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ECHOLOCATION CALL FREQUENCY DIFFERENCES BETWEEN GEOGRAPHIC ISOLATES OF *RHINONICTERIS AURANTIA* (CHIROPTERA: HIPPOSIDERIDAE): IMPLICATIONS OF NASAL CHAMBER SIZE

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Most previous studies considering intraspecific differences in bat echolocation call frequency among geographic groups have related this to morphological features not directly involved in producing the characteristics of the emitted signal. We related a pattern of intraspecific call differences to the size of nasal chambers (estimated from radiographs of museum specimens), expecting chamber dimensions to be functionally coupled with the sound source. Such a relationship is potentially informative in the context of competing hypotheses that account for call frequency differences. Allopatry has been a precursor to differences in echolocation call frequency between isolated populations of the Australian endemic orange leaf-nosed bat (*Rhinonicteris aurantia* (Gray, 1845); Pilbara isolate: 120.99 ± 1.91 kHz; compared with Kimberley region: 114.65 ± 1.98 kHz and Northern Territory: 114.62 ± 2.10 kHz). Correlations with morphological features not directly involved in signal production or modification were either moderate (nose-leaf width) or absent (forearm length). Overall nasal volume was shown to be relatively smaller in the Pilbara population, which had higher average call frequency. This relationship was expected given the suggested function of nasal chambers in impedance matching. The finding is significant because nasal chamber size was the only character observed to vary in a species that was otherwise conserved morphologically, suggesting adaptation and not a simple scaling relationship with body size that might be more indicative of drift. We consider that the combination of patterns from echolocation call frequency and associated morphological features, as well as neutral DNA markers, provide adequate support for recognition of the separate populations of *R. aurantia* for conservation, according to more recent concepts that consider ecological as well as genetic characters when allocating groups to evolutionarily significant units.

Key words: allopatry, ecologically adaptive trait, geographic groups, nasal chamber, Pilbara, resting frequency

Resting or search-phase echolocation calls are being used increasingly in conjunction with morphometric and molecular data sets in studies of bat taxonomy and speciation (e.g., Barratt et al. 1997; Jacobs et al. 2006; Kingston et al. 2001; Kingston and Rossiter 2004; Miller-Butterworth et al. 2005). Differences in characteristic call frequency have been noted among geographically isolated intraspecific groups or sister species of constant frequency (CF)-emitting bat (examples and reviews in Taniguchi 1985; Coles 1993; Francis and Habersetzer 1998; Guillén et al. 2000; Heller and von Helversen 1989; Jones et al. 1994). However, we still do not understand whether such differences that develop in allopatry are the result of adaptation to contrasting ecological factors, sexual selection, or simple drift (Guillén et al. 2000; Jones and Barlow 2004), nor do we understand the importance of the magnitude of any differences. In situations where there are clear call-frequency differences and concomitant reciprocal monophyly in molecular markers, taxonomic resolution might be straightforward despite morphological crypsis (e.g., Jacobs et al. 2006). In cases where species or subspecific groups might have only recently diverged, taxonomic resolution might be less straightforward, and the inclusion of an acoustic data set in combination with others might be informative. Furthermore, it might be relevant to consider geographic isolates separately in conservation categories. Recently, ecologically adaptive traits have been promoted for use in defining evolutionarily significant units for

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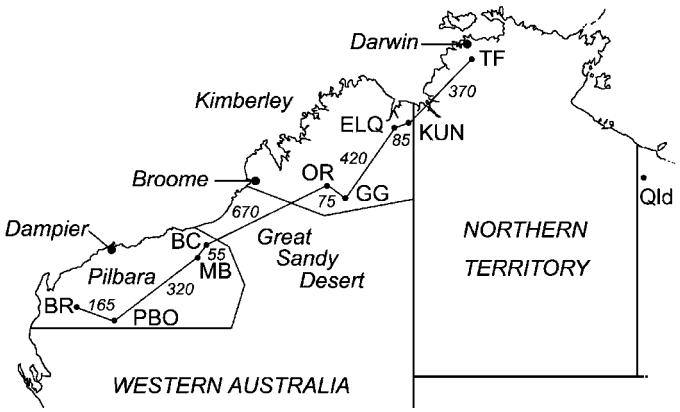

Fig. 1.—Sampling locations in northern Australia. Approximate distances between locations are given (km). Locations are: BR: Barlee Range (western Pilbara group); PBO: Paraburdoo (central Pilbara group); MB: Marble Bar; BC: Bamboo Creek (both of these eastern Pilbara group); OR: Oscar Range; GG: Geike Gorge; ELQ: El Questro Station; KUN: Kununurra; TF: Tolmer Falls in Litchfield National Park, Northern Territory. Source of the Queensland morphological specimens is indicated (Qld).

conservation efforts below the species level, rather than relying solely on criteria derived from neutral genetic markers (Crandall et al. 2000; Fraser and Bernatchez 2001). Thus, determining the reasons for call variation among geographic groups is important for understanding the process of speciation in bats, for determining the utility of any differences among groups in decisions of taxonomy, and will be useful for defining evolutionarily significant units for conservation.

When considering call-frequency variation within and among species, many studies have 1st attempted to find some relationship with morphological features. It is well recognized that in interspecific comparisons, call frequency correlates negatively with body size, and the size of various other morphological features. Interspecific comparisons of resting frequency within the genera *Hipposideros* and *Rhinolophus* demonstrate a negative correlation between call frequency and morphological features such as overall body size (as represented by forearm length—Francis and Habersetzer 1998; Heller and von Helversen 1989; Jones 1995, 1996), nose-leaf width (Robinson 1996), pinna size (Guppy and Coles 1988; Zhao et al. 2003), and cochlear size (Francis and Habersetzer 1998). However, although such features correlate to varying degrees with frequency, it is the dimensions of the vocal tract that provide a functional explanation for the emitted frequency (Hartley and Suthers 1988, 1990; Suthers et al. 1988). This is exemplified by the lack of a general relationship between external morphometrics and call frequency within CF-emitting bat species. For some species, call frequency varies with body size in the same way as the among-species comparisons, and also with factors such as sex and age to which body size is related. In other species, there is no correlation or else the correlation with sex or age is the reverse, indicating that body size does not strongly constrain CF in the Hipposideridae and Rhinolophidae (Guillén et al. 2000—see examples in Coles [1993]; Huffman and Henson [1991]; Jones et al. [1992, 1993, 1994]; Jones and Ransome [1993]; Long and Schnitzler [1975]; and Suga et al. [1987]; and examples and reviews in Siemers et al. [2005]).

If call frequency varies among geographic groups as a result of drift, we would expect that most morphological characters would vary consistently in their correlation with call frequency according to a simple scaling relationship with body size. For example, if one population has a relatively smaller body size and correspondingly higher emitted frequency, we might expect that the morphological components of the echolocation system were also of relatively small size. However, if a particular average call frequency is somehow ecologically adaptive or socially informative, then the dimensions of the vocal tract components should be related to the emitted frequency, and relatively independent of other characters related to body size. In our study, we considered the Australian endemic orange leaf-nosed bat (*Rhinonicteris aurantia* (Gray, 1845); Hipposideridae), which has been shown to be morphologically conserved across northern Australia, with the exception of subtle differences in the size of the rostrum where the nasal chambers are located (Armstrong 2002, 2005). *R. aurantia* occupies a relatively continuous range across the Kimberley region of Western Australia and the Top End of the Northern Territory. An isolated population also occurs in the Pilbara region of Western Australia, separated from the Kimberley region by the 500 km expanse of the Great Sandy Desert (Fig. 1). Preliminary observations of *R. aurantia* in the Pilbara region suggested that the CF component was comparatively high (N. L. McKenzie, pers. comm.), and a recent phylogeographic study indicated possible genetic disjunctions (Armstrong 2006).

Nasal chambers are a suitable morphological correlate with call frequency for several reasons: they are functionally coupled to the sound source and hence their size plays a direct role in the character of the signal emission; they are the only part of the vocal tract that can be recognized in the skull (the remainder being soft tissue not available to a study of museum cranial specimens); and they have been virtually ignored since the study of Hartley and Suthers (1988), which suggested a role for them and highlighted the importance of the shape and dimensions of the nasopharynx in signal emission. Most studies since then have focused upon the correlations between call frequency and indirectly related external morphological features. Thus, the aims of our study were to determine the degree of call frequency difference, and confirm patterns of rostral size difference (cf. Armstrong 2002), between isolates of *R. aurantia*; determine whether there was evidence that the size of sound-producing components could be decoupled from the remainder of the body; and comment on how such patterns should be considered in decisions of taxonomy and conservation. We expected that our data would illustrate that call differences could occur for reasons other than a simple scaling with body size, rather than resolving the mechanism or reason for echolocation frequency differences.

Materials and Methods

Call collection and processing.—Calls of bats were recorded at 4 locations in the Pilbara region (values are sample sizes;

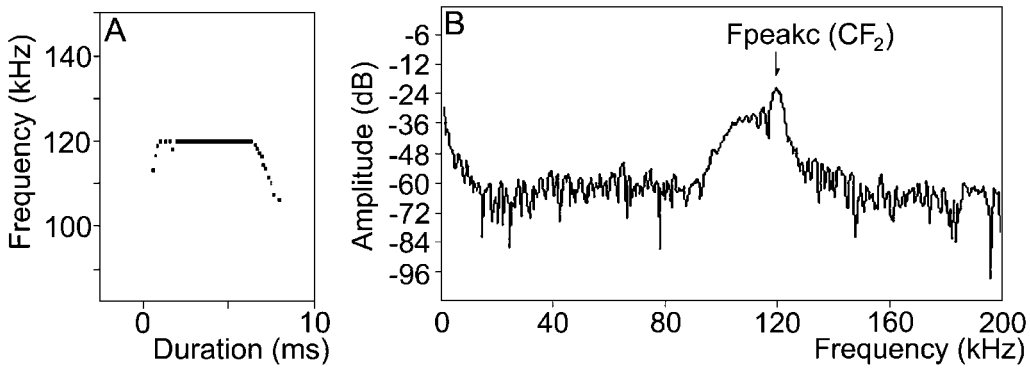

FIG. 2.—A) Zero crossings analysis of a call from handheld *Rhinonicteris aurantia*. B) Frequency analysis spectrum of a resting-frequency call showing the frequency at which there was a peak number of cycles (F_{peakc}) at approximately 120 kHz. The frequency-modulated (FM) component also is recognizable at lower amplitude approximately 20 kHz below the constant-frequency (CF_2) peak. Background noise makes up the remainder. Fast Fourier transformation size is 2,048, Blackmann–Harris window function; harmonic information was not retrieved by the U30 detector.

Barlee Range [western Pilbara]—55, Paraburdoo [central Pilbara]—2, Marble Bar [eastern Pilbara]—5 and Bamboo Creek [also eastern Pilbara]—11), 4 locations in the Kimberley region (Oscar Range near Tunnel Creek—30, Geike Gorge—23, these raw data are from Coles and Guppy [1989]; plus El Questro Station—2 and Kununurra—1; the latter 2 eastern Kimberley), and 1 location in the Northern Territory (Tolmer Falls, Litchfield National Park—34; Fig. 1). Our capture and handling methods were consistent with those recommended in the guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998). The majority of recordings were made in July. Recordings were made from handheld individuals because, when stationary, CF–frequency-modulated (FM) bats emit calls with a characteristic resting frequency where the CF component is extremely stable, and interpulse variation is small (e.g., Suga et al. 1987). This resting frequency is individually distinctive and almost coincides with the acoustic fovea (Schnitzler et al. 1976). This minimizes measurement errors due to variations in the flight speed and direction of the bat relative to the microphone (especially when compensating for Doppler-shifted echoes—Coles 1993; Coles and Guppy 1989), facilitating comparison of the CF components. Bats emitted calls spontaneously when extracted from a holding bag. Care was taken to ensure that the microphone was always directly in front of the nose leaf at a distance of about 15 cm. Ideally, recordings of individuals are made immediately after capture to avoid the effects of handling time and changes in body temperature (Huffman and Henson 1991); however, this was not possible. Time between capture and recording (maximum of 6 h) was therefore considered as a factor in statistical analyses. Nose-leaf width and forearm length were measured with Mitutoyo dial calipers (Tokyo, Japan; accuracy of calipers $\pm$ 0.02 mm).

Calls were recorded with either a S25 or a U30 bat detector (Ultra Sound Advice, London, United Kingdom) onto a ferrous metal analog audiotape using a Sony Professional Walkman WM-D6C (Tokyo, Japan). Two individuals from Marble Bar were recorded with an Anabat II bat detector (Titley Electronics Pty. Ltd., Ballina, New South Wales, Australia). Frequency division ratios were set at 20 for the U30 and S25 detectors and 16 for the Anabat II detector. For the S25 and U30 detectors, a calibrated pure tone of 25 kHz from a Tandy ultrasonic dog-trainer device Y-2310 (Sydney, Australia) was recorded onto the tape with the bat calls. Calibration tones of 40 kHz were recorded with the call of each individual when using the Anabat detector. Measurements of call frequency were made in Cool Edit 2000 (Syntrillium, now Adobe Audition, http://www.adobe.com) and UltraByte (Kriscomp Pty. Ltd., Australia, not commercially available) software (including the signals recorded with the Anabat detector). Audio signals were sampled at a rate of 44.1 kHz with a 16-bit (mono) conversion. A single variable (the frequency at which there was a peak number of cycles [F_{peakc}], which corresponds to the frequency at which amplitude or power is greatest; Fig. 2) was measured from pulses with sufficient amplitude and a distinct, narrow peak. Emissions from lethargic bats were excluded (resulting outlier values are stated but not used in analyses). Likewise, we observed that measurements of pulse duration and resting-frequency intensity from handheld bats were not consistent and therefore not used in our analysis. F_{peakc} values were determined by a frequency analysis in the power spectrum window of Cool Edit 2000. A Blackmann–Harris window function with a fast Fourier transformation size of 2,048 points gave adequate resolution (21.5 Hz) of F_{peakc}. Statistical analyses are described in the ''Results'' because the results from 1 analysis determined the nature of the next.

Skull radiographs and measurements.—The rarity of this species precluded comparisons of echolocation and skull morphology in the same individuals. Thus, the same museum specimens measured in Armstrong (2002) were used: Pilbara region of Western Australia—10, Kimberley region of Western Australia—22, Northern Territory—26, and Queensland—5 (most specimens collected within approximately the last 50 years). Details including museum accession numbers, locations, sex, and ages are listed in the appendix of that paper. Armstrong (2002) commented that relatively coarse measurements taken with calipers could have been a reason for the very subtle differences detected in several measurements of the rostrum. We therefore chose an approach that could simultaneously achieve greater accuracy, could be used in cranial

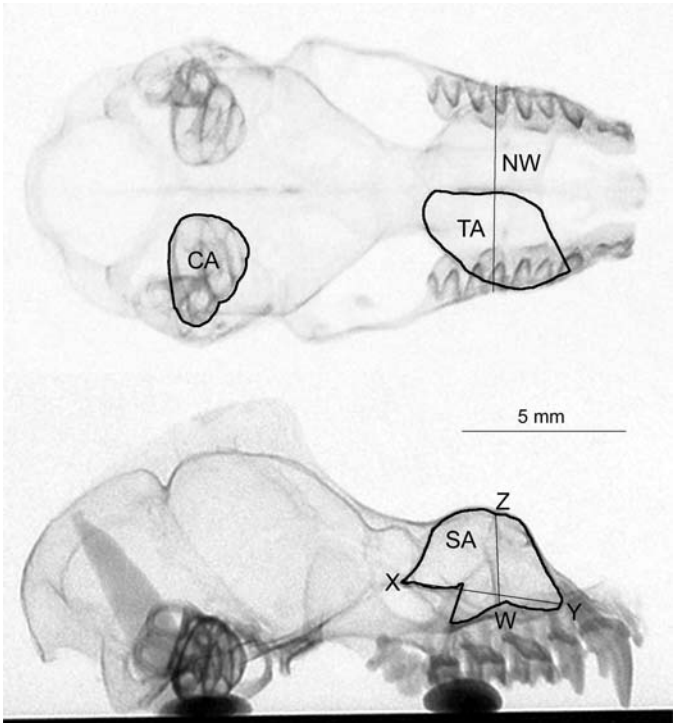

FIG. 3.—Measurements made from skull radiographs (see ''Materials and Methods'' for explanation of abbreviations; transverse and sagittal views respectively; distance X–Y represents the measure nasal capsule length (CL); Z–W represents nasal capsule height (CH).

specimens, and focused on morphology directly involved in echolocation. X-ray radiographs were taken from above (transverse) and from the side (sagittal) with a Stanford 90-30 X-ray machine at 185 volts, 55 kilovolt peak for 36 s. Radiographs were retaken if the skull appeared to be viewed from an oblique angle. A scale was included with the radiographs for later calibration of measurements. Sagittal views were obtained by fixing skulls with plasticine to a small length of angled aluminum. Radiographs were developed by hand, and then scanned into a computer using a scanning resolution of 1,200 dpi at a scale of 1:1. Measurements of various lengths and areas were made using ImageJ freeware (Abramoff et al. 2004; Rasband 1997–2005). Six variables were measured: transverse area of nasal capsule (TA), cochlear area (CA), nasal width across rostrum (NW), sagittal area of nasal capsule (SA), nasal capsule length (CL), and nasal capsule height (CH; Fig. 3). The 3 linear measurements defined the size of the nasal capsule in 3 dimensions and were equivalent but not homologous to the 3 significant measures of rostrum size in Armstrong (2002): condylocanine length, nasal breadth, and rostral height. Percent measurement error (the variability of repeated measurements of a particular character taken on the same individual, relative to its variability among individuals in a particular group) was assessed by taking 3 measurements of each variable (Bailey and Byrnes 1990). This allowed us to assess the utility of each variable, which was important because some X-ray images were less clear than others because of differences in bone density or other factors.

Statistical analyses were undertaken using SPSS version 11.5 (SPSS Inc. 2002) and were similar to those in Armstrong (2002). Plots of standardized residuals were checked for outliers and homoscedasticity. Levene's *F*-tests also were used to check for variance homoscedasticity. No transformations were required. Missing values resulting from specimen damage were replaced by the population means to maintain adequate overall sample size in multivariate analyses. These included 3 values of CA from the Kimberley region and 2 from the Queensland region, and 1 value each of TA and NW from the Kimberley region. Sexual dimorphism and age effects were examined using multivariate analysis of variance (MANOVA) with location, sex, and age as independent variables. The MANOVA was performed separately to examine the 3-way and 2-way interactions (type III sums of squares). No significant interactions were detected and thus all variables were used in a 3rd MANOVA examining the main effects only (type II sums of squares). For variables with significant terms for sex or age, individual sample values were corrected using the regression coefficients (parameter estimate, B) for that variable. This general linear model was then re-run to check the corrections and determine the variables with significant terms for the factor location. Finally, a stepwise discriminant function analysis was performed (using Mahalanobis distance to enter and remove variables) to examine the effect of location only, which included variables with significant location terms.

RESULTS

Call description.—The pulse structure of *R. aurantia* was characterized as FM-CF-FM, with a very brief upward FM sweep (not always detectable in either zero crossings analysis or analysis using Cool Edit), a CF component of about 5- to 8-ms duration (data not shown), and a steep terminating FM sweep of about 20 kHz (Fig. 2). Harmonic components could not be examined because these are not recorded by frequency division or heterodyne detectors (Sales and Pye 1974).

Geographic variation in echolocation frequency.—Variation in F_{peakc} was examined within individuals, within populations, and among populations. The variation due to morphological covariates also was examined. Before analyses, 2 individuals from Barlee Range and 3 individuals from Tolmer Falls were removed as outliers because of the long interval between their capture and recording, their lethargic behavior while recording, and the low quality of their output.

First, a repeated-measures analysis of variance was performed on data from the Pilbara region and Northern Territory to compare the amount of variation in F_{peakc} within individuals between populations. Averages from 5 clean sequences (calculated from 2–7 pulses per sequence) were included from each individual. A Greenhouse–Geisser approximation was used to compensate for violating assumptions of sphericity. There was no significant variation within individuals in either population ($F = 1.70$, $d.f. = 4, 400$, $P = 0.16$), indicating that F_{peakc} emitted by handheld bats was relatively stable. The standard deviation within each bat averaged 0.30 kHz (range 0–1.03 kHz), indicating that variation produced by the bat (or resulting

TABLE 1.—A) Population sample size (*n*) and mean ± *SD* and range in parentheses for call frequency (F_{peakc}), forearm length (FA), and nose-leaf width (NLW). B) Mean ± *SD* and range of F_{peakc} for each subpopulation.

A: Population	*n*	F_{peakc} (kHz)	FA (mm)	NLW (mm)
1 Pilbara, Western Australia	71	120.99 ± 1.91 (116.3–126.0)	46.5 ± 1.2 (43.9–49.5)	7.88 ± 0.38 (7.12–8.68)
2 Kimberley, Western Australia	56	114.64 ± 1.98 (110.4–119.0)	—	—
3 Northern Territory	31	114.62 ± 2.10 (109.9–117.8)	46.8 ± 0.85 (44.1–48.5)	8.42 ± 0.31 (7.70–8.80)

B: Population	Subpopulation or colony	*n*	$\bar{X}$ ± *SD*	Range
1	Western Pilbara (Barlee Range)[a]	53	120.86 ± 1.88	116.3–125.6
1	Central Pilbara (Hamersley Range)	2	120.12 ± 0.64	119.7–120.6
1	Eastern Pilbara (Bamboo Creek, Marble Bar)	16	121.55 ± 2.07	119.7–126.0
2	Oscar Range	30	113.40 ± 1.21	110.4–115.6
2	Geike Gorge	23	116.12 ± 1.54	111.6–117.6
2	Eastern Kimberley (El Questro, Kununurra)	3	115.80 ± 3.24	112.5–119.0
3	Litchfield National Park[b]	31	114.62 ± 2.10	109.9–117.8

[a] Outliers from Barlee Range: 114.22, 114.81 kHz. Samples sizes from the field as stated in the ''Materials and Methods'' include these individuals, but not the values of *n* above.
[b] Outliers from Litchfield National Park: 106.73, 107.08, 107.60 kHz. Samples sizes from the field as stated in the ''Materials and Methods'' include these individuals, but not the values of *n* above.

from measurement error) was generally much less than 1 kHz. A significant difference in F_{peakc} was also detected between the Pilbara and Northern Territory populations ($F = 226.05$, $d.f. = 1, 100$, $P < 0.0001$), with higher F_{peakc} in the Pilbara population ($\bar{X} = 120.99$ kHz ± 0.23 *SE*, $n = 71$) than the Northern Territory population ($\bar{X} = 114.62$ ± 0.35 kHz, $n = 31$).

Among-population variation was then examined in more detail by expanding the data set to include the Kimberley region. Only 1 measure was available from each Kimberley individual, which was the average value of several pulses from 1 stable sequence with strong signals and good signal to noise ratio. Because the amount of variation within individuals from the Pilbara region and Northern Territory was found to be comparatively small, data from the Kimberley region could be included to compare populations. An average value was calculated from the 5 repeated measures in the Pilbara and Northern Territory data sets. A univariate general linear model was performed to compare F_{peakc} between Pilbara, Kimberley, and Northern Territory. F_{peakc} was again significantly different between populations ($F = 202.7$, $d.f. = 2, 158$, $P < 0.0001$), with the Pilbara population having a higher frequency than both of the other populations (Pilbara and Northern Territory: mean ± *SE* as above; Kimberley: $\bar{X} = 114.65 \pm 0.26$ kHz, $n = 56$; Table 1).

A 2nd univariate general linear model was performed to determine if there were significant differences in CF within populations because the distance between some Pilbara and Kimberley locations is equivalent to that between southern Kimberley and Northern Territory locations (approximately 600–800 km; Fig. 1). Although the effect of isolation of the Pilbara population was evaluated in the previous analysis, this test examined the distinctiveness of calls between colonies within the same range. Again, a single average value was used for each individual. There was a significant difference between subpopulations ($F = 85.7$, $d.f. = 6, 158$, $P < 0.0001$; means in Table 1). Post hoc multiple comparison tests (Bonferroni and Tukey honestly significant difference) confirmed that the differences were between all colonies from the Pilbara region and all those further north. Both tests also identified a significant difference between colonies from Geike Gorge and Oscar Range (only) in the Kimberley region, illustrating a pattern of subdivision within the Kimberley underlying the pattern of difference between isolated populations (Fig. 4). Although there was no significant difference between any Kimberley population and the Northern Territory, individuals from the Oscar Range produced calls with an average F_{peakc} that was 2.7 kHz lower than those at Geike Gorge.

The relationship between call frequency and various independent factors or covariates was 1st examined separately for the Pilbara region and Northern Territory data sets to avoid variation due to location. A univariate general linear model indicated no significant differences in call frequency between sexes in either population. Likewise, simple regression indicated that there was no significant relationship between call frequency and nose-leaf width, although there was a significant positive relationship with forearm length in the Pilbara region ($F = 5.4$, $d.f. = 1, 70$, $P = 0.023$), but not in the Northern

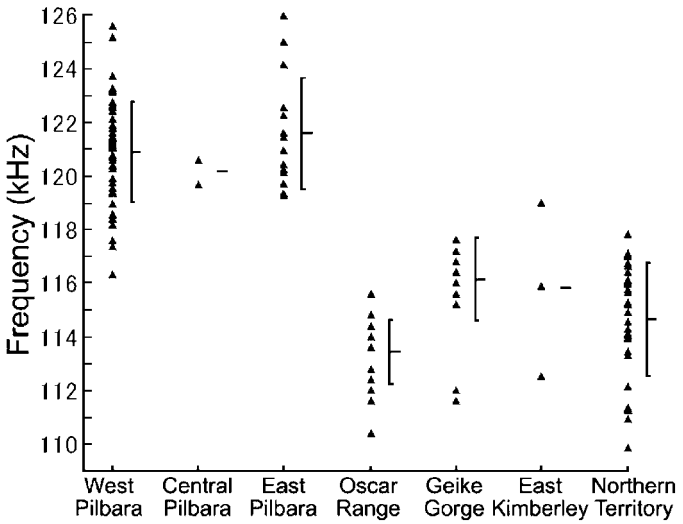

FIG. 4.—Scatterplot of peak frequency (F_{peakc}) values from each sampling location. Bars indicate mean ± 1 *SD* (except for colonies with low sample size).

TABLE 2.—Measurements (mm) of 3 linear and 3 area variables from skull radiographs of *Rhinonicteris aurantia*. Means were calculated excluding missing value estimates; *SD*: 1 standard deviation; *n*: sample size; %ME: percent measurement error; Var Comp: variance component as represented by percent among-skull variance (100 minus %ME); see ''Materials and Methods'' for explanation of variable names.

Location	Parameter	CA	CH	CL	NW	SA	TA
Pilbara	$\bar{X} \pm$ SD	4.84 ± 0.20	2.73 ± 0.05	4.43 ± .09	5.77 ± 0.09	8.79 ± 0.36	7.43 ± 0.40
	Minimum−maximum	4.57−5.18	2.66−2.82	4.26−4.53	5.60−5.91	8.16−9.22	6.69−8.06
	n	10	10	10	10	10	10
Kimberley	$\bar{X} \pm$ SD	5.03 ± 0.17	2.81 ± 0.11	4.52 ± 0.15	5.99 ± 0.12	9.20 ± 0.45	7.93 ± 0.43
	Minimum−maximum	4.72−5.42	2.60−3.02	4.13−4.72	5.78−6.20	8.14−10.07	7.14−8.77
	n	19	22	22	21	22	21
Northern Territory	$\bar{X} \pm$ SD	4.98 ± 0.14	2.82 ± 0.07	4.48 ± 0.12	5.92 ± 0.13	9.19 ± 0.34	7.91 ± 0.41
	Minimum−maximum	4.71−5.21	2.69−2.92	4.16−4.68	5.60−6.17	8.47−10.16	6.93−8.68
	n	26	26	26	26	26	26
Queensland	$\bar{X} \pm$ SD	5.07 ± 0.16	2.73 ± 0.05	4.38 ± 0.16	6.01 ± 0.14	8.91 ± 0.33	8.32 ± 0.36
	Minimum−maximum	4.88−5.18	2.65−2.78	4.13−4.54	5.83−6.20	8.52−9.34	7.81−8.77
	n	3	5	5	5	5	5
	%ME	6.04	8.33	3.57	1.00	3.28	3.45
	Var Comp	93.96	91.67	96.43	99.00	96.72	96.55

Territory. The correlation between call frequency and the time between capture and recording was examined with a Spearman rank correlation test. No significant correlation was found in either data set indicating that handling time was unlikely to have influenced call frequency. Finally, univariate general linear model and Spearman rank correlation tests conducted on a combined data set (Pilbara and Northern Territory) indicated a significant difference in nose-leaf width between populations (smaller in the Pilbara region; $F = 46.7$, $d.f. = 1, 100$, $P < 0.001$; Table 1), and therefore a significant negative correlation with call frequency (Spearman's rho $= -0.43$, $P < 0.001$), but there was no significant difference in forearm length between bats from the Pilbara region and Northern Territory, or relationship between forearm length and call frequency.

Morphological analyses.—Means (before corrections for sex and age) and other summary parameters are given in Table 2. Measurement error can be considered to be low in the case of all variables, and is unlikely to affect conclusions regarding significant differences among groups. Three variables had significant main effects terms for both sex and age: NW, SA, and TA. These variables were corrected with the individual univariate regression coefficients before further analysis. All 6 variables had significant main effects terms for location (Table 3). Most pairwise comparisons indicated that Pilbara specimens were slightly smaller than those from both the Kimberley and Northern Territory (Tables 2 and 3), but particularly for the variables NW and TA. The relatively small sample size from Queensland limits definitive conclusions about this population; however, Pilbara specimens were smaller in most cases. Bonferroni corrections indicated that there was no difference in any pairwise comparison for the variable CL.

Discriminant function analysis was calculated for both sexes combined using all variables. The stepwise procedure included the 2 variables NW and TA in the analysis. Function 1 was most highly correlated with NW and TA, respectively, and explained most of the variation (Table 4). Cross-validation analysis resulted in 47.6% of cases being classified correctly. With regard to the Pilbara isolate, 80% of cases were classified correctly, and the remaining populations had a relatively lower rate of correct classification because of their greater overlap (45.4%, 34.6% and 60% for the Kimberley region, Northern Territory, and Queensland, respectively; Fig. 5). There was overlap in the discriminant function analysis scatterplot; how-

TABLE 3.—Univariate *F*-tests on the independent factors sex, age, and location for radiograph variables. Two- and 3-way interactions were all not significant. Pairwise comparisons are based on 2-tailed Tukey's honestly significant difference tests with Bonferroni corrections for the factor location (direction of size difference is indicated). NS not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; P: Pilbara; K: Kimberley; NT: Northern Territory; Q: Queensland.

Variable	Sex	Age	Location	Comparison
CA	0.27NS	2.85NS	3.20*	P < K
CH	2.15NS	2.42NS	4.90**	P < K = NT
CL	3.40NS	2.27NS	3.04*	NS
NW	4.06*	3.90*	9.14***	P < K = NT = Q
SA	4.10*	4.42*	5.04**	P < K = NT
TA	5.94*	3.90*	7.89***	P < K = NT = Q

TABLE 4.—A) Standardized canonical discriminant function coefficients for the 2 variables nasal width (NW) and transverse capsule area (TA), eigenvalues, percent of variance explained, and canonical correlations. B: Pooled within-group correlations between discriminating characters and canonical discriminant functions. Variables are ordered by the size of the correlation within the function and an asterisk (*) indicates the largest absolute correlation between each variable and any discriminant function.

	Function 1	Function 2
A		
NW	0.69	−0.99
TA	0.43	1.13
Eigenvalue	0.61	0.11
% of variation explained	85.2	14.8
Canonical correlation	0.62	0.31
B		
NW	0.93*	−0.36
TA	0.82*	0.57

ever, the Pilbara specimens were mostly smaller, and overlapped for the most part with a few Northern Territory specimens (Fig. 5).

Discussion

Call frequency differences.—A clear difference in average pulse frequency was apparent between the Pilbara and northern isolates of *R. aurantia*. Handheld *R. aurantia* from the Pilbara region emitted relatively high frequencies with little overlap in variation (approximately 3 kHz), and there was no significant difference across the northern range that comprises both the Kimberley region of Western Australia and the Northern Territory. One value from near Kununurra in the present study was relatively high (119 kHz), suggesting that further sampling should be undertaken to determine if this is atypical. Previously, McKenzie et al. (1995) recorded resting frequencies of 110–111 kHz in this locality. Other outlier values also could be confirmed in future with more efficient recording equipment that reduces field processing time. Some small but significant differences between colonies within the Kimberley were apparent but this was overshadowed by the greater degree of difference between the 2 isolates. In addition to sampling bias, there are perhaps 2 explanations for the difference between the colonies at Geike Gorge and Oscar Range. First, there may be a sex- or age-related bias between the individuals in this colony. Unfortunately, such data were not available from these colonies; however, resting frequency had no relationship with sex in the Pilbara or at Tolmer Falls. An alternative explanation involves the relative isolation of colonies that has led to slightly different mean frequencies simply through drift. There is some suggestion from matrilineal DNA markers that Pilbara colonies are semi-independent units (Armstrong 2006); however, the exodus observed in some colonies during the wet season in the Northern Territory demonstrates that there may indeed be regular gene flow between major roosting sites (Churchill 1991, 1995) which would counteract the effect of drift.

Morphological correlates.—It is useful to consider various macrostructures of bats in several categories that vary in their degree of influence on emitted signals. Structures that primarily have a functional role in signal production include the size of the larynx and vocal membranes. Other features have a functional relationship that is matched or coupled, such as the dimensions and arrangement of the diverticula and chambers in the vocal tract, and the size and width of the nares (Hartley and Suthers 1987, 1988, 1990; Möhres 1953; Suthers et al. 1988). Structures that do not modify or process the signal in some way, but are involved in applying it to the environment, such as the dimensions of the nose leaf (e.g., Hartley and Suthers 1987; Schnitzler and Grinnell 1977) might be considered separately from the 2nd group. Lastly, there are other structures whose size correlates to some degree with both the emitted signal and those structures coupled to signal emission, but whose relationship is an indirect result only. Examples are mostly from inter-species comparisons and include the dimensions of the external muzzle in CF-emitting bats (Goudy-Trainor and Freeman 2002; see below) and overall body size as indicated by mass and forearm length (e.g., *Rhinolophus hipposideros*—Jones et al. 1992; among species—Robinson 1996). The latter might be expected to have a lower correlation than those that are coupled. It is in this context that we considered measurements of nasal chamber variables, nose-leaf width, and forearm length.

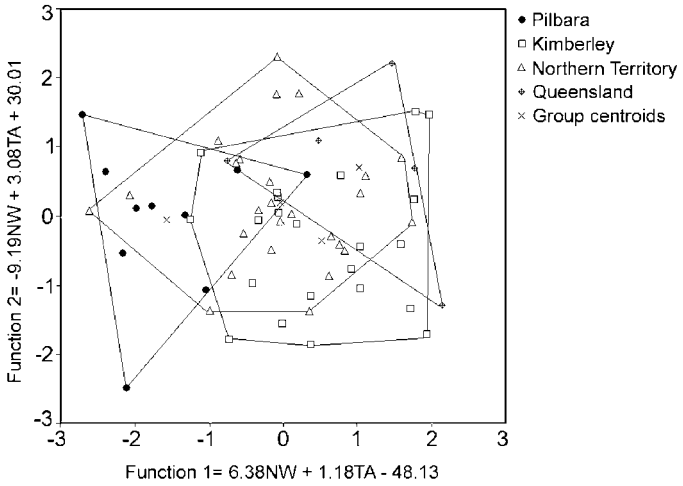

Fig. 5.—Scatterplot of functions 1 and 2 from discriminant function analysis on the variables nasal width (NW) and transverse capsule area (TA).

Overall body size, as indicated by forearm length, exhibited almost no relationship to call frequency, because forearm length was similar in sexes and between regions (Pilbara and Northern Territory). There was a slight positive relationship between call frequency and forearm length in the Pilbara population. Nose-leaf width was moderately correlated with call frequency when compared between regions. Broader nose-leaves were present in individuals from the Northern Territory and these were associated with lower call frequency. The latter result contrasts with the study of Armstrong (2002), who observed no significant difference in nose-leaf width between the Pilbara and the 2 northern populations. In that study, small differences (<1 mm) in nose-leaf width between populations were only observed between Kimberley and Northern Territory, and attention was called to the possible variation caused by the measurement of cutaneous features that may deform in preserved specimens. Examination of our data in this study demonstrates a much lower correlation between external characters that are not directly involved in signal production and modification, but this does not necessarily indicate the lack of a relationship between form and function, as suggested by Goudy-Trainor and Freeman (2002). Much closer relationships are likely to be found between morphological features that are directly involved in shaping signal characteristics. One example of this, although still involving an external feature, comes from the study by Goudy-Trainor and Freeman (2002). They found nasal-emitters had a shorter external snout, which is not surprising because, at least in *Rhinolophus hildebrandti*, the distance between the nares and the nasal chamber is equivalent to half the wavelength of the emitted frequency

(Hartley and Suthers 1988), although the functional significance of this is not known.

Cochlear size was not greatly different between isolates (Armstrong 2002), in contrast to some examples presented by Francis and Habersetzer (1998), who found a relationship between cochlear size and echolocation frequency. They concluded that there is moderate plasticity in the relationship between size and frequency based on some exceptions they observed in other species. The results of our study support their argument that different call frequencies could be accommodated through differences in the internal structure of cochlea (Francis and Habersetzer 1998:175). The sound-reception organs are not directly responsible for its production, and thus their overall dimensions may not change greatly according to a small change in foveal frequency (Huffman and Henson 1993a, 1993b). However, it might be expected that the dimensions of such internal features would be more tightly correlated with call frequency than external features such as forearm length.

Measurements from skull radiographs indicated significant differences between the Pilbara and Kimberley–Northern Territory isolates in terms of overall nasal volume. The Pilbara population was smaller on average in most dimensions, but most clearly rostral width. This pattern corresponds to both the direction of the difference and the magnitude, to that of call-frequency differences given that relatively high frequencies are generally implicated with smaller skull features (Francis and Habersetzer 1998). The pattern from the radiographs also agreed with the assertion of Armstrong (2002), who found that the Pilbara population had relatively smaller measurements of the rostrum (rostral length, breadth, and height; made with calipers), but that the 2 northern groups were similar. Thus, although the same limited skull collection was used (significantly more samples have not become available since Armstrong's 2002 study), the patterns could be confirmed with a technique of greater precision, and in features that might be more closely correlated with echolocation frequency.

Differences in nasal chamber size were subtle, but this was significant given that population isolates are similar in terms of most other morphological characters. Although we observed the nasal chambers as a single voluminous space in the radiographs, we recognize that this region contains 3 discrete pairs of chambers (Korad and Joshi 1998; Suthers et al. 1988). Our interpretations reflect the whole rostrum of *R. aurantia* based on illustrations in these studies. The greatest differences appeared to be in the width of the rostrum (NW, which had relatively low percent measurement error) and TA, suggesting that the nasal chambers were wider in the northern isolate, but particularly the lateral nasal chamber pair (Suthers et al. 1988). The lack of a large difference in measurements of nasal capsule height (CH) or sagittal area SA suggested that the dorsal nasal chamber had not changed in size or height. Fenton (1986) also observed changes in the size of lateral inflations in *Hipposideros*, but unfortunately did not correlate call frequency and nasal width. Both the width of the lateral nasal chambers and the height of the dorsal chambers could probably increase to a certain degree to respond to changes in call signal characteristics, being relatively free of restrictions that other skull components may impose. However, there might be different tendencies in the families Hipposideridae and Rhinolophidae. The dorsal nasal chambers of *R. aurantia* or many other hipposiderids are not particularly bulbous (K. N. Armstrong, in litt.). In contrast, species of *Rhinolophus* show dorsal nasal chamber height that varies from low (e.g., members of the *pusillus* group) to extremely high and bulbous (e.g., *R. creaghi* and *R. malayanus*—Csorba et al. 2003). There has been no comprehensive study on nasal chambers.

Function of the nasal chambers.—The dimensions of the nasal chambers relate specifically to the emitted signal, because they have a suggested function in impedance matching (Hartley and Suthers 1988), which relates to signal intensity rather than frequency. The nasal chamber components have a mechanical analogy, being represented as a small chamber lying between a piston baffle (the larynx) and a throat (the nares). Such small chambers play a role in capacitance, which is 1 type of impedance (Beranek 1954:65–67). If the impedance of the sound source is larger than the impedance terminating the nasal tract at the nares opening, radiation efficiency is reduced, with a high percentage of the signal power being reflected back toward the source. Placement of a small chamber between the source and the point of emission at the nares may reduce the degree of mismatch and cause an increase in radiated signal power. This is suggested by experiments such as those of Hartley and Suthers (1988), because the 2nd harmonic is attenuated and the frequency remains unchanged when the chambers are filled with dental cement. In terms of our observations, we do not suggest that the slight size differences in nasal chamber between isolates of *R. aurantia* relate to frequency directly. Instead, we suggest that parts of the nasopharynx (soft tissue not able to be measured here) are of the correct dimension to filter the fundamental and higher harmonics, and that nasal chamber size has been selected for accordingly to provide the correct overall impedance.

Relationship of call frequency and nasal chamber volume.—Given that we still have a vague understanding of nasal chambers, and also that their complex structure is difficult to model mathematically (Hartley and Suthers 1988; Suthers et al. 1988), we cannot model the effect of the volume change on signal characteristics here. However, we can attempt to demonstrate a negative correlation between call frequency and overall nasal chamber volume using a rearrangement of the equation used by Suthers et al. (1988), which describes the frequency dependency of a cavity on its volume, aperture area, and neck length (Table 5; see Appendix I for description of terms):

$$F = \frac{c}{2\pi}\sqrt{\frac{A}{VL'}} \quad (1)$$

Applying the values in Table 5, and rearranging the formula, based on the assumption that the dimensions of the neck are the same in both isolates, we found that volume differs by about 10%, and that there is a negative correlation between frequency and estimated relative volume. Changes of similar magnitude were evident between Pilbara and northern populations in measurements of NW (3–4%) and TA (6%; Table 2). The values of

volume obtained are not likely to represent real volumes of nasal cavity space, but both the direction of change and its relative magnitude are informative and correspond with predictions if chamber neck dimensions are not significantly different. Earlier, we suggested that the anterior and lateral median swellings comprising the nasal and maxillary bones could simply expand to increase chamber size. This is most likely; however, if our assumption regarding neck length (L) and radius (a) is incorrect and these values are actually smaller in the Pilbara group, then the volumes become more similar and the correlation would eventually become positive.

Although this may not be an accurate model for nasal cavities, equation 1 does describe the basic relationship between tuned frequency and cavity volume in physical structures. Thus, we suggest that in *R. aurantia* there has been morphological evolution concomitant with that of population average emitted frequency, which suggests the echolocation apparatus might not scale with body size, and is indicative of a relationship between form and function (cf. Goudy-Trainor and Freeman 2002). Whether such changes that must be represented in the genome result from adaptation, simple drift, or a more complex relationship between the 2 needs to be examined experimentally. Our suggestion of the decoupling of the echolocation system would also need to be confirmed in a situation where both body size and call frequency vary.

Considerations for conservation.—In light of small population size and threatening processes identified in the isolated Pilbara population (Armstrong 2001; Hutson et al. 2001; McKenzie et al. 1999), we considered that there is sufficient evidence of divergence (morphological—Armstrong 2002; this study; genetic—Armstrong 2006; ecological, as inferred from echolocation frequency—this study) to ascribe it separate evolutionarily significant unit status. This assertion is based partly on the conclusion that the call differences are adaptive in some way, given the apparent decoupling of the nasal apparatus. Call frequency must therefore be partly dependent on the genetic basis of the morphological features involved (Rübsamen 1987). Ecologically adaptive traits are being promoted increasingly for use in defining units for conservation efforts below the species level, rather than relying solely on criteria derived from neutral genetic markers (Crandall et al. 2000; Fraser and Bernatchez 2001). However, lack of resolution about whether intraspecific differences in the emitted frequency of bats is the result of local adaptation, drift, or some interaction between the 2 (Jones and Barlow 2004) limits the usefulness of call frequency difference by itself. A conclusion on whether differences between geographical isolates are adaptive requires further examination and the identification of the ecological factors involved. A comprehensive discussion of possible sources of disruptive ecological selection cannot be undertaken here, although the factor most widely suspected in such cases is atmospheric relative humidity. It appears to be an obvious candidate in *R. aurantia*, given the differences in climate between the regions (Gentilli 1972; Tinley 1991). However, the notion of Guillén et al. (2000) that bats adapt call frequency to humidity levels to maintain an optimum target detection distance was not supported in a separate study (K. N. Armstrong and L. J. Kerry, in litt.), and so the cause remains to be described. Despite the clarification that nuclear markers and further understanding on the reason for call frequency differences would provide, in ascribing evolutionarily significant unit status we gave greater weight to the inferred long period of isolation (Armstrong 2006); the consistency in the patterns from acoustic, genetic, and morphological data sets; and the large potential for further local adaptation if they can be conserved in the shorter term, which is based on the precautionary principle.

Table 5.—Input values for equation 1, and the resulting output volumes. Values of F used were approximates for each isolate. See Appendix I for explanation of terms.

Variable	Pilbara	Northern	Unit
F	121,000	115,000	Hz
c	343	343	ms^{-1} (at 20°C)
A	7.85×10^{-7}	7.85×10^{-7}	m^2
L'	1.85×10^{-3}	1.85×10^{-3}	m
V	8.64×10^{-11}	9.56×10^{-11}	m^3

Conclusions.—Our study demonstrates significant differences in average CF (resting frequency) between individuals in the Pilbara region and more northern populations of *R. aurantia*, a pattern that fits well with the subtle differences in snout size (Armstrong 2002) and radiographs of nasal chambers. Taken together with the phylogeographic evidence from mitochondrial DNA markers (Armstrong 2006), we suggest that the direction of phyletic evolution in the population isolates is different, and that the echolocation system is responding separately to evolutionary forces. Examination of our data draws attention to the function of the nasal chambers and highlights the importance of making correlations with functionally important components of the skull that may not scale with or relate to the variation in other features. The imaging approach used here that allowed estimation of nasal chamber size might give some idea of call frequency for cranial-only museum specimens or fossils.

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APPENDIX I

Explanation of terms in equation 1 describing the relationship of cavity volume and its tuned frequency (see ''Discussion'').

F = tuned frequency of the cavity (Hz)
c = speed of sound in air (20°C; ms^{-1})
A = area of aperture leading to the chamber (m^2; calculated using a = radius of neck opening = 0.5 mm, which is approximately half the diameter of the nasal tract; we estimated this proportion from the model of Suthers et al. [1988], which represents the distance between the primary and secondary palates in our radiographs)
L' = effective neck length of aperture from the open end to the body of the cavity (m; as obtained with an end correction $L' = L + 1.7a$, where a = radius of the neck opening; L was estimated from the model in Suthers et al. (1988), where it can be seen that this distance is approximately equal to the diameter of the nasal tract)
V = volume of the cavity (m^3; in our case an estimate of relative volume)