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Dendropsophus minutus (Anura: Hylidae) of the Guiana Shield: using DNA barcodes to assess identity and diversity

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Abstract

Herein we discuss the geographic structure of genetic variation of the widely distributed lesser treefrog (*Dendropsophus minutus*) from the Guianas in a preliminary examination of the status of Guianan populations referred to this taxon. Current sampling is insufficient to determine the status of all lineages of this complex within South America, but our results do indicate the presence of cryptic species within this relatively small portion of the 'species' range. Our findings reveal a geographic discontinuity of genetic structure within this region that appears to be correlated with elevation. We also present evidence that supports previous assertions that the conventional DNA barcode locus (COI) is not suitable for amphibians.

Key words: *Dendropsophus minutus*, DNA barcoding, Guiana Shield

Resumen

Se discute la estructura geográfica de la variación genética en la ranita trepadora (*Dendropsophus minutus*), ampliamente distribuida en Sudamérica. Las muestras analizadas provienen de las Guayanas en una evaluación preliminar del estatus de las poblaciones incluidas en este taxon. El muestreo actual no es suficiente para determinar el estatus de todos los linajes de este complejo de America del Sur, sin embargo nuestros resultados indican la presencia de especies crípticas dentro de esta proción relativamente pequeña del intervalo de distribución de esta 'especie'. Nuestros resultados revelan una discontinuidad geográfica de la estructura genética dentro de esta región que parece estar correlacionada con la altitud. También mostramos evidencia que apoya las observaciones previas de que el locus COI, convencionalmente usado en DNA barcoding, no es adecuado para ser utilizado en amphibios.

Introduction

In 1979 John Lynch observed that fewer than 5% of known Neotropical forest amphibian species were "widely distributed". He predicted that within a decades time, intensive study of taxonomy and distributions would reveal that there are very few species whose distributions are truly so extensive. While it took slightly more time than Lynch had anticipated, his theory has largely been supported by intensive phylogeographic analyses of many of these widespread 'species' (Camargo *et al.* 2006; Fouquet *et al.* 2007; Heyer *et al.* 1996). It appears that the single largest obstacle currently preventing the continued study of these 'species' is the lack of a comprehensive sample of molecular material that will permit the analysis of geographic structure of genetic variation. For many of these widespread 'species', the lack of differentiable, diagnostic phenotypes as well as trained taxonomists able to interpret these characters has left the task incomplete. DNA barcoding provides a means whereby todays taxonomists can streamline the sorting process and focus their attention on populations/lineages that merit consideration for further examination as potential species.

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Historically *Dendropsophous minutus* (Peters) has been taxonomically problematic (Kaplin 1994). It was first described as *Hyla minuta* by Peters in 1872 from a locality near Rio de Janeiro, Brazil. Recent phylogenetic analysis of the Hylidae by Faivovich *et al.* (2005) reassigned *H. minuta* to the genus *Dendropsophous* (necessitating a change in the species epithet). Variation in color and pattern in this species has led many authors to assign specific rank to various populations. At present eight previously recognized species (*H. velata* [Cope, 1887], *H. bivitta* [Boulenger, 1888], *H. goughi* [Boulenger, 1911], *H. pallens* [Lutz, 1925], *H. suturata* [Miranda Ribeiro, 1926], *H. emrichi* [Mertens, 1927] and *H. minuta bivitta* [Barrio, 1967]) are considered synonyms of *D. minutus* (Kaplin 1994, Lutz 1973).

While *D. minutus* has long been considered a species complex, the clarification and delimitation of this species has only begun recently. Cochran referred to *D. minutus* as a "small and perplexing species" and reported that it has "given rise to great confusion in collections owing to the insufficiency of Peters' original description and the lack of any figure of the cotypes" (Cochran 1955). Cochran concluded with a statement that effectively indicated that the true limits of this taxon would require the study of many more specimens from across the distribution.

Some authors have regarded the observed sympatry of phenotypic variants of *D. minutus* as justification for considering coloration and pattern as intraspecifically variable and of little use to taxonomic delimitation (Kaplan 1994). More recent work has shown that what is currently recognized as *D. minutus* includes populations that differ significantly in ecological (Cardoso & Haddad 1984) and morphological characteristics (Kaplin 1994). Cardoso and Haddad (1984) studied the acoustical properties of vocalizations in three different populations, visiting the type localities of *D. minutus*, *H. bivitta*, and *H. velata*. The latter two are at present considered to be synonyms of *D. minutus* (Cochran 1955). One of the objectives of this study was to verify the species status of *D. minutus* (Cardoso & Haddad 1984). Although they recognized a great deal of variation in vocalizations and identified three distinct vocalization patterns they concluded that these indicated emerging species not meriting specific recognition. Conversely, Köhler and Lötters (2001) based their description of *D. delarivai* largely on characteristics of the advertisement call. It is unfortunate that the vocalization characteristic that Köhler and Lötters (2001) advocate as taxonomically informative (pulse repetition rate) is not reported by Cardoso and Haddad (1984) as their geographically widespread sampling and (including the type locality) would provide a great deal of insight into taxonomic diversity within this 'species'.

At this point, the only thing that is clear about the taxonomy of *D. minutus* is that there is a great deal of inter- and intraspecific variation in advertisement call, phenotype, and ecology. Kaplin (1994) astutely suggested that variation in these characters alone will do little to resolve relationships and identity in what is almost certainly a species complex.

Lynch (1979) was one of the first to suggest that forest inhabiting amphibian species considered to be widespread in the Neotropics (spanning the entirety of Amazonia) would likely prove to be complexes of cryptic species. Of the taxa cited by Lynch as examples of this, most have since been divided into multiple species (e.g. *Ameerega picta*: Caldwell & Myers 1990; Haddad & Martins 1994 and *Leptodactylus*: Camargo *et al.* 2006; Heyer *et al.* 1996) and ongoing molecular studies of others (e.g. Fouquet *et al.* 2007) continue to support this idea. *Dendropsophus minutus* is one of the most widespread of all Neotropical treefrogs, occurring in the lowlands east of the Andes from Trinidad and Tobago, to Argentina and ranging in elevation from sea level to 1800m.

Within the Guiana Shield, populations assigned to *D. minutus* range in elevation from sea level to > 1600m (McDiarmid & Donnelly 2005) and their designation as *D. minutus* remains suspect (Donnnelly *et al.* 2005). These animals are one of the few (seven total) herpetofaunal taxa in the Shield to exhibit such elevational plasticity, and are predicted to exhibit geographic differentiation associated with localized adaptation (McDiarmid & Donnelly 2005). Geographic structure within Neotropical fauna associated with altitude (particularly within the Guiana Shield) has been the subject of a great deal of attention of late (Noonan & Gaucher 2005; 2006; Rull 2005; 2006). We have analyzed variation in the mitochondrial *cytochrome oxidase* subunit I

(COI) of 73 individuals of *D. minutus* from the Guianas in order to test for the presence of geographic variation (as has been suggested by McDiarmid & Donnelly, 2005) and to clarify the taxonomy of populations currently assigned to *D. minutus*.

Materials and methods

Sampling and amplification

Samples of *D. minutus* from the Guianas were obtained directly through the fieldwork of BPN, or from borrowed/collaboratively collected material. Tissue grants were obtained from P. Gaucher, C. Marty, M. Blanc, and A. Fouquet. Localities of samples are listed in Table 1 and illustrated in Fig. 1a. Due to collection restrictions, some individuals are not represented by voucher specimens and were sampled by toe clipping. A total of 73 samples from 9 Guianan populations were sampled. Tissues were taken from toe clips, liver or muscle and preserved in 95% ethanol and then stored at –80º C prior to DNA extraction.

Genomic DNA was isolated using the high-salt precipitation method of Crandall *et al.* (1999). Amplification of a ~800 bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) was carried out using the novel primer HmCO1-R (5' CGTCACTCAGTACCAAACCCCC 3') and the previously published CO1AXen-H (5' TGTATAAGCGTCTGGGTAGTC 3') (Mable & Roberts 1997). Amplifications were performed in 13µl reaction volumes using TaKaRa hotstart *Taq* DNA polymerase and 10X reaction buffer (100 mM Tris-Hcl [pH8.3], 500 mM KCl, 15 mM $MGCl_2$). Amplification followed standard, previously reported profiles (Noonan & Gaucher, 2005). PCR products were purified with Millipore MANU030 PCR cleanup plates. The purified double-stranded products were used directly in 1/32 volume dideoxy-termination sequencing reactions using BigDye Terminator v3.1 (Applied Biosystems). Unincorporated dye terminators were removed by Sephadex cleanup. Sequences were edited and aligned with Sequencher v. 4.1 (Gene Codes Corp) and checked by eye. Alignment was unambiguous with no apparent insertions or deletions.

Sequence Analysis

As this study is aimed at detecting the presence of geographic structure among populations of *D. minutus* as well as possible cryptic species, we have not conducted rigorous phylogenetic analyses. Nor have we attempted to estimate any demographic parameters. These will be addressed in future work as additional samples become available. Phylogenetic relationships of all observed mtDNA haplotypes (including redundant haplotypes) were estimated using PAUP* version 4.0b10 (Swofford 2001) employing distance based Neighbor Joining (NJ) methods.

Results and discussion

Phylogenetic analysis reveals the presence of two distinct lineages within the Guianas, one of which is restricted to the highlands of the western portion of this region (Fig. 1b; D) and the other spans the entirety of the area examined with some obvious geographic structure to the observed genetic variation (AB&C). Sequences have been deposited in GenBank (EF587765-EF587837). The 9% uncorrected sequence divergence between the western, highland lineage (D) and the widespread group (ABC) is surprisingly high, particularly given the geographic proximity of these two lineages (both occur in the area surrounding Imbaimadai, Guyana). Within the more widespread lineage, group B corresponds to northern lowland populations in French Guiana and Suriname and group A is represented by individuals collected from Sipaliwini village in southern Suriname. All individuals included in group C were collected from the same highland area (near Imbaimadai) as group D. It is notable that while these highland populations were collected on a single expedi-

tion within a few days time, there was no indication that these animals were different in their ecology/behavior, nor was their physical appearance indicative of two distinct taxa (Fig. 2). Subsequent examination of specimens has also failed to reveal obvious, diagnostic characteristics associated with these two lineages.

The results of this preliminary assessment of phylogeographic structure of Guianan *D. minutus* reveal geographic patterns that somewhat parallel the results of previous work on anurans in this region (Noonan & Gaucher 2005; 2006). These previous studies have also found differentiation between the costal lowlands and interior uplands of Suriname/French Guiana. Differences among the ABC lineages are all less than 1.9% (uncorrected, pairwise sequence divergence) and there is no evidence to suggest that these should, at present, be afforded formal, taxonomic recognition. The lineage here referred to as group D is highly divergent from the ABC lineage and likely represents a distinct, cryptic species. However, as we are unable to identify morphological characteristics that substantiate the delimitation of these two groups and we do not have recordings of vocalizations from the Imbaimadai region we have refrained from making formal taxonomic changes. It must also be noted that the lack of material from the type locality of *D. minutus* prevents us from identifying which of these two groups is referable to that name (if any). It is entirely possible that both of these lineages present in the Guianas are distinct from *D. minutus*.

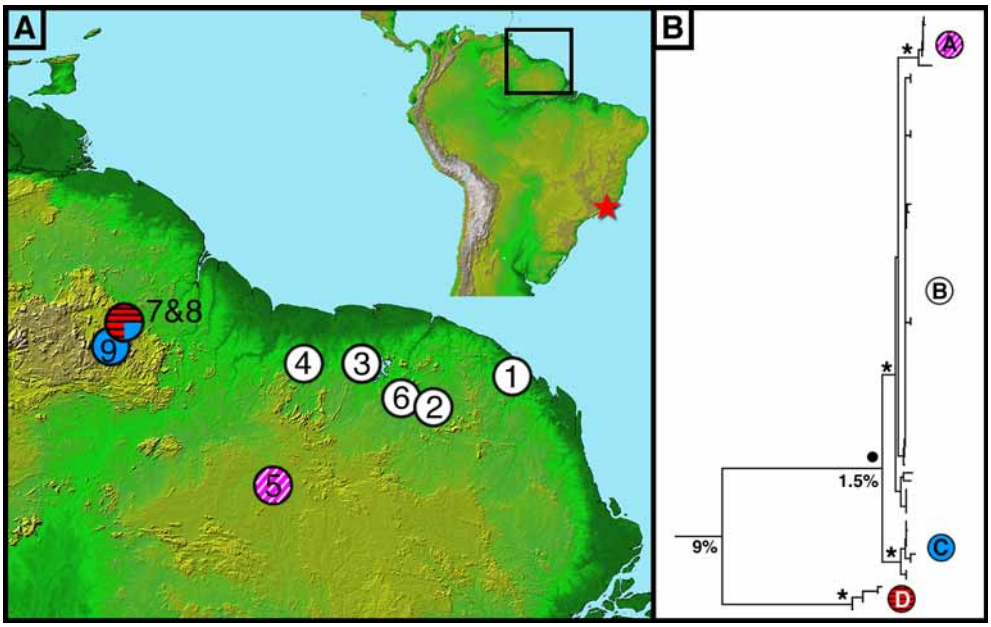

FIGURE 1. **A**: Geographic distribution of populations sampled for this study. Inset map indicates region focused on (Guianas) as well as the type locality of *D. minutus* (red star). Numbers correspond to localities listed in *Specimens Examined* section. **B**: Neighbor Joining tree illustrating relationships among major lineages of '*D. minutus*' from the Guianas. Colored circles correspond to like colored localities in **A**. Bootstrap support values above 70% and 99% are indicated by a circle and asterisk respectively. Interrealtionships among A, B & C are poorly resolved and average pariwise sequence divergence (uncorrected) among these lineages does not exceed 1.5%. Average divergence between D and ABC is 9%.

Conclusion

At the onset, this study aimed to implement DNA barcoding analysis of the taxonomic composition of Guianan '*D. minutus*'. We encountered a number of problems in our attempts to amplify the universal barcode locus (COI) for this taxon that necessitated the development of a novel primer. Furthermore, a number of individuals for which we have samples from the Amazon basin (not included in this study) would not amplify for COI (though we have been able to amplify nuclear DNA from these samples). It seems that our study provides

a perfect example of both the potential of DNA barcoding to reveal individuals/populations of interest (e.g. as a sorting tool) while also supporting the suggestion of Vences *et al.* (2005) that the COI locus is not universally suitable for anurans.

While our study adds to the growing body of literature demonstrating the fallacy of widespread Neotropical anuran species (Lynch, 1979), additional genotypic and phenotypic data (vocalization recordings) will be needed before the taxonomic quagmire of *D. minutus* can be fully resolved. Based on our results within the relatively restricted area of the Guianas, it seems that the recognition of multiple species currently covered by the umbrella of *D. minutus* awaits only the collection of additional material.

FIGURE 2. Phenotypic variation among populations of '*D. minutus*' in the Guianas. Clockwise from top left: near Imbaimadai, Guyana; road to Apura, Suriname; Lely Mt., western Para, Suriname; near Imbaimadai, Guyana.

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Specimens examined

French Guiana: (1) Kaw Mt. (4.49° N; -52.03° W): PG 359-373; (2) Mt. Kotika (3.93° N; -54.2° W): PG 422, 423; 425-438. Suriname: (3) Brownsberg (4.94° N; -55.17° W): BPN 867-871; (4) Road to Apura (5.19° N; -55.65° W): BPN 934-941; (5) Sipaliwini Village (2.03° N; -56.12° W): BPN 1003-1005; BPN 1024-1026; (6) Lely Mt. (4.27° N; -54.74° W): BPN 1035-1039; BPN 1043; BPN 1070; Guyana: (7) Mt. Thomasing (5.74° N; -60.3° W): BPN 1175, 1296; (8) Imbaimadai (5.72° N; -60.27° W): BPN 1157, 1217; (9) Upper Mazaruni (5.63° N; -60.25° W): BPN 1237, 12551260.