8 species) not sorted by species. Now the DFA correctly classified 74.2% of calls by sequence, with FME, LF and HF used to discriminate between sequences (Table 6). These results could have been used to support the view that we were dealing with 24 rather than eight species.

To examine the effect of sample size, we compared the results of DFA performed on

TABLE 1. Descriptive statistics of call features, where *n* = sample size (number of calls), DUR = duration in ms, FME = frequency with most energy in kHz, HF = the highest frequency in kHz, and LF = the lowest frequency in kHz. Means ± 1SD are shown, followed by CV (coefficient of variation), and min–max (the range). For *S. leptura*, 50 calls were divided into high and low frequency groups, resulting in two groups of 25 calls each. For *S. bilineata*, 100 calls were divided by geographic location; 50 from Belize and 50 from Brazil. These calls were further divided into high and low frequency groups, resulting in 25 high calls from Belize, 25 low calls from Belize, 25 high calls from Brazil, and 25 low calls from Brazil

Taxon	*n*	DUR	FME	HF	LF
Emballonurid	50	6.7 ± 1.0	58.7 ± 0.5	59.6 ± 0.6	56.6 ± 1.1
		14.3	0.9	0.9	1.9
		4.7–8.3	57.7–59.8	57.8–60.6	54.0–58.7
S. bilineata	25	9.6 ± 0.7	48.0 ± 0.4	48.6 ± 0.6	45.9 ± 0.7
High (Belize)		7.8	0.8	1.2	1.5
		7.9–11.2	47.3–48.6	46.9–49.2	44.6–47.4
S. bilineata	25	9.7 ± 0.9	45.7 ± 0.5	46.4 ± 0.5	44.0 ± 0.9
Low (Belize)		9.6	1.2	1.2	2.1
		7.9–11.4	44.8–46.6	45.5–47.3	41.8–45.7
S. bilineata	25	8.6 ± 0.9	45.9 ± 0.4	46.6 ± 0.8	43.0 ± 0.99
High (Brazil)		9.1	1	1.7	2.3
		7.3–10.4	45.2–46.8	45.9–50.0	40.6–44.6
S. bilineata	25	9.0 ± 1.3	42.7 ± 0.7	43.8 ± 0.3	39.2 ± 2.1
Low (Brazil)		13.9	1.6	0.7	5.4
		6.3–11.5	41.2–43.8	43.0–44.4	33.9–42.0
S. leptura	25	7.5 ± 0.9	53.6 ± 0.7	54.5 ± 0.8	51.4 ± 1.5
High		11.7	1.3	1.5	3
		5.3–9.0	52.4–55.0	53.3–56.1	48.4–54.1
S. leptura	25	7.5 ± 1.1	50.4 ± 0.9	51.4 ± 0.9	47.2 ± 2.7
Low		14.2	1.7	1.7	5.8
		5.5–10.0	49.0–51.9	50.0–53.9	40.1–50.3
P. davyi	30	6.7 ± 0.9	71.5 ± 4.7	79.2 ± 6.7	69.2 ± 2.3
		13.1	6.6	8.5	3.3
		4.9–8.0	68.5–84.5	63.0–85.7	64.9–79.0
T. brasiliensis	50	14.2 ± 2.0	27.3 ± 1.8	31.1 ± 3.4	26.0 ± 1.7
		14.4	6.7	10.8	6.5
		9.1–19.0	23.6–31.0	24.2–40.0	22.6–28.7
N. macrotis	50	17.4 ± 2.6	15.0 ± 2.0	19.5 ± 4.4	13.6 ± 1.9
		14.7	13.1	22.6	13.8
		10.0–24.0	11.6–19.4	12.7–29	10.7–18.0
Molossus sp.	50	11.7 ± 3.4	36.9 ± 4.0	38.6 ± 4.7	34.3 ± 4.3
		28.9	11	12.1	12.5
		5.7–18.0	28.8–46.4	31.8 –53.1	23.5–45.0
P. macrotis	180	8.6 ± 3.8	42.1 ± 1.9	43.4 ± 1.9	40.3 ± 2.4
		44.4	4.4	4.4	6.0
		2.4–22.0	37.7–46.0	40.4–47.9	31.8–45.2

data from three and from five sequences (Table 5). While the analysis with three sequences correctly classified call sequences to species in 93.8% of cases, with 5 sequences correct identifications declined to 89.5%. In all cases, the most important call features were FME and LF (Table 6). In comparing the effect of three and five sequences on classification, we found that species 100% correctly classified with data from three sequences were similarly classified with data from five sequences. However, for most other species, the incidence of correctly classified results decreased with increased samples.

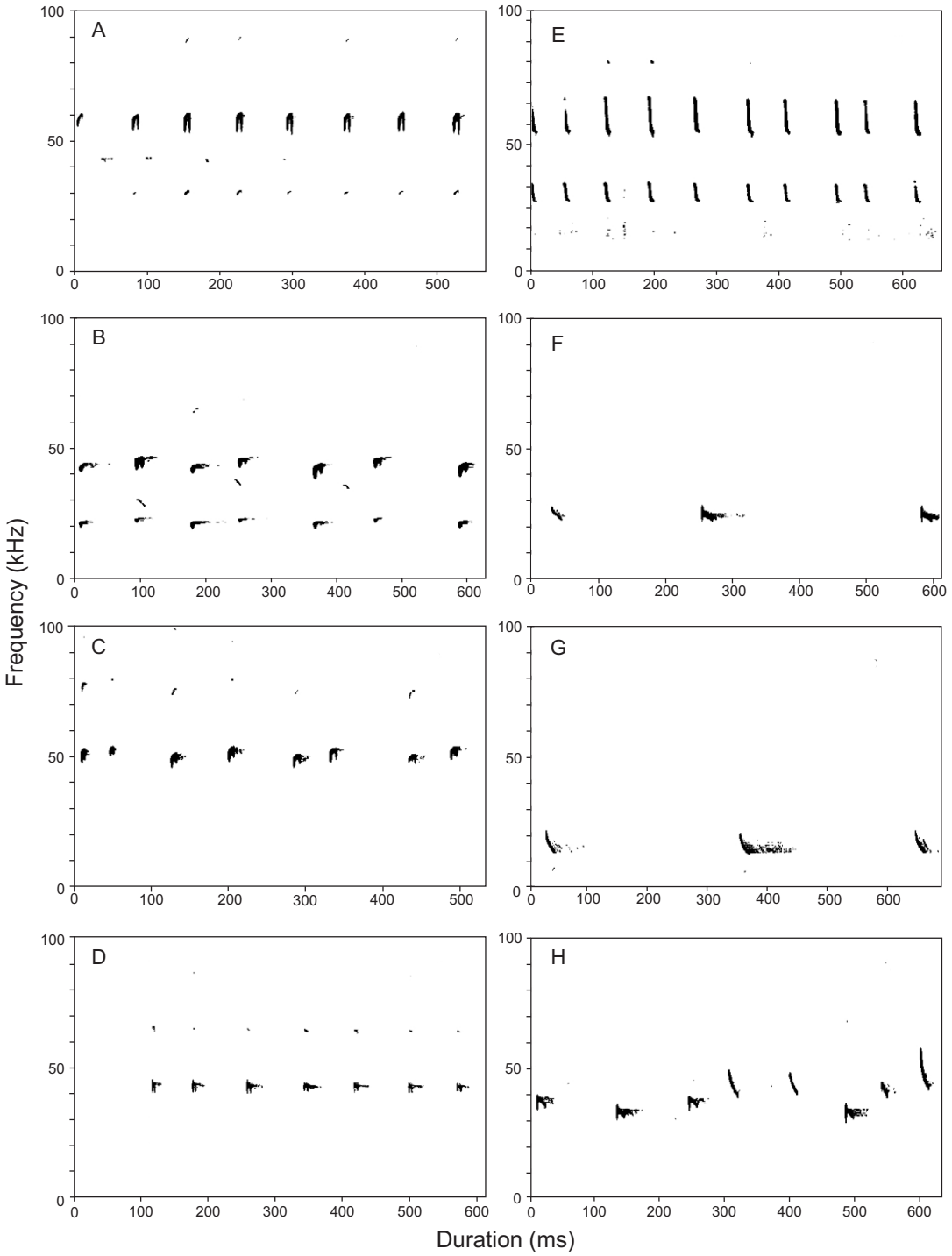

FIG. 2. Search phase patterns of frequency change over time for each species: A) emballonurid, B) *S. bilineata*, C) *S. leptura*, D) *P. macrotis*, E) *P. davyi*, F) *T. brasiliensis*, G) *N. macrotis*, H) *Molossus* sp.

TABLE 2. Cluster analysis results for all eight taxa. Each cluster done twice, once with all four call parameters (DUR, LF, HF and FME) and once with only two call parameters (LF and FME). Letters define the cluster groups. Cluster groups: f and g (*); e, f and g (**); f and g (***); and g (****) are in the last four sequences

Taxon	Hierarchical clustering		K-mean clustering	
	All four parameters	LF and FME only	All four parameters	LF and FME only
Emballonurid	e	a	a	c
S. leptura	a	b	a	c, h
S. bilineata	b	b	d	h
P. davyi	h*	h**	b***, f	a****
T. brasiliensis	c	c	h	b
N. macrotis	d	d	c	d
Molossus sp.	b	b	d, e, h	e, h
P. macrotis	b	b	d, e	h

However, correct classification of a species by DFA does not necessarily mean low variability in echolocation calls. A species such as *N. macrotis* with very variable calls (Table 1) was 100% correctly classified by DFA using data from three and from five sequences (Table 5). *Nycteris macrotis* had the lowest frequency values (HF, LF and FME) in our sample (Table 1) contributing to its consistent classification by DFA. Correct classification of species by DFA could merely indicate distinct species means.

Multinomial logistic regression (MLR) analyses with data from 3 and 5 sequences provided the same patterns of results as DFA. With three sequences per species, we obtained 96.7% correctly classified cases, while with five sequences per species, the MLR showed 91.8% correct (Table 7). Further, with a blind MLR analysis, we obtained 91.7% correctly classified cases, again suggesting separation by sequence rather than by species. With all the parameters taken into account, the error associated with each MLR model was significantly reduced (for 3 sequences, $\chi^2 = 1420.9$, *d.f.* = 28, $P < 0.001$; for 5 sequences, $\chi^2 = 973.1$, *d.f.* = 28, $P < 0.001$; for the blind analysis $\chi^2 = 1454.6$, *d.f.* = 92, $P < 0.001$) indicating that the model for each analysis was appropriate.

Effects of Call Variability on DFA and MLR Results

Four examples illustrate levels of intraspecific variation we encountered in echolocation calls and how they emerged from MANOVA and DFA analyses.

First, descriptive statistics (Table 1) and MANOVA (Table 4) indicated that echolocation calls of *S. bilineata* differed significantly between sites in Belize and Brazil. Using data from five sequences of 10 calls each from Belize and Brazil, a DFA showed that 92% of the two groups of calls of *S. bilineata* were correctly classified by seven geographic locations and that the most important parameters distinguishing the groups were LF and FME (Table 6).

Second, durations of echolocation calls recorded from *P. macrotis* varied with time of night (Fig. 3). Calls recorded early in the evening were significantly shorter than those recorded later (5.67 ± 1.87 ms versus 11.54 ± 2.93 ms, respectively), t (151.4) =

TABLE 3. Non-parametric correlation (Spearman's r) for all four call parameters. Two-tailed test used. All correlations significant at $P < 0.001$

Parameter	LF	HF	FME
DUR	-0.791	-0.808	-0.801
LF		0.984	0.989
HF			0.994

TABLE 4. MANOVA results, computed using $\alpha = 0.0125$. In all cases $P < 0.001$ and observed power = 1.00

Analyzed variant	Wilks' λ	*F*	Hypothesis *d.f.*	Error *d.f.*	Partial ε
8 species 3 sequences	0.005	96.9	28	827.1	0.73
24 sequences (blind analysis)	0.000	53.9	92	845.6	0.85
8 species 5 sequences	0.009	127.6	28	1331.9	0.69
S. bilineata (Brazil and Belize)	0.427	31.9	4	95.0	0.57
P. macrotis (dusk and late)	0.341	84.1	4	174.0	0.66

-16.06, $P < 0.001$. A DFA using data from 18 sequences revealed that 95.5% of the two temporally distinct groups of calls from *P. macrotis* were correctly classified, and that DUR was the most important feature (Table 6).

Third, we used the echolocation calls of *S. leptura* to assess the impact of variation in call frequencies on DFA-based identifications because in any sequence of search phase calls, this species alternates calls dominated by higher and lower frequencies. We grouped data from five sequences of echolocation calls of *S. leptura* as if the alternating calls represented two different hypothetical sequences consisting of either high or low frequency calls (Table 1). Had we not been aware of the bats' behavior during recording and not known of call alternation from the literature, we could have presumed that the call sequences represented two different species. A DFA classification indicated that 100% of the manipulated sequences were correctly classified as high or low frequency based primarily on FME.

Fourth, calls of a *Molossus* species were particularly variable in call parameters and patterns of frequency change over time. This was especially evident from the calls produced as a bat approached and attacked a flying insect (Fig. 4A). We also observed that search phase calls varied both in shape and in frequency parameters (Figs. 2H and 4B; Table 1). Analysis of call data by DFA and MLR using three and five sequences showed that this was the species most often misclassified of any in our samples (Tables 5 and 7).

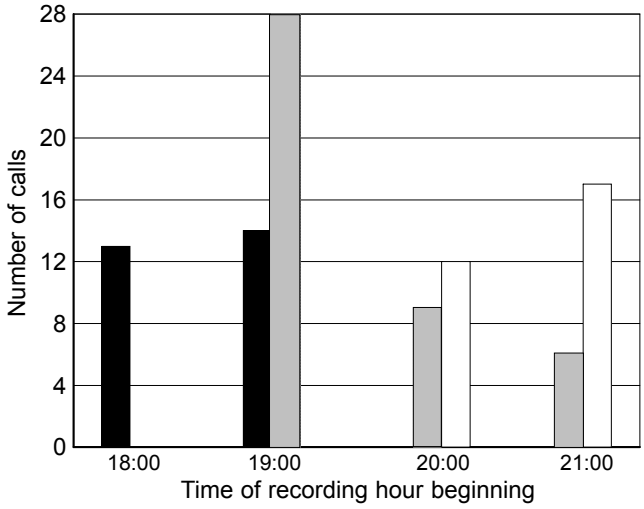

FIG. 3. Frequency distribution of *P. macrotis* echolocation calls of different durations recorded for the 60 min period beginning at 18:00 h, 19:00 h, 20:00 h, and 21:00 h. Calls ≤ 5 ms long are shown by solid black bars, 6–10 ms long calls as gray bars, and calls ≥ 11 ms as open bars

TABLE 5. Discriminant function analysis classification data showing (top) data for 8 species, 3 sequences of 10 calls each (93.8% of original grouped cases and 92.1% of cross-validated grouped cases correctly classified) and (bottom) 8 species, 5 sequences of 10 calls (but not for *P. davyi* where three sequences of 10 calls were used) (89.5% of original grouped cases correctly classified, 88.9% of cross-validated grouped cases correctly classified). Rows are the observed categories of the dependent (species) and columns are the predicted categories of the dependent. The percentage of cases on the diagonal is the percentage of correct classifications

Taxon	Emballonurid	*S. leptura*	*S. bilineata*	*P. davyi*	*T. brasiliensis*	*N. macrotis*	*Molossus* sp.	*P. macrotis*
				3 sequences of 10 calls each				
Emballonurid	100.0	.0	.0	.0	.0	.0	.0	.0
Saccopteryx leptura	6.7	90.0	3.3	.0	.0	.0	.0	.0
S. bilineata	.0	.0	90.0	.0	.0	.0	.0	10.0
Pteronotus davyi	3.3	.0	.0	96.7	.0	.0	.0	.0
Tadarida brasiliensis	.0	.0	.0	.0	100.0	.0	.0	.0
Nyctinomops macrotis	.0	.0	.0	.0	.0	100.0	.0	.0
Molossus sp.	.0	.0	3.3	.0	.0	.0	80.0	16.7
Peropteryx macrotis	.0	.0	6.7	.0	.0	.0	.0	93.3
				5 sequences of 10 calls each				
Emballonurid	100.0	.0	.0	.0	.0	.0	.0	.0
S. leptura	4.0	92.0	4	.0	.0	.0	.0	.0
S. bilineata	.0	.0	86.0	.0	.0	.0	.0	14.0
P. davyi	6.7	.0	.0	93.3	.0	.0	.0	.0
T. brasiliensis	.0	.0	.0	.0	100.0	.0	.0	.0
N. macrotis	.0	.0	.0	.0	.0	100.0	.0	.0
Molossus sp.	.0	.0	8.0	.0	4.0	.0	64.0	24.0
P. macrotis	.0	.0	2.0	.0	.0	.0	16.0	82.0

TABLE 6. Results of discriminant function analysis using parameters distiniguishing: the 8 species (3 sequences); 24 sequences (blind analysis); 8 species (5 sequences); *S. bilineata* from Belize and from Brazil; and *P. macrotis* at dusk and late at night

Parameter	Wilks' λ	*F*	*d.f.* 1	*d.f.* 2	*P*-level
8 species (3 sequences)					
Duration	0.356	60.0	7	232	<0.001
Low frequency	0.015	2133.1	7	232	<0.001
High freqeuncy	0.031	1019.2	7	232	<0.001
Frequency with max energy	0.018	1791.3	7	232	<0.001
24 sequences (blind analysis)					
Duration	0.072	120.6	23	216	<0.001
Low frequency	0.013	711.6	23	216	<0.001
High freqeuncy	0.022	412.4	23	216	<0.001
Frequency with max energy	0.013	736.6	23	216	<0.001
8 species (5 sequences)					
Duration	0.380	86.9	7	372	<0.001
Low frequency	0.026	1978.6	7	372	<0.001
High freqeuncy	0.040	1286.7	7	372	<0.001
Frequency with max energy	0.021	2464.4	7	372	<0.001
S. bilineata from Belize vs. *S. bilineata* from Brazil					
Duration	0.836	19.2	1	98	<0.001
Low frequency	0.505	96.2	1	98	<0.001
High freqeuncy	0.604	64.2	1	98	<0.001
Frequency with max energy	0.574	72.7	1	98	<0.001
P. macrotis at dusk versus late at night					
Duration	0.411	254.1	1	177	<0.001
Low frequency	0.999	0.2	1	177	0.629
High freqeuncy	0.660	91.4	1	177	<0.001
Frequency with max energy	0.752	58.3	1	177	<0.001

DISCUSSION

In summary, we used modest sample sizes of echolocation calls of eight species of high intensity, echolocating bats readily acoustically distinguishable from one another by human observers. Using four simple call features, we illustrated how DFA and MLR can be affected by the way in which data are presented to either analysis, by sample sizes, and by call variability. 'Blind' loading of echolocation call data can generate the impression of greater than actual diversity. Thus blind loading could occur intentionally (as in our case) or in the absence of detailed knowledge about variation in echolocation calls.

We agree with many researchers that it is possible to use features of echolocation calls to identify species of high intensity echolocating bats present in an area (Fenton and Bell, 1981; Barclay, 1983; Ahlén, 1990; O'Farrell and Miller, 1999; Jones *et al*., 2000; Rydell *et al*., 2002). Cluster analyses, DFA and MLR can confirm subjective arrangements of species by the features of their echolocation calls. However, our analyses also show weaknesses inherent in DFA and MLR, statistical analyses commonly used to objectify and normalize subjective echolocation call analysis (or the analysis of other calls — e.g., Boughman and Wilkinson, 1998; Wollerman and Wiley, 2002).

Statistical Concerns

Analyses using MANOVA can be affected by sample size, equal variances, and

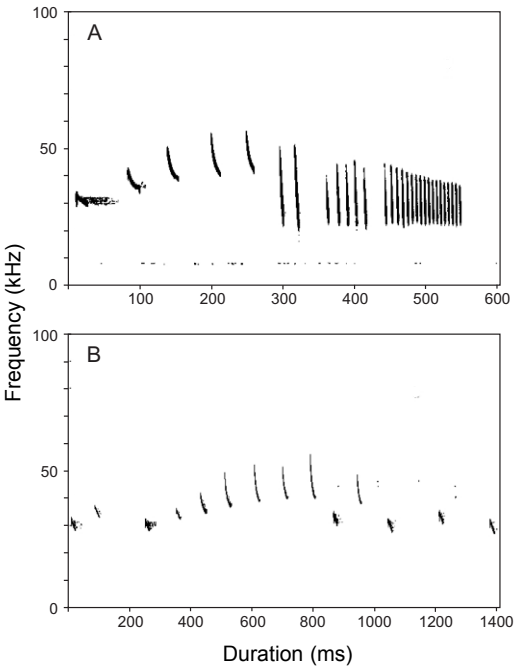

FIG. 4. Calls of *Molossus* sp.: A) feeding buzz, B) search phase

normality. Timm (2002) indicated the importance of not only determining that variables contain data that are normally distributed from the perspective of univariate statistics (e.g., by using procedures such as the Shapiro-Wilks' tests) but also that these variables exhibit multivariate normality. This exercise can be done using tests such as Mardia's test of multivariate skewness, multivariate chi-square, and Q-Q plots. As is the case in ANOVA, equal variances are assumed and violation of this assumption by unchecked data reduces the reliability of the results. These problems may be overcome by transforming the data (Tabachnick and Fidell, 2001). Most important, and most often overlooked, is the effect of small sample size and correlation of variables on power and significance. The assumptions of MANOVA stipulate that users must have more cases in every cell than there are dependent variables. Further, homogeneity of variance is also assumed for MANOVA and, if this assumption is violated, covariance matrices become untestable and overeall results unreliable (Tabachnick and Fidell, 2001). Furthermore, power is negatively affected by small sample sizes which can generate Type II errors (i.e., accepting the null hypothesis when it is in fact false). Finally, the power of MANOVA is negatively affected by positively correlated dependent variables, when these correlations are zero, and even when they are only slightly negative (Tabachnick and Fidell, 2001). This last point is especially important given the relationships between call duration, bandwidth, and frequency of maximum energy. Such relationships commonly observed within different call designs of the same or congeneric species are studied. They also emerge when distantly related species converge on similar echolocation call designs reflecting similar habitats/foraging tactics (for review see Schnitzler and Kalko, 2001).

It is clear that both sample size and the way that data are loaded into the statistical programme can affect the outcome of DFA. A further complication arises from the interpretation of a DFA output. First, acceptable levels of overall percent correct classifications at the species level range from 64–96% in the literature (e.g., Obrist, 1995; Pearl and Fenton, 1996; Vaughan *et al.,* 1997; Parsons and Jones, 2000; Russo and Jones, 2002), reflecting a variety of species and call variables. There appears to be no 'rule' about what is an 'acceptable' level for accuracy in DFA analyses. We showed that percentages of correct classification by DFA are readily altered by data loading and sample size. Second, DFA identifies differences between call parameters used in the model, not between species.

Effects of Call Variability

Our data on call variability reiterate those of many previously published studies

TABLE 7. Results of a multinomial logistic regression classification analysis showing the data for (top) 8 species, 3 sequences of 10 calls each (overall percent correct classified cases 96.7%) and (bottom) 8 species, 5 sequences of 10 calls each, except for *P. davyi* (overall percent of correct classified cases 91.8%). Rows are the observed categories of the dependent (species) and the columns are the predicted categories of the dependent. The percentage numbers indicated on the last column are the percentage of correct classification for each species

Taxon	Emballonurid	*S. leptura*	*S. bilineata*	*P. davyi*	*T. brasiliensis*	*N. macrotis*	*Molossus* sp.	*P. macrotis*	Percent correct
8 species, 3 sequences of 10 calls each									
Emballonurid	30.0	.0	.0	.0	.0	.0	.0	.0	100.0
S. leptura	.0	30.0	.0	.0	.0	.0	.0	.0	100.0
S. bilineata	.0	.0	30.0	.0	.0	.0	.0	.0	100.0
P. davyi	.0	.0	.0	30.0	.0	.0	.0	.0	100.0
T. brasiliensis	.0	.0	.0	.0	30.0	.0	.0	.0	100.0
N. macrotis	.0	.0	.0	.0	.0	30.0	.0	.0	100.0
Molossus sp.	.0	.0	.0	.0	.0	.0	25.0	5.0	83.3
P. macrotis	.0	.0	.0	.0	.0	.0	1.0	29.0	96.7
8 species, 5 sequences of 10 calls each									
Emballonurid	50.0	.0	.0	.0	.0	.0	.0	.0	100.0
S. leptura	.0	50.0	.0	.0	.0	.0	.0	.0	100.0
S. bilineata	.0	.0	44.0	.0	.0	.0	.0	6.0	88.0
P. davyi	.0	.0	.0	30.0	.0	.0	.0	.0	100.0
T. brasiliensis	.0	.0	.0	.0	50.0	.0	.0	.0	100.0
N. macrotis	.0	.0	.0	.0	.0	50.0	.0	.0	100.0
Molossus sp.	.0	.0	2.0	.0	.0	.0	36.0	12.0	72.0
P. macrotis	.0	.0	1.0	.0	.0	.0	10.0	39.0	78.0

and raise important questions about the value of acoustic libraries of echolocation calls (e.g., O'Farrell and Miller, 1997; Rydell *et al*., 2002) in the absence of careful documentation of variability. Like others, we agree that issues associated with variability erode the accuracy of identifications based on single calls (Parsons and Jones, 2000; Russo and Jones, 2002). Furthermore, in examining the effect of sample size on the outcomes of DFA and MLR analyses, we found that increasing the number of call sequences decreased correct classification. This was most probably due to a higher total variance inherent in increased sample sizes.

Geographic variation in echolocation calls is well known (e.g., Thomas *et al*., 1987; Brigham *et al*., 1989; Barclay, 1999) and our example extends the data base to *S. bilineata* from Brazil and Belize. Geographic variation can complicate identification of species by calls, making it vital to know where calls were recorded. Furthermore echolocation calls of *S. leptura* illustrate how an unknown species producing variable patterns of echolocation calls could be mistaken for more than one species when recorded but not observed during recording.

We were surprised to learn that time of night could influence bat echolocation calls, but this clearly is indicated by our data on *P. macrotis.* We can offer no concrete explanation of our observations which do put a different perspective on the issue of call duration as a variable character (Parsons and Jones, 2002). However, we speculate that these differences may reflect task (e.g., transit flights versus foraging) or insect abundance.

Others have documented variability in the echolocation calls of *Molossus* spp., whether in the larger *M. ater* (O'Farrell and Miller, 1999), or the smaller *M. molossus* (Kossl *et al*., 1999). Like them, we found obvious variations in call shapes and in actual values for LF and HF. Such spectacular within-species variability reaffirms the importance of a priori documentation of the bat species present in the study area and of observing bats whose calls are being recorded.

The inherent variability of echolocation calls is the main problem in distinguishing bat species by their calls. For example, Obrist's (1995) analysis showed that only a species using much lower frequency calls (*Euderma maculatum*) was consistently distinguished from species using calls of progressively higher frequencies (*Lasiurus cinereus, Eptesicus fuscus, Lasiurus borealis*) even though most calls were readily assigned to species.

Statistical analyses such as DFA are powerful instruments to quantify data on echolocation calls but, by themselves, are not objective instruments because their outcomes can confirm subjective classifications and reflect the way data are loaded for analysis. We recognize the value of using echolocation calls to identify the species of high intensity echolocating bats, especially those difficult to sample by capture. However, in areas with many sympatric species the task of species recognition by echolocation calls can be challenging. When there are many species, statistical procedures could lead to ambiguous classifications of some species by their calls.

Researchers in many parts of the world use a variety of bat detectors to monitor what bats say. The results of this work have important implications for conservation as well as for basic science. Effective identification of echolocating bats by their calls hinges on knowing what call features are important. Unlike some other procedures, DFA identifies the call parameters most important in discriminating between signals, facilitating quantifying data and sharing them among researchers.

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