Molecular Diagnosis of Atlantic Forest Mammals Using Mitochondrial DNA Sequences: Didelphid Marsupials

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Abstract: Most living species of New World marsupials are classified in the family Didelphidae, with 98 species and 18 genera currently recognized. We sequenced fragments of two mitochondrial genes of didelphid marsupials from the Atlantic Forest of eastern South America, a biodiversity hotspot. We evaluated sequence divergences within and among species and contrasted the efficiency of cytochrome c oxidase subunit I (COI) with cytochrome b (CytB) in species-level diagnosis. The average intraspecific genetic divergence of COI and CytB was 2.0% and 1.9%, respectively; which was about five times lower than the comparison among species of the same genus (11.2 and 10.8%). In both genes, divergence levels among closely related species are usually higher than within species. The barcoding gap is similar in COI and CytB, indicating that either gene can be used in molecular diagnoses of didelphid species. DNA barcodes are a welcome addition to traditional taxonomic methods when viewed as additional diagnostic characters in the context of integrative taxonomy.

Keywords: Cytochrome b, Cytochrome oxidase I, DNA barcodes, Integrative taxonomy, Mammals.

INTRODUCTION

Most living species of New World marsupials are classified in the family Didelphidae, which has a geographic range from southeastern Canada to southern Argentina. They are important components of the Neotropical mammalian fauna, usually comprising the third most diverse group (after bats and rodents) at a given rainforest locality [1]. There were 91 species and 18 genera of didelphid marsupials recognized in a recent taxonomic compilation [2]. In the face of such diversity, the paucity of studies on systematic limits and appropriate species diagnosis is remarkable [3]. Nevertheless, didelphid taxonomy has been very dynamic in recent decades. The number of recognized species increased 36% from 69 to 94 in 12 years when we compare subsequent editions of the most influential taxonomic reference to living mammals of the world [4, 5]. This increase is mainly due to the description of new species, or the split of widespread taxa formerly lumped into one polytypic species. Recent systematic revisions have even resulted in the description of new didelphid genera, such as *Chacodelphys* and *Cryptonanus* [6, 7]. In addition, several classification schemes above the species level have been proposed throughout the years, but robust phylogenetic analyses based on abundant molecular and morphological data and dense taxon sampling has only just become available [8].

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Knowledge about species diversity, as well as the evolutionary relationships among species can only be acquired with appropriate taxonomic identification at the species level. Traditional morphological analyses have been used extensively for centuries as a successful tool for diagnosing species [9]. However, many species have phenotypic plasticity, sexual dimorphism or ontogenetic changes in morphological characters, hindering species identification [10]. Cryptic species are common in many groups and their identification has always been a challenge, but the advent of relatively inexpensive and rapid DNA sequencing has given biologists a new tool for detecting and differentiating morphologically similar species [11]. In these cases, reliable species identification can be achieved with the analysis of a short segment from the genome, or a specific DNA barcode [12]. DNA barcodes offer additional diagnostic characters, and may be exceptionally helpful in species identification, especially when integrated with traditional morphological approaches [13].

The cytochrome c oxidase subunit I (COI) mitochondrial region has emerged as the standard barcode region for animals, including mammals (www.mammaliabol.org). The selection of this gene fragment is based on general characteristics of mitochondrial DNA, the standard choice of genome to use in phylogeographic studies. These features include rapid accumulation of mutations, lack of introns, high number of copies per cell, negligible recombination rate, and haploid inheritance [14]. Most importantly, the efficiency of DNA barcodes depends on the existence of a large barcoding "gap" between intra- and interspecific variation [15]. Interspecific divergences in COI sequences are significantly

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higher than intraspecific variation in many groups of animals [16, 17], but there are exceptions [18, 19]. COI sequences have been used in diagnosing species in many animal groups, including mammals [20, 21]. Only a few didelphid species have been characterized using standardized barcoding protocols in one study [21], based on 32 specimens from northern South America (mainly from Surinam and Guyana) and a few scattered points in Central America. In the present paper, we expand the COI sequence database of didelphid marsupials to species from the Atlantic Forest of eastern South America, one of the top biodiversity hotspots in the world [22]. Our main goals were to evaluate sequence divergences within and among didelphid species and to compare the efficiency of COI in species diagnosis with the mitochondrial cytochrome b gene (CytB), the traditional marker in species level taxonomy of mammalian species [23].

MATERIAL AND METHODS

DNA was extracted from tissue samples (muscle or liver) fixed in ethanol and housed at Universidade Federal do Espírito Santo (UFES), Vitória, Brazil and Museu de Biologia Professor Mello Leitão (MBML), Santa Teresa, Brazil. We followed the taxonomy of Gardner [2], and not the most recent revision by Voss and Jansa [8]. Voss and Jansa [8] treated *Micoureus* as a subgenus of *Marmosa* in order to keep *Marmosa* as a monophyletic genus. In contrast, we keep *Micoureus* as a full genus, which leaves *Marmosa* paraphyletic, but future studies will probably resolve this situation by recognizing additional genera. The monophyly of *Micoureus* is well supported [8] and warrants recognition at the taxonomic level of genus and not subgenus.

The COI gene was sequenced from 73 specimens belonging to 11 species and 10 genera of didelphid marsupials. Additional COI sequences used in the present paper are from Borisenko *et al.,* [21] and are available online at the Barcode of Life Data System (BOLD; www.barcodinglife.org), in the projects 'Small mammal survey in Bakhuis, Suriname (ABSMS)' and 'Small mammal survey in Bakhuis reference sequences (ABSMC)'. Total taxonomic diversity for COI was 26 species in 10 genera. CytB sequences were obtained from 70 specimens belonging to 12 species of 10 didelphid genera. There were 55 of 89 specimens that had both COI and CytB sequenced. The results presented in this paper are part of the project 'Barcoding Atlantic Forest Opossums (BATFO)', which is also available in BOLD. All sequences are deposited in GenBank (www.ncbi.nlm.nih.gov) and are associated to voucher specimens (Appendix), which were identified to the species level using morphological characters. Detailed museum and locality data of voucher specimens are also available in BOLD.

DNA was extracted using a salt protocol [24], and the product was quantified in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.). Samples with high concentration were diluted to 100 ng/μL with ddH_2O. Both COI (657 bp) and CytB (801 bp) sequences were amplified through the polymerase chain reaction (PCR). The 25 μL PCR reaction solution included 2.5 μL of 10× PCR buffer, 1.0 μL of $MgCl_2$ (50 mM), 0.5 μL of dNTP mixture (10 mM each oligonucleotide), 0.3 μL of Platinum Taq DNA polymerase (Invitrogen), 0.3 μL of each primer (10 μM), and 2 μL of DNA template (100 ng/μL). For the COI amplification, we used the forward primer LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'), and the reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') [25] under the following PCR profile: 94 °C for 5 min, followed by 39 cycles of 94 °C for 30 s, 44 °C for 45 s, 72 °C for 45 s, and a final cycle of 72 °C for 5 min. For the CytB amplification, we used the forward primer MVZ05 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and the reverse primer MVZ16 (5'- AAATAGGAARTATCAYTCTGGTTTRAT-3') [26] under the following PCR regime: 94 °C for 5 min, followed by 39 cycles of 94 °C for 30 s, 48 °C for 45 s, 72 °C for 45 s, and a final cycle of 72 °C for 5 min. PCR products were purified using enzymes ExoSap-IT (USB Corporation) and cycle sequenced using BigDye Terminator 3.1 (Applied Biosystems, Inc.) during 25 cycles of 95 °C for 30 s, 50 °C for 15 s, 60 °C for 4 min. After precipitation in isopropanol/ethanol, the product was sequenced in both directions using an automated capillary sequencer ABI 310 (Applied Biosystems, Inc.).

COI and CytB sequences were aligned using ClustalW in MEGA 4.0 [27]. Neighbor-joining (NJ) trees based on the Kimura two-parameter distance model (K2P) were generated in MEGA 4.0 and support for each node was estimated using 100 bootstrap replicates. Sequences were submitted to species identification using the Identification Engine in BOLD (BOLD-IDS). This engine accepts sequences from the 5' region of the mitochondrial gene COI, and returns a species-level identification when one is possible. Inter and intraspecific divergences were calculated using K2P in BOLD.

RESULTS

In the overall COI NJ tree (Fig. **1**), specimens of the same species always grouped together with high bootstrap support (99%), except for *M. demerarae*. Two specimens from Ecuador (ROM104494 and ROM105521) identified in BOLD as *M. demerarae* grouped together, but not with the remaining *M. demerarae*. Species of the same genus also grouped together, except for *Marmosops*, but with lower bootstrap support (<81%; Fig. **1**). There is generally no support for intergeneric groupings, with the exception of the large opossums *Didelphis*, *Philander*, and *Chironectes* at 79-91% (Fig. **1**). Individuals of the same species also always grouped together in the CytB NJ tree (Fig. **2**), with high bootstrap support (>98%). Species of *Monodelphis* also clustered together with high bootstrap support (81%), and *Philander frenatus* and *Didelphis aurita* formed a well-supported group (93%).

The average intraspecific COI divergence was 2.0%, which was more than five times less than interspecific variation within each genus (11.2%), and almost ten times less than the divergence among genera of the same family (19.5%) (Table **1**). *Micoureus demerarae* showed the highest average intraspecific divergence (5.0%) followed by *Gracilinanus microtarsus* (4.2%), whereas *Didelphis aurita* showed negligible intraspecific variation (0.1%). Some species showed extreme values of intraspecific COI divergences, ranging from 0 to 9.2% in *Metachirus nudicaudatus*, 0 to 8.4% in *M. demerarae*, and 0.5 to 7.2% in *G. microtarsus*.

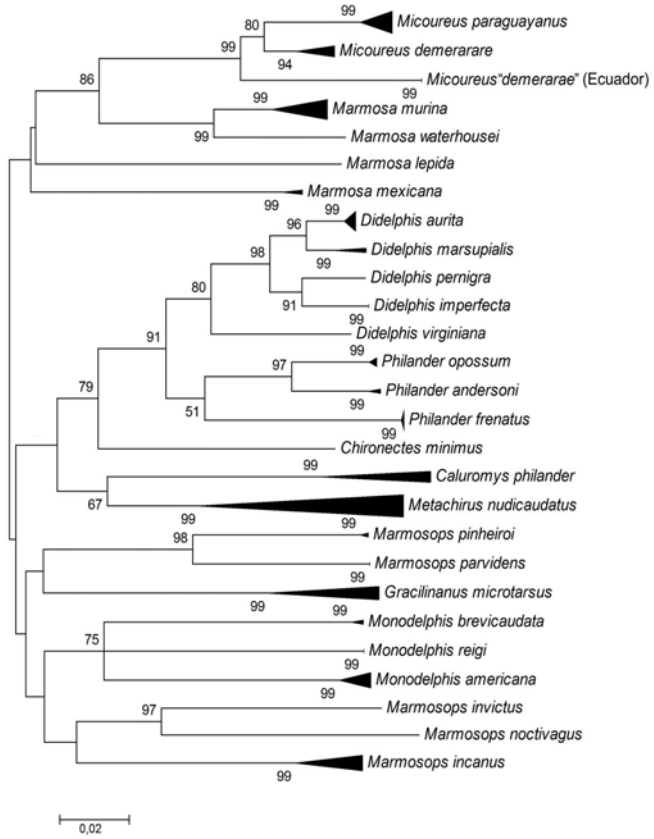

Fig. (1). Neighbor-joining tree constructed using sequences of the cytochrome oxidase subunit I (COI) gene of didelphid species. Numbers represent bootstrap support values (%).

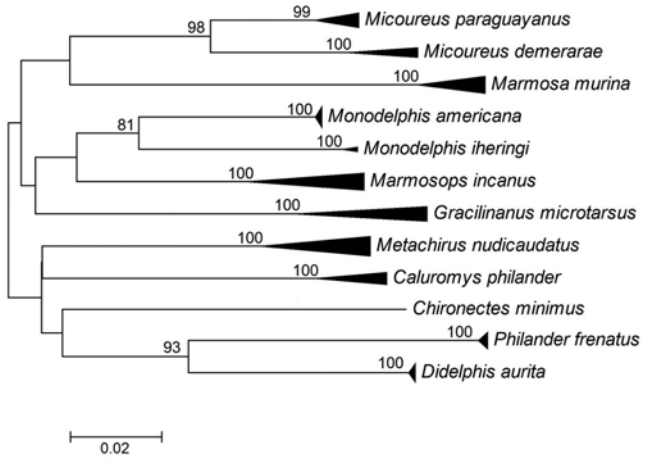

Fig. (2). Neighbor-joining tree constructed using sequences of the cytochrome b (CytB) gene of didelphid species. Numbers represent bootstrap support values (%).

The average intraspecific CytB divergence (1.9%) was virtually the same as the COI gene (Table **1**). The average CytB divergence among species of the same genus (10.8%) was more than five times higher than the intraspecific divergence, and the average distance among genera of the same family (21.3%) was more than ten times higher than the intraspecific distance. The lowest average CytB divergence was found within *Monodelphis americana* (0.2%) and the highest in *Marmosops incanus* (5.0%). The highest value within species was observed in *M. incanus* (9.1%).

The taxonomic identification of all COI sequences matched with identifications on BOLD-IDS database. How-

Table 1. Genetic Distances of the Cytochrome c Oxidase Subunit I (COI) and the Cytochrome b (CytB) Genes of Ten Species and within Three Taxonomic Ranks of Didelphid Marsupials. n = number of Sequences

Species/Rank	COI Genetic Distance (%)				CytB Genetic Distance (%)			
	n	Minimum	Maximum	Mean	n	Minimum	Maximum	Mean
Caluromys philander	5	0.3	4.7	3.1	5	0.5	6.2	3.8
Didelphis aurita	9	0.0	0.3	0.1	8	0.0	0.8	0.4
Gracilinanus microtarsus	6	0.5	7.2	4.2	6	0.0	8.7	3.7
Marmosa murina	9	0.0	4.0	2.4	7	0.0	5.6	2.9
Marmosops incanus	7	0.2	4.6	3.1	7	0.5	9.1	5.0
Metachirus nudicaudatus	10	0.0	9.2	3.9	8	0.4	6.1	2.5
Micoureus demerarae	7	0.0	8.4	5.0	4	0.8	4.3	3.1
Micoureus paraguayanus	10	0.2	2.3	1.3	6	0.4	2.6	1.6
Monodelphis americana	7	0.0	2.0	0.7	9	0.0	0.4	0.2
Philander frenatus	9	0.0	0.6	0.3	7	0.0	1.3	0.4
Within species	99	0.0	9.2	2.0	69	0.0	9.1	1.9
Among species	106	2.5	20.9	11.2	69	8.3	13.5	10.8
Among genera	107	10.3	24.9	19.5	70	12.9	25.9	21.3

ever, two specimens of *M. demererae* from Ecuador (ROM 104494 and ROM 105521) probably represent a different species not currently on BOLD (see Lim, this issue).

DISCUSSION

In the current paper, we added 3 genera (*Caluromys*, *Chironectes*, and *Gracilinanus*) and 8 species (*Caluromys philander, Chironectes minimus, Gracilinanus microtarsus, Marmosops incanus, Micoureus paraguayanus, Monodelphis americana, Philander frenatus*, and *Didelphis aurita*) to the published COI database in BOLD, increasing the taxonomic coverage of didelphid marsupials to 10 genera and 26 species, which represents over half of the genera and one third of the current species recognized in this group. In addition, we expanded sample sizes within species and especially the geographic coverage to include the Atlantic Forest of eastern South America, an area of unique biodiversity with many endemic species [22].

Previous molecular studies suggested genetic distances among congeneric species are usually above 2–3% for both COI and CytB [28, 29]. In didelphids, the average CytB divergence among congeneric species reaches more than 15% in some genera, such as *Marmosa* and *Micoureus* [30], which is similar to the interspecific divergences found here. But the overall average within species was relatively high (1.9–2.0%) for both genes. These intraspecific divergences are, however, slightly overestimated because of our sampling strategy. Since our sample size was small (usually 5–10 specimens/species), our priority was to sample sequence variation across the geographic range of each species, so we chose specimens from distant localities whenever possible. This probably inflated the intraspecific distances when compared to a large sample of specimens randomly taken from the species distribution.

Our COI results suggest that the two *M. "demerarae"* from Ecuador (ROM104494 and ROM105521) are likely to represent another species because they did not group with the other *M. demerarae* in the NJ tree and they show relatively high genetic distances from them (7.6–8.3%). In addition, current data indicates that *M. demerarae* does not occur in Ecuador [2]. This is the kind of situation where molecular data are very helpful in diagnosing species, but the true identity of the two ROM specimens can only be confirmed by the examination of the voucher specimens in a comprehensive taxonomic revision of the genus.

Some didelphid species have an old evolutionary history, and we expect more genetic variation and eventually more phylogeographic structure in older diverged than more recently diverged species [31]. For example, one specimen of *M. nudicaudatus* from Surinam (ROM117525) and another one from Guyana (ROM111938), showed very high divergence values (8.6–9.0%) when compared to the remaining specimens (<2.8%). The high genetic divergences of *M. nudicaudatus* from the Guyana shield, when compared to specimens from the Brazilian shield, has been pointed out by other authors [3, 30, 32]. These high levels of genetic divergence associated with deep phylogeographic discontinuities across its range, coupled with morphological and morphometric analyses of museum specimens (Carlos L. G. Vieira, unpublished data) suggest that *Metachirus nudicaudatus* is a composite and should be split into several species.

Although high values of intraspecific genetic divergences suggest that more than one species might be involved, additional data do not always confirm this trend. For example, when analyzing COI sequences of *G. microtarsus*, two specimens from southeastern Brazil (LPC822 and YL01) inflated the divergence values (4.8–7.1%), in relation to comparisons without these specimens (0.4–2.3%). Using CytB sequences, Costa *et al.*, [33] proposed the possibility of two species within *G. microtarsus*, but a recent reassessment of morphological and genetic variation across the range of *G. microtarsus* (Simone L. Freitas, unpublished data) found incongruence among mitochondrial DNA sequences and morphological character variation, concluding that this taxon should be treated as a single species with deep phylogeographic structure.

The present study has shown that both COI and CytB are useful in providing characters for molecular diagnosis of didelphid species, confirming that divergence levels among closely related species are usually higher than within species in both mitochondrial genes. The barcoding gap is almost the same for COI and CytB, indicating that either gene can be used in molecular diagnosis of didelphids. Recent research [34] suggests that mitochondrial bioenergetics plays a key role in multiple basic cellular processes and could be a candidate genetic mechanism of speciation. Under this scenario, mitochondrial sequences could be responsible for undermining the reproductive compatibility within a species when they conflict with sequences in the nucleus, which differ by an order of magnitude in their mutation rates [34]. This is a new perspective, which associates the DNA barcoding pattern with the process of speciation, but it still remains to be confirmed [35]. Nonetheless, DNA barcodes are a welcome addition to traditional taxonomic methods when viewed as additional diagnostic characters in the context of an integrative taxonomy [9, 13].

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APPENDIX. GenBank Accession Numbers for Cytochrome c Oxidase Subunit I (COI) and Cytochrome b (CytB) Sequences Generated in this Study with Association to Species, Sample Number and Barcode Number Catalogued in the Barcode of Life Data System (BOLD).

Species	Sample ID	Barcode ID	Genbank Accession Numbers	
			COI	CytB
Caluromys philander	LPC494	BATFM001-09	GU112788	GU112877
Caluromys philander	LPC705	BATFM002-09	GU112787	GU112876
Caluromys philander	LC97	BATFM363-09	GU112784	GU112873
Caluromys philander	YL267	BATFM003-09	GU112786	GU112875
Caluromys philander	SLF65	BATFM004-09	GU112785	GU112874
Chironectes minimus	LC195	BATFM005-09	GU112789	GU112878
Didelphis aurita	LPC1035	BATFM006-09	GU112796	–
Didelphis aurita	LPC993	BATFO001-09	–	GU112880
Didelphis aurita	LGA1285	BATFO002-09	–	GU112879
Didelphis aurita	LPC806	BATFM007-09	GU112790	GU112881
Didelphis aurita	LC94	BATFM364-09	GU112795	–
Didelphis aurita	LPC970	BATFM008-09	GU112792	GU112883
Didelphis aurita	LPC984	BATFM009-09	GU112794	–
Didelphis aurita	SLF01	BATFM010-09	GU112793	GU112884
Didelphis aurita	YL450	BATFM011-09	GU112798	GU112886
Didelphis aurita	LPC864	BATFM012-09	GU112791	GU112882
Didelphis aurita	YL297	BATFM013-09	GU112797	GU112885
Gracilinanus microtarsus	LPC1091	BATFM014-09	GU112804	GU112892
Gracilinanus microtarsus	LPC1204	BATFM015-09	GU112803	GU112891
Gracilinanus microtarsus	LPC1074	BATFO003-09	–	GU112889
Gracilinanus microtarsus	LPC955	BATFM016-09	GU112802	–
Gracilinanus microtarsus	YL01	BATFM017-09	GU112801	–
Gracilinanus microtarsus	YL237	BATFM018-09	GU112800	GU112890
Gracilinanus microtarsus	LPC822	BATFM019-09	GU112799	–
Gracilinanus microtarsus	YL515	BATFO004-09	–	GU112888
Gracilinanus microtarsus	LGA1326	BATFO005-09	–	GU112887
Marmosa murina	LPC542	BATFM020-09	GU112811	GU112899
Marmosa murina	SLF280	BATFM021-09	GU112810	GU112898
Marmosa murina	YL580	BATFM022-09	GU112809	GU112897
Marmosa murina	LC29	BATFM023-09	GU112808	GU112896
Marmosa murina	LPC396	BATFM024-09	GU112807	GU112895
Marmosa murina	SLF08	BATFM025-09	GU112806	GU112894
Marmosa murina	YL265	BATFM026-09	GU112805	GU112893
Marmosops incanus	LPC1080	BATFM027-09	GU112818	GU112906

(APPENDIX) Contd....

Species	Sample ID	Barcode ID	Genbank Accession Numbers	
			COI	CytB
Marmosops incanus	LPC201	BATFM029-09	GU112816	GU112904
Marmosops incanus	LPC954	BATFM030-09	GU112815	GU112903
Marmosops incanus	YL444	BATFM031-09	GU112814	GU112902
Marmosops incanus	LC81	BATFM028-09	GU112817	GU112905
Marmosops incanus	LC22	BATFM032-09	GU112813	GU112901
Marmosops incanus	LC49	BATFM033-09	GU112812	GU112900
Metachirus nudicaudatus	LPC935	BATFM035-09	GU112826	GU112914
Metachirus nudicaudatus	MBML2447	BATFO006-09	–	GU112907
Metachirus nudicaudatus	LPC997	BATFM036-09	GU112825	–
Metachirus nudicaudatus	YL268	BATFM037-09	GU112824	GU112913
Metachirus nudicaudatus	YL82	BATFM366-09	GU112820	GU112909
Metachirus nudicaudatus	YL77	BATFM365-09	GU112821	GU112910
Metachirus nudicaudatus	YL577	BATFM038-09	GU112823	GU112912
Metachirus nudicaudatus	LPC548	BATFM039-09	GU112819	GU112908
Metachirus nudicaudatus	YL35	BATFM040-09	GU112822	GU112911
Micoureus paraguayanus	LPC1006	BATFM042-09	GU112839	GU112924
Micoureus paraguayanus	LPC326	BATFM044-09	GU112838	GU112923
Micoureus paraguayanus	YL449	BATFM047-09	GU112837	–
Micoureus paraguayanus	LPC792	BATFM049-09	GU112836	GU112922
Micoureus paraguayanus	LC61	BATFM041-09	GU112840	–
Micoureus paraguayanus	LPC1046	BATFO007-09	–	GU112919
Micoureus paraguayanus	YL39	BATFM052-09	GU112833	GU112921
Micoureus paraguayanus	YL75	BATFM367-09	GU112832	GU112920
Micoureus paraguayanus	YL81	BATFM368-09	GU112831	–
Micoureus paraguayanus	MBML2370	BATFM050-09	GU112835	–
Micoureus paraguayanus	MBML2375	BATFM051-09	GU112834	–
Micoureus demerarae	LPC218	BATFM043-09	GU112830	–
Micoureus demerarae	LPC446	BATFM045-09	GU112829	GU112918
Micoureus demerarae	LPC723	BATFM048-09	GU112827	GU112916
Micoureus demerarae	LPC514	BATFM046-09	GU112828	GU112917
Micoureus demerarae	LPC209	BATFO008-09	–	GU112915
Monodelphis americana	LC60	BATFM053-09	GU112847	–
Monodelphis americana	LPC1114	BATFM054-09	GU112846	GU112933
Monodelphis americana	LPC1130	BATFM056-09	GU112845	GU112932
Monodelphis americana	LPC1181	BATFM057-09	GU112844	GU112931
Monodelphis americana	LPC999	BATFM058-09	GU112843	GU112930

(APPENDIX) Contd....

Species	Sample ID	Barcode ID	Genbank Accession Numbers	
			COI	CytB
Monodelphis americana	MBML2704	BATFM062-09	GU112842	GU112929
Monodelphis americana	MBML2710	BATFM063-09	GU112841	–
Monodelphis americana	LPC991	BATFO009-09	–	GU112928
Monodelphis americana	LPC1045	BATFO010-09	–	GU112927
Monodelphis americana	LPC990	BATFO011-09	–	GU112926
Monodelphis americana	LPC1028	BATFO012-09	–	GU112925
Monodelphis iheringi	LPC1124	BATFM055-09	–	GU112935
Monodelphis iheringi	MBML2346	BATFM061-09	–	GU112934
Philander frenatus	LPC1127	BATFM064-09	GU112856	GU112942
Philander frenatus	LPC944	BATFM065-09	GU112855	GU112941
Philander frenatus	ORG01	BATFO013-09	–	GU112937
Philander frenatus	YL232	BATFM066-09	GU112854	–
Philander frenatus	YL52	BATFM067-09	GU112850	–
Philander frenatus	YL107	BATFM369-09	GU112848	GU112938
Philander frenatus	YL573	BATFM068-09	GU112853	GU112940
Philander frenatus	YL579	BATFM069-09	GU112852	–
Philander frenatus	LPC877	BATFM070-09	GU112851	GU112939
Philander frenatus	YL572	BATFM071-09	GU112849	–
Philander frenatus	LGA1196	BATFO014-09	–	GU112936

CONFLICT OF INTEREST

None declared.

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