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BARCODING

DNA barcoding of Neotropical bats: species identification and discovery within Guyana

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Abstract

Sequence diversity in the cytochrome *c* oxidase subunit 1 gene has been shown to be an effective tool for species identification and discovery in various groups of animals, but has not been extensively tested in mammals. We address this gap by examining the performance of DNA barcodes in the discrimination of 87 species of bats from Guyana. Eighty-one of these species showed both low intraspecific variation (mean = 0.60%), and clear sequence divergence from their congeners (mean = 7.80%), while the other six showed deeply divergent intraspecific lineages suggesting that they represent species complexes. Although further work is needed to examine patterns of sequence diversity at a broader geographical scale, the present study validates the effectiveness of barcoding for the identification of regional bat assemblages, even highly diverse tropical faunas.

Keywords: cytochrome *c* oxidase subunit 1, species diversity, tropics

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Introduction

DNA barcoding seeks to advance both species identification and discovery through the study of patterns of sequence divergence in a standardized gene region. A segment near the 5′-terminus of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene has been selected as the barcode region for members of the animal kingdom (Hebert *et al*. 2003). Its effectiveness has been validated for various animal groups and most investigated species (> 94%) possess distinct barcode arrays, with low intraspecific variation and high divergences from closely allied taxa (Ward *et al*. 2005; Hajbabaei *et al*. 2006a). Barcode sharing has been found between a few congeneric species, largely among taxa that are known to hybridize (Kerr *et al*., in press). Most prior barcode studies have generated hypotheses concerning overlooked (cryptic) species (Hebert *et al*. 2004a), many of which have subsequently been recognized as having morphological and ecological differences (Ward *et al*. 2005; Hajibabaei *et al*. 2006a).

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The earliest DNA barcode studies involved investigations of sequence variation in local faunas (Hogg & Hebert 2004; Ball & Hebert 2005), but these are now leading to continental or global barcode campaigns for a few groups such as birds, fish and Lepidoptera (Marshall 2005). Although the efficacy of DNA barcoding has gained increasing validation, prior work on mammals has been restricted to two studies of primate species, most represented by a single individual (Lorenz *et al*. 2005; Hajibabaei *et al*. 2006b). Bats (order Chiroptera) are an obvious target for analysis as approximately 20% (1116 of 5416) of all mammal species belong to this order (Wilson & Reeder 2005). Moreover, although most mammal species are thought to have been described, the incidence of overlooked taxa is likely to be high within bats due to their cryptic behaviour and morphology.

In a previous survey of bats, sequence diversity in cytochrome *b* (cyt *b*) established that most species show mean intraspecific divergences that are less than 2.5% (Ditchfield 2000; Bradley & Baker 2001). Because rates of evolution in cyt *b* and COI are roughly similar, these earlier investigations provide a useful benchmark for our work. Here we build on earlier genetic work with bats and previous DNA barcoding studies by examining patterns of COI divergence in a highly diverse Neotropical bat fauna. Bats follow the

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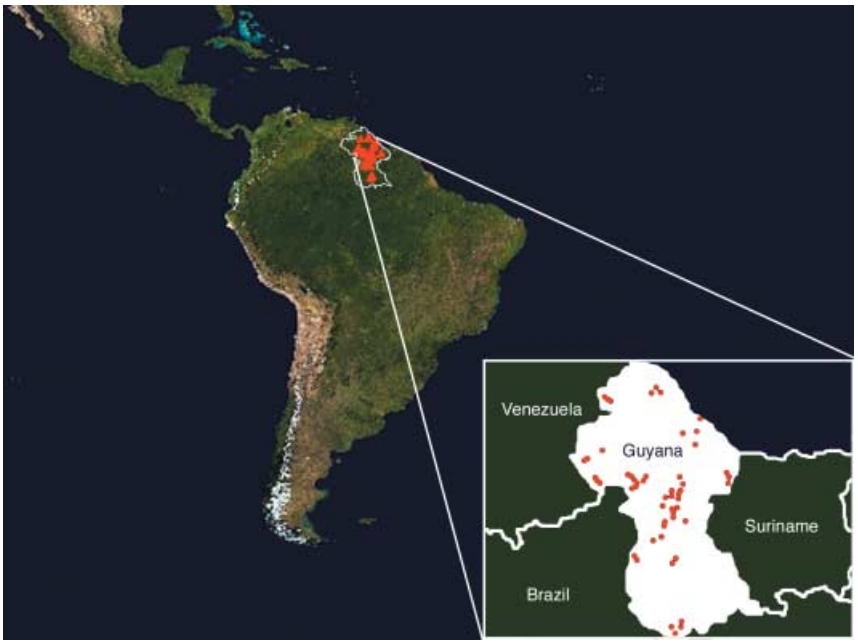

Fig. 1 Samples were collected from across Guyana including inland and costal sites. Full details of the sampling location including GPS coordinates and elevation information are available for each specimen within the 'Bats of Guyana' project in the Published Projects section of the Barcode of Life Data Systems (BOLD, www.barcodinglife.org) and following the 'view all records' link and then clicking on the 'specimen page' links.

usual pattern of increased taxon diversity in tropical regions (Willig & Selcer 1989) and thus represent a good group with which to test earlier predictions that barcoding will fail in the tropics (Moritz & Cicero 2004). Neotropical bats are a particularly good test system as this region has historically been regarded as containing the highest microchiropteran species density in the world (Willig & Selcer 1989), provoking detailed taxonomic studies (e.g. Simmons & Voss 1998; Barquez & Diaz 2001; Lim & Engstrom 2001; Lim *et al*. 2003). We focus on Guyana because of both recent taxonomic work (Lim & Engstrom 2001) and the availability of vouchered specimens. Our investigation seeks to assess the effectiveness of DNA barcoding for species discrimination in this fauna.

Methods

Samples

We sampled tissue from 840 vouchered (skin and skeleton or whole in alcohol) specimens held at the Royal Ontario Museum, representing 87 species, 47 genera and 7 bat families which had previously been collected from a variety of locations within Guyana (Fig. 1). Taxonomic designations followed Wilson & Reeder (2005) with the following exceptions; we retain *Artibeus planirostris* as a unique species from *Artibeus jamicensis* following Lim *et al*. (2004) and we retain the name *Artibeus bogotensis* due to taxonomic revisions in progress. We also retained a *Molossus* sp. designation for one specimen because Lim & Engstrom (2001) concluded that it was either a species unknown from Guyana or an undescribed taxon. Details on each specimen including sampling location are available within the 'Bats of Guyana' project in the Published Projects section of the Barcode of Life Data Systems (BOLD, www.barcodinglife.org). Specimen information [global positioning system (GPS) coordinates of collection, institution-holding voucher, voucher number, etc.] is found by following the 'view all records' link and then clicking on the 'specimen page' for each specimen. Similarly, sequence information and trace files are found under the 'sequence page' linked to each specimen.

DNA isolation, amplification and sequencing

A 1-mm^3 piece of frozen tissue (liver, heart or kidney) from each specimen was placed directly into 96-well plates containing lysis buffer and proteinase K. Subsequent DNA extraction employed a glass fibre protocol (Ivanova *et al*. 2006). The 658-bp target region of COI was amplified using two primer cocktails and visualized in a 96-well E-Gel (Invitrogen). Cocktail 1 was C_VF1di,/C_VR1di (Ivanova *et al*. 2006), while cocktail 2 (C_VF1LFt1/C_VR1LRt1), was an improved version including M13-tailed versions of the primers and an additional primer pair, LepF1_t1 and LepRI_t1 in the following ratio; 10 pmol/μL, VF1_t1: VF1d_t1: LepF1_t1: VF1i_t1 (1:1:1:3) or VR1_t1: VR1d_t1: LepRI_t1: VR1i_t1 (1:1:1:3) (Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN, pers comm). All primer sequences are available from the Canadian Center for DNA Barcoding by visiting the website at www.dnabarcoding.ca/clareetal2006.php. The polymerase chain reaction (PCR) mix included 6.25 μL of

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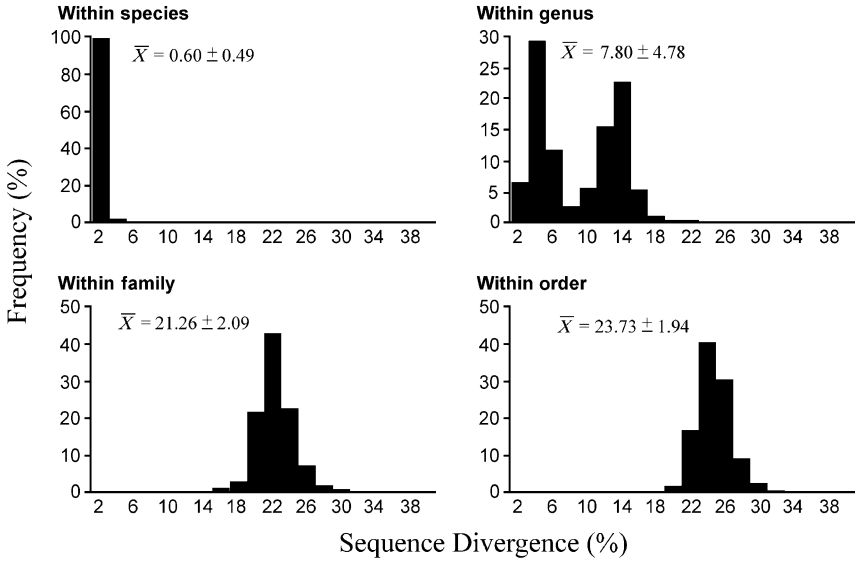

Fig. 2 Frequency histograms of mean (± SE) COI sequence divergences (K2P) at various levels of the taxonomic hierarchy for bat species from Guyana.

10% trehalose, 1.25 μL 10× PCR buffer, 0.625 μL (2.5 mM) $MgCl_2$, 0.125 μL (10 μM) forward and reverse primer cocktail, 0.625 μL (10 mM) DNTPs, 0.625 μL *Taq* polymerase and 4 μL H20 + template DNA (Hajibabaei *et al*. 2005). PCRs were run under the following thermal cycle conditions: 1 min at 94 °C followed by 5 cycles of 30 s at 94 °C, 40 s at 50 °C, and 1 min at 72 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 55 °C, and 1 min at 72 °C, and finally 10 min at 72 °C and PCR products were sequenced on an ABI 3730 (Hajibabaei *et al*. 2005).

Samples producing single clear amplicons from cocktail 1 were sequenced with VF1d and VR1d (Ivanova *et al*. 2006), while those from cocktail 2 were sequenced with M13F and M13R (Messing 1983) using BigDye version 3.1 on an ABI PRISM 3730 capillary sequencer (Applied Biosystems).

Data analysis

Sequences were aligned using SEQSCAPE version 2.1.1 (Applied Biosystems) and edited manually. Sequences and original trace files are available in the 'Bats of Guyana' project on BOLD and on GenBank (accession nos EF079971–EF080810). Sequence divergences were calculated using the Kimura two-parameter (K2P) model of base substitution (Kimura 1980). A neighbour-joining (NJ) tree of K2P sequence distances showing intraspecific variation was created using BOLD and a NJ tree of interspecific divergence including bootstrap analysis (500 replications) was performed using MEGA3 (Kumar *et al*. 2004). K2P sequence divergences for all levels in the taxonomic hierarchy were determined using the 'Distance Summary' tool on BOLD. Species that split into two or more distinct groups with high bootstrap support and sequence divergences greater than 2.5% between them are hypothesized to represent provisional species following the observations of Ditchfield (2000) and Bradley & Baker (2001). Such groups were differentiated by adding a suffix (PS1, PS2) to the current species name. Regression analysis was used to assess the relationships between mean and maximum intraspecific sequence divergence and sample size, and between sequence divergence and geographical distance for individuals of each species.

Results

A COI amplicon was recovered from all 840 individuals and more than 97% of the sequence reads were greater than 600 bp in length (most 657 or 658 bp). The mean K2P sequence distance within species was 0.60%, while the mean divergence between congeners was 13× higher at 7.80% (Fig. 2; Table S1, Supplementary material). Regression analysis indicated that mean divergence values were not significantly correlated to sample size (adjusted $R^2 = 0.013$ $P = 0.161$) and that maximum divergence was only weakly related to sample size (adjusted $R^2 = 0.105$, $P = 0.003$) (Fig. 3). Geographic distance also explained very little of the sequence divergence among conspecific individuals (adjusted $R^2 = 0.016$, $P < 0.001$). Six species showed deep intraspecific variation, forming two or more intraspecific barcode groups with greater than 2.5% mean divergence between them (Table 1, Fig. 4).

A NJ tree of sequence divergences (K2P) at the COI region indicated that most genera formed cohesive units (Fig. 4; Figure S1, Supplementary material for a complete NJ tree showing intraspecific variation). However, levels of sequence divergence between congeneric taxa varied substantially, appearing to approximate a bimodal distribution. At the extremes, two genera showed very low divergences among species — *Molossus ater* and *Molossus*

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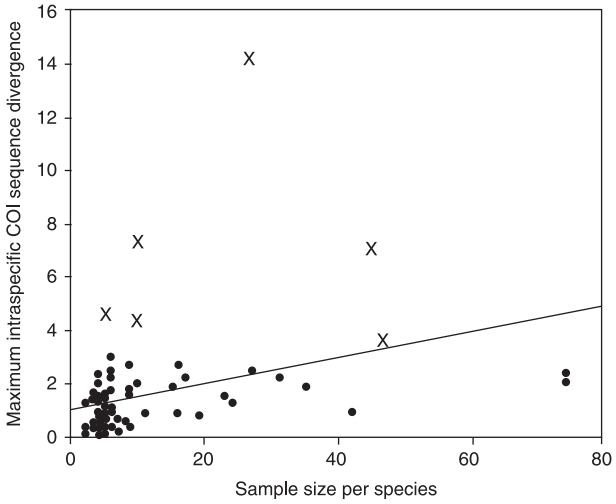

Fig. 3 The relationship between maximum intraspecific sequence divergence (K2P) at COI and the number of individuals analysed for each species (adjusted $R^2 = 0.105$, $P = 0.003$). Taxa showing deep intraspecific divergences that clustered into two or more groups are indicated with an X. (see Table 1 for details)

molossus were 2.18% divergent, while *Carollia brevicauda* and *Carollia perspicillata* were just 1.2% divergent. By comparison, *Peropteryx leucoptera* and *Peropteryx kappleri* showed almost 20% sequence divergence.

Discussion

Several previous studies on vertebrates have raised concerns regarding the acquisition and ease of interpretation of DNA barcode data. For example, Vences *et al*. (2005) encountered difficulties in amplifying the barcode region from amphibian lineages, but they did not use a primer set that was designed for this group. Their difficulty contrasts with the results on other vertebrate groups, including birds and fishes, where amplification of the barcode region has proven straightforward (Hebert *et al*. 2004b; Ward *et al*. 2005). While we encountered early difficulties in barcode recovery for bats, development of a primer cocktail enabled amplification of all species included in our study. Because the component primers in this cocktail were tailed with M13, sequencing reactions were straightforward. The application and extension of this formulation strategy not only promises a solution to barcode amplification for groups such as the amphibians, but also the generation of cocktails that amplify the barcode region for broad assemblages of life.

Aside from difficulties in PCR amplification of the barcode region, concerns have been raised in relation to interpretational problems derived from the inadvertent amplification of COI nuclear pseudogenes of mitochondrial origin (NUMTs, Bensasson *et al*. 2001). In practice, the ~650-bp length of the barcode amplicon provides substantial protection against this as most NUMTs are < 200 bp in length (Richly & Leister 2004). In addition, because of the higher copy number of mitochondrial COI sequences, prior barcode studies have shown that NUMTs are detected in a very small percentage of species (Kerr *et al*., in press). Moreover, when detected, NUMTs regularly show indels or diagnostic mutations (e.g. stop codons) that reveal their presence. Rigorous inspection of trace files, especially those with low PHRED (quality) (Ewing & Green 2006) scores linked to heterozygous peaks or uncalled bases, can also be used to filter possible NUMTs. Detection allows a shift in analytical strategy to suppress their amplification. In the present study, we detected no signs of pseudogenes, a fact that may be correlated with the unusually small genome sizes of bats (Van Den Bussche *et al*. 1995; Gregory 2002).

Prior barcode studies have established that more than 95% of species possess diagnostic sequence arrays for the barcode region. For example, Ward *et al*. (2005) found that all 207 Australian fish species that they examined had a diagnostic barcode sequence array. Few species assemblages

Table 1 Six bat species from Guyana with large sequence divergence (K2P) between lineages at COI. Lineages without bootstrap support represent single specimens

	Scientific name	Number of lineages	% Mean divergence	Bootstrap values* between lineages
1	*Carollia brevicauda*	2	2.5	79/99
2	*Myotis riparius*	3	4.2, 13.8, 12.7	100/100/100
3	*Noctilio albiventris*†	2	6.5	100/100
4	*Phylloderma stenops*	2	4.3	n/a/100
5	*Platyrrhinus helleri*	3	3.3, 4.2, 3.4	100/100/100
6	*Trachops cirrhosus*	3	5.4, 4.2, 6.3	100/n/a/100

*500 replicates; †prior taxonomic work suggests this may be a species complex in other areas of its range (Lewis-Oritt *et al*. 2001; Wilson & Reeder 2005).

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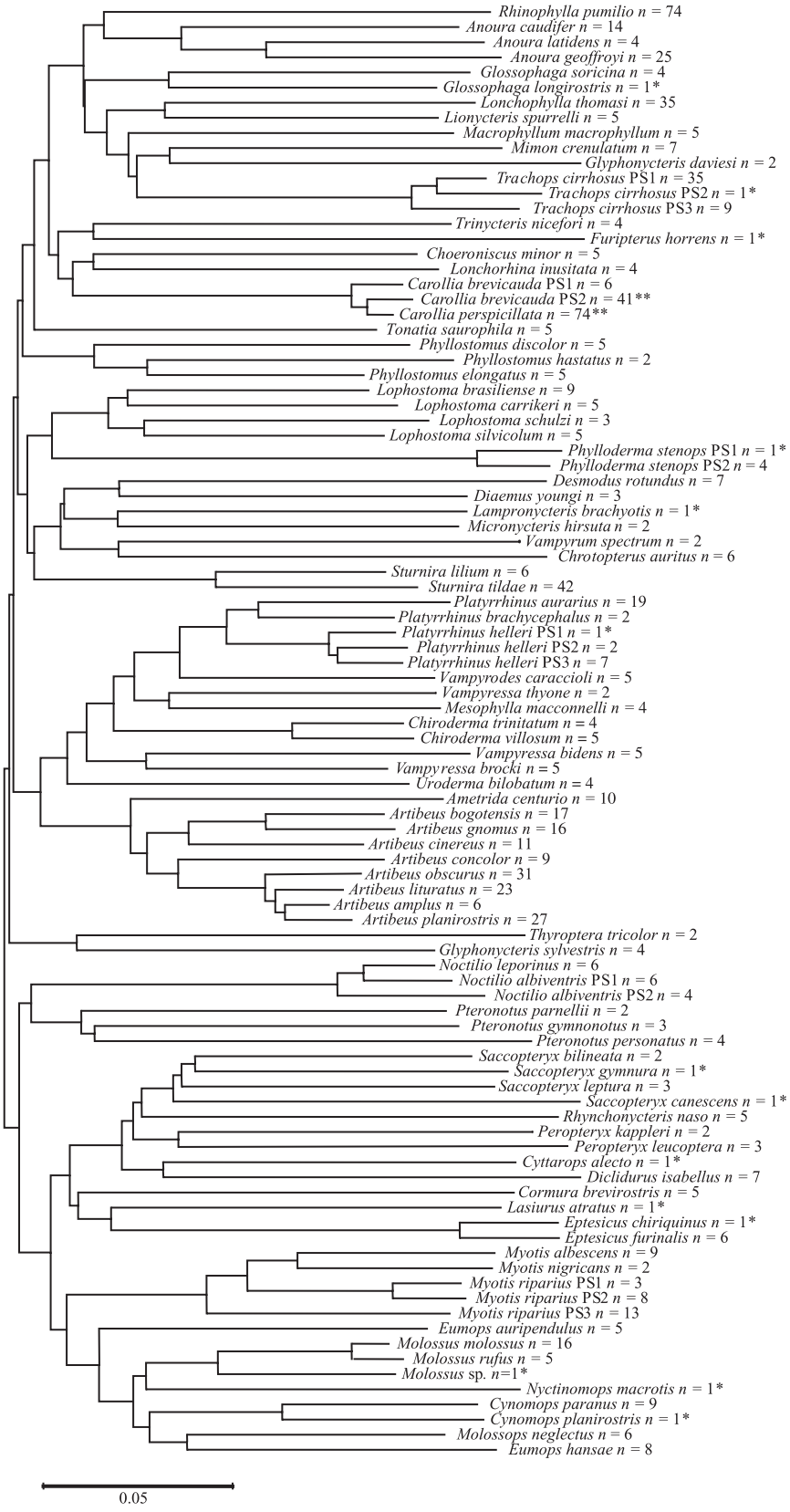

Fig. 4 A neighbour-joining tree of COI sequence divergences (K2P) in 87 bat species from Guyana. All species branches have bootstrap values greater than 95 barring marked exceptions. The number of specimens is indicated following each species name. * bootstrap values unavailable ($n = 1$); ** bootstrap values less than 95.

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have been surveyed on a continental scale, but work on North American birds revealed that nearly 95% of recognized species have distinct barcode arrays (Kerr *et al*., in press). The few cases where barcodes failed to separate bird species involved either closely allied allopatric taxa whose status as distinct species is uncertain or sister taxa that hybridize (Kerr *et al*., in press). Although no prior investigation has assessed the effectiveness of DNA barcoding in tropical vertebrates, a study on more than 500 species of Costa Rican Lepidoptera established that 98% of these species had diagnostic barcode arrays (Hajibabaei *et al*. 2006a).

Our investigation reinforces the conclusions of earlier barcode studies on animals. All of the bat species that we examined possessed a diagnostic array of COI sequences, enabling their identification. In 81 of 87 species, sequences formed a single cohesive cluster that was clearly divergent from those of congeneric taxa, as evidenced by the 13-fold higher mean sequence divergence among congeners than among members of a species. The other six species showed a different pattern of variation — sequences fell into two or three clusters, each showing substantial sequence divergence from their sister clusters. Similar cases of deep sequence variation within 'species' have been regularly encountered in prior DNA barcode surveys and they provide *prima facie* evidence that the taxon/taxa under investigation represents a species complex. The incidence of overlooked taxa recognized in this fashion ranges from lows of 3% in groups that have seen much taxonomic work such as birds (Hebert *et al*. 2004b) or 5% in the Lepidoptera (Hajibabaei *et al*. 2006a) vs. > 200% in groups that have received little attention (Blaxter *et al*. 2005). With an incidence of 6.9%, the bats of Guyana show a higher level of overlooked taxa than most other vertebrate groups, but not dramatically so. At least one of the six taxa we identified has been previously recognized as a probable species complex in other geographical areas (Lewis-Oritt *et al*. 2001; Wilson & Reeder 2005).

We did not observe any striking anomalies in the patterning of barcode variation within bats although levels of sequence variation within species were higher than those in most other groups. For example, treating the provisional species as separate taxa, intraspecific variation in Guyanese bats averaged 0.60% vs. 0.27% in North American birds (Hebert *et al*. 2004b), 0.39% in marine fishes (Ward *et al*. 2005) and 0.46% in Lepidoptera (Hajibabaei *et al*. 2006a). The higher level of variation in bats reflected a general shift in the pattern of intraspecific diversity. Within other groups, most species showed a single dominant sequence and rare variants around it. By contrast, in some Guyanese bat species, every individual that we examined had a different sequence. This elevated variation may reflect some unique aspect of mitochondrial evolution in bats.

In summary, we have established the effectiveness of DNA barcoding for identification of the bat fauna of Guyana, despite its high diversity. By extension, our results suggest that DNA barcode libraries will create highly effective identification systems for any regional bat fauna. We further conclude that the assembly of these local libraries will generate a substantial number of hypotheses regarding overlooked species. In this study, sequence divergence was unrelated to the geographical distance between collection sites for specimens. However, this result is not unexpected, given that these localities were never more than 700 km apart, a distance representing just a small segment of most species ranges. Hence, the present investigation provides no guide to the further diversity that will be revealed when barcode data are gathered on bats from different geographical regions. However, a substantial number of additional taxa will surely be revealed.

Although bats show a high incidence of cryptic species and substantial difficulties in species identification, similar complexities occur in other mammal orders such as rodents and insectivores. Viewed from this context, the assembly of a DNA barcode library for the global mammal fauna will not only aid recognition of currently overlooked species, but will also lead to the development of an automated identification system that will be particularly valuable for taxa in these groups. The latter tool will further be a useful practical resource for varied ecological, biodiversity and evolutionary investigations. With just over 5000 species, the global mammal fauna represents a smaller challenge than the campaigns that seek to barcode all birds and fishes by 2011. As a consequence, despite a delayed start, it seems likely that a comprehensive barcode inventory for all mammals can reach closure by the same timeline.

Acknowledgements

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Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEN/MEN1657/MEN1657sm.htm

Fig. S1 Neighbour-joining tree (K2P distances) for all specimens

Table S1 Species included in this study and genetic variation descriptions

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