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DNA barcoding of an invasive mammal species, the small Indian mongoose (*Herpestes javanicus*; E. Geoffroy Saint-Hillaire 1818) in the Caribbean and Hawaiian Islands

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Abstract

Background and aim: The use of DNA barcodes has been proposed as a promising tool for identifying species. The efficacy of this tool for invasive species requires further exploration. The species status of the small Indian mongoose, an exotic invasive in several parts of the world, has been contentious due to morphological similarity with its congeners in its natural habitat. Although the small Indian mongoose is recognized as *Herpestes javanicus*, this nomenclature has been used interchangeably with *Herpestes auropunctatus*.

Materials and methods: Here, we demonstrate the utility of using DNA barcoding approaches with mtDNA cytochrome *b* to discriminate between the two species and other sympatric members of the genus *Herpestes* (*Herpestes naso*, *Herpestes urva*, and *Herpestes edwardsii*). Using the diagnostic DNA positions we obtain, we can identity specimens of nonnative populations of the small Indian mongoose from the Caribbean and Hawaiian Islands to their species of origin.

Results: A singe diagnostic site accomplishes the identification of *H. javanicus* versus *H. auropunctatus*.

Conclusion: Our results indicate that the nonnative mongoose populations from the Caribbean and Hawaiian Islands are *H. auropunctatus*, and not *H. javanicus*.

Keywords: *cytochrome* b*, small Indian mongoose,* Herpestes javanicus*, invasive species, Caribbean, Hawaii*

Introduction

Mitigating the risks posed by introduced invasive species hinges on the ability to recognize, correctly identify, and monitor these nonnative organisms (Sakai et al. 2001). Understanding the taxonomic status, species origin, and geographic distribution of an invasive species is critical to hindering their establishment or expansion and could assist in screening for species invasiveness. Therefore, confirming the identification and distribution of invasive species is often necessary in order to make sound management decisions. However, distinguishing among morphologically cryptic invasive species, or for species with ambiguous systematic assignments, can be a difficult task.

DNA barcoding is a valuable diagnostic and broadly applicable approach for rapidly determining species identity (DeSalle 2006, 2007; Rubinoff 2006; Rach et al. 2008). This technique uses short DNA sequences from standardized gene regions as a species identifier for comparison against described species, which can be developed from new or existing databases. Due to the technique's success and accuracy, DNA barcoding methods have been developed for a host of invertebrate and vertebrate species (e.g. Hebert et al. 2004; Hajibabaei et al. 2006; Lemer et al. 2007). For invasive species, DNA barcoding can provide data to address the classification of morphologically cryptic species, to diagnose or identify new invasive species, and to determine the association

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of nonnative populations to source populations (Armstrong and Ball 2005), which, when integrated within morphological character data, provides a strong framework for characterizing species in novel environments. In particular, applications of DNA barcoding for invasive species identification have been demonstrated to be effective (Smith et al. 2003; Armstrong and Ball 2005; DeSalle et al. 2005; Siddall and Budinoff 2005; Scheffer et al. 2006).

The small Indian mongoose, currently recognized as *Herpestes javanicus* (Wozencraft 2005), occurs naturally in the southern and southeastern regions of Asia, although it has been introduced to various parts of the world, including several islands in the Pacific and Indian Ocean and Caribbean and Mediterranean Seas (see reviews in Hoagland et al. 1989; Tvrtkovic and Krystufek 1990; Simberloff et al. 2000). Since its introduction, this predator's pervasiveness remains a serious threat to the preservation of insular biodiversity. Originally, the species was described as two separate taxa—the Javan mongoose, *H. javanicus* (E. Geoffroy Saint-Hilaire 1818), and the small Indian mongoose, *Herpestes auropunctatus* Hudgson 1836—although, recently, the names have been used interchangeably to represent a single species (e.g. Coblentz and Coblentz 1985; Vilella 1998; Thulin 2002, 2006) or to identify a subspecies (as in *Herpestes javanicus auropunctatus*; e.g. Wolcott 1953; Pascal et al. 2004). Veron et al. (2007) examined the evolutionary relationships among *H. javanicus*/*H. auropunctatus* and *Herpestes edwardsii* in their study, and provide phylogenetic evidence supporting the existence of the small Indian and Javan mongoose as two distinct species. According to the authors, *H. javanicus*–*H. auropunctatus* is not monophyletic: sequences from the species throughout its range separate into different clades, with the small Indian mongoose (*H. javanicus*) clade more closely related to the Indian gray mongoose, *H. edwardsii*, than to the Javan mongoose (*H. auropunctatus*). In fact, their data suggest that the easternmost edge of the small Indian mongoose's range ends at Myanmar, where the Javan mongoose's distribution begins (Veron et al. 2007). As suspected, mongooses from introduced populations grouped with *H. auropunctatus* (Veron et al. 2007). Consequently, we have used this taxonomic scheme to establish diagnostics to more precisely identify nonnative specimens collected from Jamaica and Hawaii.

Here, we analyze the nucleotide sequences of a fragment of the mitochondrial cytochrome *b* (*CYTB*) gene and apply character-based DNA barcoding methods (DeSalle et al. 2005; Rach et al. 2008) for five closely related sympatric Herpestidae species. Whereas the common target sequence for DNA barcoding is the cytochrome *c* oxidase subunit 1 (Hebert et al. 2003), the *CYTB* gene has been shown to function as an effective and suitable barcode region for discriminating among a diverse range of taxa at the species level (e.g. rabbitfishes, Lemer et al. 2007; or cetaceans, Amaral et al. 2007). Establishing a DNA-based identifications system for discriminating between *H. javanicus* and *H. auropunctatus* will improve our overall knowledge of the distribution of these species in both native and nonnative invasives, and thereby assist in the selection of appropriate management strategies for populations of the introduced mongoose species in its nonnative range.

Materials and methods

Samples

Tissue from 56 putative *H. javanicus* specimens deposited and maintained in the Ambrose Monell Cryo Collection at the American Museum of Natural History were sampled for the present study, representing individuals from multiple localities and islands in the species' introduced range in the Caribbean and Hawaiian Islands (Table I). Collection and research permits were legally obtained from relevant agencies and are available upon request. Taxonomic identification of the mongoose species in the introduced range followed Wozencraft (2005). Twenty-five sequences from other *H. javanicus* specimens from the species' native range, as well as 12 sequences from four native sympatric congeners (genus *Herpestes*), were obtained from GenBank (http://www.ncbi.nih.gov/Genbank; Table I).

DNA isolation, CYTB *amplification and sequencing*

Total genomic DNA was extracted from 56 skeletal tissue fragments collected in the field between 2002 and 2005 using the DNeasy tissue kit (QIAGEN, Valencia, California, USA) and eluted in water. Individual DNA extracts were subjected to PCR amplification of a 427 bp target region of the *CYTB* gene using primers modified from Kocher et al. (1989; SIMcytbF, 5′-Gaccaacatccgcaaatcaca-3′; SIMcytbR, 5′-Ggctcctca-gaatgatatttgacctca-3′).

The amplification reactions were carried out in a 25 μl mixture (1 μl genomic DNA, 0.48 μM each primer, 200 μM each dNTP, 0.5 units *Taq* polymerase [Fisher Scientific, Pittsburgh, PA, USA], and 1 × associated 10 × Fisher PCR buffer [500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.0]) on Mastercycler Gradient and Gradient *S* thermocyclers (Eppendorf, Hauppauge, New York, USA). The PCR conditions consisted of an initial denaturation step of 5 min at 94°C followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 49°C for 30 s, and extension at 72°C for 1 min, ending with a final a 5-min elongation at 72°C. PCR products were electrophoresed in 1.5% TBE agarose gels, stained with ethidium bromide, and visualized under UV light. PCR products were cleaned using the AMPure bead purification system (Agencourt Bioscience, Danvers, MA, USA) and used in 10 μl cycle sequencing

Table I. Tissue samples and GenBank *CYTB* sequences included in the molecular study.

Species	GenBank	Locality	Reference
H. javanicus	FJ848667	Jamaica, Hawaii	Present study
H. javanicus	FJ848668	Jamaica, St Croix USVI, Puerto Rico, Vieques, Hawaii	Present study
H. javanicus	FJ848669	Jamaica	Present study
H. javanicus	FJ848670	Jamaica	Present study
H. javanicus	FJ848671	Jamaica	Present study
H. javanicus	FJ848672	Jamaica	Present study
H. javanicus⋆	AB050128	Japan	Referred to in Veron et al. (2007)
H. javanicus⋆	AB050129	Japan	Referred to in Veron et al. (2007)
H. javanicus⋆	AB050130	Japan	Referred to in Veron et al. (2007)
H. javanicus⋆	AB050131	Japan	Referred to in Veron et al. (2007)
H. javanicus⋆	AF522338	Guyana	Referred to in Veron et al. (2007)
H. javanicus⋆	AY170108	Caribbean Islands	Referred to in Veron et al. (2007)
H. javanicus⋆	AY873843	Fiji	Referred to in Veron et al. (2007)
H. javanicus⋆	DQ519064	Myanmar	Veron et al. (2007)
H. javanicus⋆	DQ519065	Guyana	Veron et al. (2007)
H. javanicus⋆	DQ519067	Bangladesh	Veron et al. (2007)
H. javanicus⋆	DQ519068	Pakistan	Veron et al. (2007)
H. javanicus⋆	DQ519069	Croatia	Veron et al. (2007)
H. javanicus⋆	DQ519070	Myanmar	Veron et al. (2007)
H. javanicus⋆	DQ519071	Pakistan	Veron et al. (2007)
H. javanicus⋆	DQ519072	Bangladesh	Veron et al. (2007)
H. javanicus⋆	X94926	India	Referred to in Veron et al. (2007)
H. javanicus	AY928675	Vietnam	Referred to in Veron et al. (2007)
H. javanicus	DQ519057	Thailand	Veron et al. (2007)
H. javanicus	DQ519058	Thailand	Veron et al. (2007)
H. javanicus	DQ519059	Thailand	Veron et al. (2007)
H. javanicus	DQ519060	Vietnam	Veron et al. (2007)
H. javanicus	DQ519061	Thailand	Veron et al. (2007)
H. javanicus	DQ519062	Thailand	Veron et al. (2007)
H. javanicus	DQ519063	Thailand	Veron et al. (2007)
H. javanicus	DQ519073	Thailand	Veron et al. (2007)
*H. urva_*	DQ519074	Taiwan	Veron et al. (2007)
*H. naso_*	AF522339	Gabon	Referred to in Veron et al. (2007)
*H. edwardsii_*	AF522336	Bahrain	Referred to in Veron et al. (2007)
*H. edwardsii_*	DQ519050	Bahrain	Veron et al. (2007)
*H. edwardsii_*	DQ519053	United Arab Emirates	Veron et al. (2007)
*H. edwardsii_*	DQ519051	United Arab Emirates	Veron et al. (2007)
*H. edwardsii_*	DQ519049	United Arab Emirates	Veron et al. (2007)
*H. edwardsii_*	DQ519052	Iran	Veron et al. (2007)
*H. edwardsii_*	DQ519054	Bangladesh	Veron et al. (2007)
*H. edwardsii_*	DQ519055	Bangladesh	Veron et al. (2007)
*H. edwardsii_*	DQ519056	Bangladesh	Veron et al. (2007)
*H. edwardsii_*	AY170107	Unknown	Referred to in Veron et al. (2007)

Notes: Presented as species, GenBank accession number (GenBank), geographic locality (Locality), and source reference (Reference). 2mu⋆*H. auropunctatus* suggested by Veron et al. (2007).

reactions consisting of 1 μM of the same primers indicated above, an annealing temperature of 50°C, and using the BigDye Sequencing Ready Reaction Kit (Applied Biosystems, Inc., Carlsbad, California, USA). Cycle sequence reactions were carried out bidirectionally, and products were cleaned with 70% isopropanol and 70% ethanol and then resuspended in Montage Injection Solution (Millipore Corp., Bedford, MA, USA).

Cleaned sequence products were resolved on an Applied Biosystems, Inc. 3730xl DNA Analyzer, and both forward and reverse strands were aligned using Sequencher 4.06 (Gene Codes Corp., Ann Arbor, MI, USA) with further edits by eye. The resulting consensus sequences were verified using NCBI's basic local alignment search tool (Altschul et al. 1990). *CYTB* gene sequences from other *H. javanicus* individuals, as well as from *H. edwarsii*, *Herpestes naso*, and *Herpestes urva*, were obtained from GenBank. Final consensus and GenBank sequences were aligned using ClustalX 1.8 (Thompson et al. 1997) and edited in MacClade 4.06 (Maddison and Maddison 2003).

Phylogenetic analysis

Maximum parsimony was employed to infer relationships within *H. javanicus*. Parsimony searches were performed using PAUP⋆ 4.08.10 (Swofford 2003) using the random stepwise addition option of the heuristic search for 10 replicates with tree-bisection–reconnection branch swapping, the collapse of

zero-length branches, equal weighting of all characters, and the comparison of best trees at or below 387 steps (based on previous observation). If searches produce multiple trees, then a strict consensus is performed to summarize the relationships. Node support was assessed using the nonparametric bootstrap (Felsenstein 1985) and more specifically using stepwise addition of heuristic searches without the multiple trees (MulTrees) option in effect and keeping a subset of trees with scores greater than 387 for 1000 bootstrap replicates.

Construction of diagnosis matrix and analysis using characteristic attribute organization system

The characteristic attribute organization system (CAOS; Sarkar et al. 2008) was implemented in order to determine character-based diagnostic DNA sites for barcoding of the targeted *Herpestes* species from the sequence dataset. The CAOS-based algorithm is a fast protocol that retains evolutionary information contained in character-state data, and is much more complementary to traditional taxonomic methods than distance-based approaches (DeSalle et al. 2005). The diagnosis matrix used in the CAOS analysis was generated from a NEXUS format matrix compiled of the sequences obtained above (see Supplementary Table S1). The DNA sequences of the mongooses introduced to the Caribbean and Hawaiian Islands are given in Supplementary Table S2. We followed the suggestion of Veron et al. (2007) that the currently recognized *H. javanicus* represents not one, but two species—*H. javanicus* and *H. auropunctatus*—and compiled the DNA sequence information for all five closely related species to the introduced mongoose (*H. javanicus*, *H. auropunctatus*, *H. urva*, *H. naso*, and *H. edwardsii*) into a NEXUS file as described for the CAOS program by Rach et al. (2008; available from http://www.genomecurator.org/CAOS/PGnome/PGnomeindex.html). The P-Gnome application option in CAOS was selected to search for characteristic attributes and generate diagnostic rules for the 427 bp stretch of the *CYTB* gene in order to classify the sequence dataset. These diagnostics were then used to search for states in the *CYTB* sequences recovered from introduced mongooses in the Caribbean and Hawaiian Islands.

Results

CYTB *results*

CYTB was sequenced from all 56 introduced mongoose specimens, and sequences ranged in length from 411 to 420 bp. This sampling regime includes mongoose individuals representing 16 localities from four Caribbean islands and two Hawaiian Islands in the species' introduced range. No insertions, deletions, or heteroplasmy within the sequences were detected. The sequences obtained in the present study corresponded in length and sequence arrangement with known *CYTB* sequences from the species (*H. javanicus*), genus (*Herpestes* spp.), and family (Herpestidae), thereby supporting the authenticity of the sequences of mitochondrial origin rather than from nuclear mitochondrial DNA pseudogenes (numts). Six different *CYTB* haplotypes were observed among *H. javanicus* individuals from the Caribbean and Hawaiian populations (Table I). All of the recovered haplotypes were observed in Jamaica, whereas only two were found in Hawaii and a single haplotype obtained from Puerto Rico, Vieques, and St Croix. When these sequences were combined with those obtained for *H. urva*, *H. naso*, *H. edwardsii*, and other *H. javanicus* from GenBank, the dataset of 68 sequences yielded 38 different haplotypes.

Phylogenetic analysis

Maximum parsimony analysis yielded nine equally parsimonious trees. A strict consensus of the trees identified a division within *H. javanicus* relating to the separation of the species as *H. javanicus* and *H. auropunctatus* supported by high bootstrap values, as suggested by Veron et al. (2007; Figure 1). *CYTB* haplotype sequences from introduced putative *H. auropunctatus* obtained for the present study grouped with the native *H. auropunctatus* clade, thereby verifying species identity for introduced populations in the Caribbean and Hawaiian Islands.

Generating diagnostics from a short stretch of the CYTB *gene*

The P-Gnome option in CAOS generated 20 pure diagnostics (unambiguous character attributes that are shared by all members of a pre-described group) from the *CYTB* dataset. We designate these diagnostics using a number that refers to the position in the *CYTB* fragment and a letter that refers to the diagnostic state of the position for each species. Of the 20 sites, 13 were diagnostic for the distant mongoose species *H. urva* (13-T, 31-C, 73-T, 145-T, 149-G, 178-C, 223-C, 226-T, 247-T, 256-T, 283-C, 289-C, and 295-C,T) and *H. naso* (4-C, 11-G, 16-C, 40-C, 106-T, 244-T, and 265-A). Four diagnostics were found for *H. javanicus* (4-T, 11-A, 40-T, and 244-C); however, a single diagnostic site (106-C) separated the species into two groups, which matches Veron et al.'s (2007) recognition of *H. auropunctatus* as a distinct species and the true identity of the small Indian mongoose. Three diagnostics were recovered for *H. edwardsii* (16-T, 149-C, and 265-G).

When the DNA sequence dataset from the short stretch of the *CYTB* gene from the introduced mongoose specimens was searched for the presence or absence of the above diagnostics, the six haplotypes from these introduced populations all possessed the

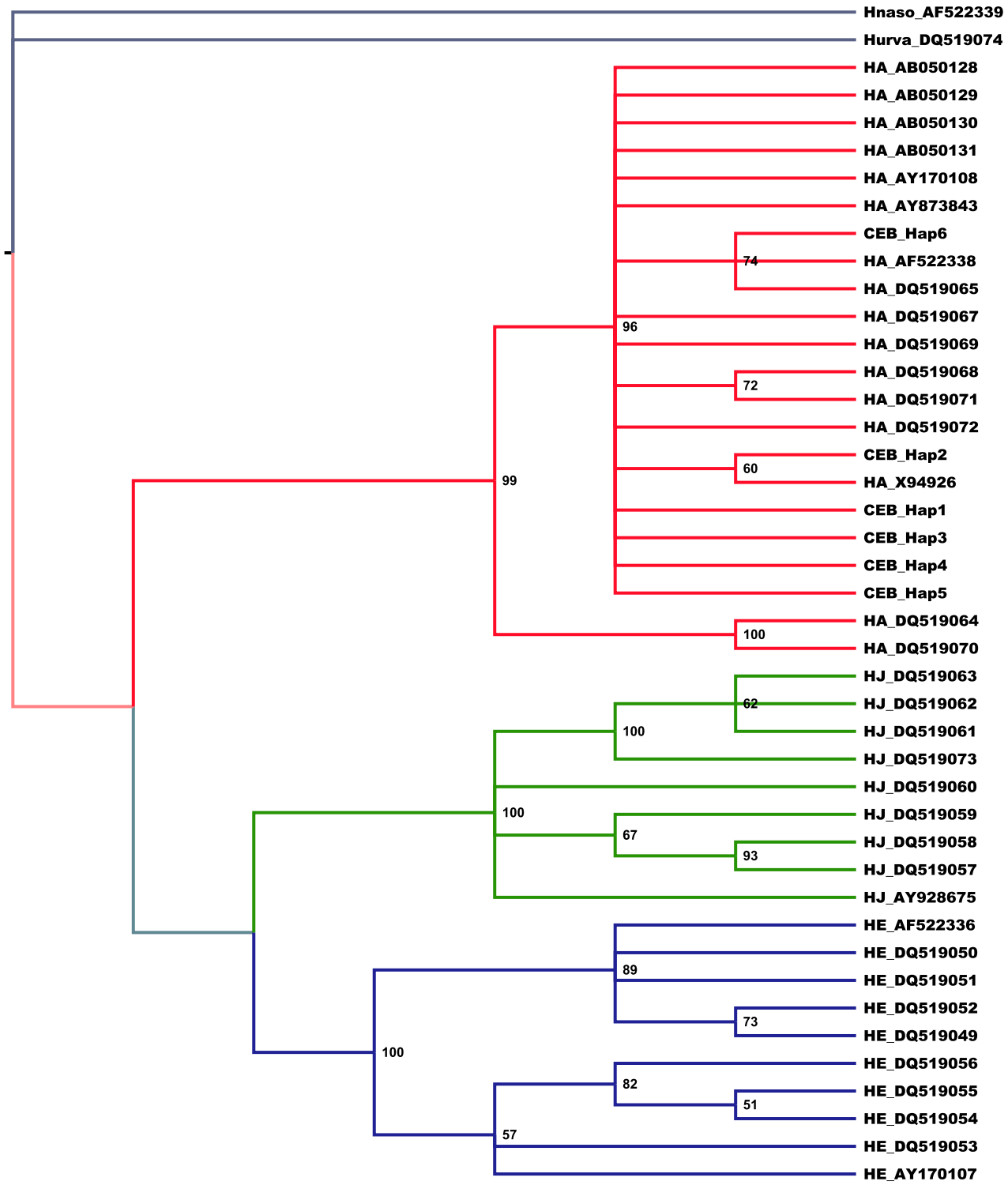

Figure 1. Strict consensus of the nine most parsimonious phylogenetic trees inferred through maximum parsimony from *CYTB* sequences. Taxa are represented by nomenclature abbreviations and GenBank accession numbers: *H. edwardsii* (HE), *H. naso* (Hnaso), *H. urva* (Hurva); annotations of HJ (*H. javanicus*) and HA (*H. auropunctatus*) refer to suggestions by Veron et al. (2007); CEB haplotype identifications refer to *CYTB* partial sequences generated during this study. Node support values (1000 bootstrap replicates) greater than 50% are shown on the respective branches.

single diagnostic character of *H. auropunctatus* and lacked the diagnostics of the other species.

Discussion

The use of *CYTB* sequences as DNA barcodes proved to be a reliable tool for the identification of the five distinct *Herpestes* species in the present study. In spite of the low intraspecific sequence variation observed, interspecific differentiation was significant enough to resolve species-level relationships and provide for the species assignments of specimens of invasive mongoose. Although CAOS is a relatively new approach (Rach et al. 2008), it was sufficiently sensitive enough to recover several diagnostics for the existing taxa at the species level for *H. naso*, *H. urva*, *H. edwardsii*, and *H. javanicus*. In addition, this character-based framework has the potential of being able to identify semi-cryptic species, such as with the discovery of a single diagnostic site identifying mongoose specimens into the *H. auropunctatus* group as described by Veron et al. (2007), thereby complementing previous morphological and molecular analyses and demonstrating the utility of combining systematics and traditional taxonomic methods for diagnosing species (DeSalle et al. 2005; DeSalle 2006, 2007; Rubinoff 2006).

The sharing of diagnostic sites of mongooses in the introduced Caribbean and Hawaiian populations with native *H. auropunctatus* is a strong indicator that the introduced animals were derived from *H. auropunctatus* individuals rather than from *H. javanicus*. Thus, consideration should be given to readdress the classification of introduced mongoose populations in the Caribbean and Hawaii as *H. auropunctatus* (the small Indian mongoose) rather than *H. javanicus*, which is commonly referred to as the small Javan mongoose.

In addition to identifying introduced mongoose specimens to species, our results provide support for the potential to go a step further to identify country of origin for introduced invasive mongoose. In the case of exotic populations of the small Indian mongoose in the Caribbean and Hawaiian Islands, it is possible not only to identify the species as *H. auropunctatus*, but also to use the observed *CYTB* sequence variation to identify possible countries of origin of the introduced species from its native range. Based on the diagnostics designated from the *CYTB* sequences, it is possible that the origin of the founding colonies of the small Indian mongoose in the Caribbean and Hawaii can be narrowed to Burma, Pakistan, and India. However, this must be taken with several caveats. Given the small sample size, limited geographic representation in the native range, and the low degree of *CYTB* sequence variability of our reference sequences to identify diagnostics, it is difficult to exclude other countries with certainty. In order to strengthen the applicability of barcodes as a tool to narrow areas of origin for introduced populations of the small Indian mongoose, it will be necessary to increase the representation of the various taxa sampled from a broader geographic region (e.g. within a country) within the species' native range so as to adequately reveal the extent of the intraspecific variation for comparison; and increase the amount of sequence information perhaps adding more rapidly evolving markers such as mitochondrial D-loop or microsatellites.

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