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DNA-based identification applied to Thamnophilidae (Passeriformes) species: the first barcodes of Neotropical birds

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RESUMO. **Identificação baseada em DNA aplicada a espécies de Thamnophilidae (Passeriformes): primeiros *barcodes* de pássaros Neotropicais.** O Consórcio "Barcode of Life" é uma iniciativa mundial dedicada a produzir um inventário molecular da biodiversidade da Terra. A subunidade I do gene Citocromo Oxidase *c* (COI) do DNA mitocondrial (mtDNA) tem sido utilizada com sucesso na identificação de espécies de aves norte-americanas. No entanto, esta técnica não foi previamente empregada na identificação de pássaros Neotropicais. Os Thamnophilidae habitam florestas Neotropicais e mais de 200 espécies pertencem a essa família. Desde 1990, 19 novas espécies foram reconhecidas nesta família. Nesse estudo, mostramos que é possível discriminar as 16 espécies de Thamnophilidae analisadas usando uma pequena seqüência do gene COI. As divergências obtidas entre espécies, mesmo as congenéricas, são muito maiores do que as observadas dentro de espécies. Apesar de se acreditar que pássaros tropicais possam apresentar alta diversidade genética intra-específica, concluímos que uma pequena seqüência do gene COI tem o potencial de ser usada como "barcode", identificando com confiança as diferentes espécies. Isto torna possível a distinção de espécies que exibem poucos caracteres morfológicos informativos, bem como a implementação de inventários rápidos, em larga escala, da pouco conhecida avifauna Neotropical.
PALAVRAS-CHAVE: *Barcode*, Thamnophilidae, DNA mitocondrial, pássaros Neotropicais.

ABSTRACT. The "Barcode of Life" Consortium is a worldwide initiative devoted to perform a molecular inventory of Earth's biodiversity. The Cytochrome Oxidase *c* subunit I (COI) gene of the mitochondrial DNA (mtDNA) has been successfully used for identification of North American bird species. However, this technique has not been previously employed to Neotropical birds' identification. Typical antbirds (Thamnophilidae) inhabit Neotropical forests and comprise over 200 species. Since 1990, 19 new species have been recognized in the Thamnophilidae family, thus making it a good candidate for taxonomic studies. In this study, we showed that it is possible to discriminate 16 Thamnophilidae species using a small COI gene region. Divergences obtained between species, even congeneric ones, were much higher than the observed within species. Although it is generally believed that tropical birds tend to show high intraspecific genetic diversity, we concluded that the COI barcodes have the potential to identify accurately Neotropical birds. It makes possible the discrimination of species that exhibit few informative morphological characters and the implementation of fast large-scale inventories of the barely known Neotropical avifauna.
KEY WORDS: Barcode, Thamnophilidae, mitochondrial DNA, Neotropical birds.

Reliable species identification is fundamental for research in the fields of systematic, zoogeography, ecology, and conservation (Isler *et al.* 1998), particularly for endemic taxa from endangered biomes. The accurate discrimination of species can only be achieved with an adequate methodology for taxonomic identification considering intra and inter-species variation. It is known that in some cases morphological analysis *per se* has not enough resolution to allow a correct identification of organisms (Hebert *et al.* 2004a). Furthermore, since classical taxonomy is frequently based on the work of specialized researchers, there are occasions when they are not easily accessible. Because of these issues, molecular markers have been used as a complementary tool for taxonomic identification (Hebert *et al.* 2003a). Molecular strategies should help and accelerate the taxonomy process, which has been so far strongly centered in morphology. Recently, molecular approaches applied to taxonomy have gained more attention with the proposal of a new initiative named the DNA Barcoding (Hebert *et al.* 2003a, Blaxter 2004).

The Consortium for the Barcode of Life (http://barcoding.si.edu) is a worldwide initiative devoted to develop an accurate and reliable tool for scientific research on the identification of plant and animal species. A broad scale molecular inventory of biodiversity was first suggested by Paul Hebert and collaborators in 2003. They successfully demonstrated that a 648 bp sequence of the 5` portion of the mitochondrial gene Cytochrome *c* Oxidase subunit I (COI) can be a reliable tool to identify animal species (Hebert *et al.* 2003b). Other initiatives have shown that COI displays a high resolution to identify species of several animal groups (Hebert *et al.* 2003a, 2004a, b), including extinct birds from New Zealand (Lambert *et al.* 2005). A previous study with 260 birds from North America revealed that most species could be properly discriminated by COI sequences (Hebert *et al.* 2004a). Another research (Hebert *et al.* 2004b) showed that the COI gene was also efficient to identify a complex of 10 species within the skipper butterfly *Astraptes fulgerator*.

Many criticisms have been raised against the barcode initiative (Mallet and Willmott 2003, Lipscomb *et al.* 2003, Seberg *et al.* 2003). However, it is noticeable that most of these criticisms are due to a misunderstanding of the main goals of this project. The barcode technique does not aim to replace the traditional taxonomy, instead it has to be used as an auxiliary tool. Since DNA sequencing is a fast, reliable and relatively cheap tool, "large-scale DNA barcoding can be used to identify and discriminate known species. Furthermore, it could be used to indicate probable new species within previously unstudied taxa", as recently pointed by Gregory (2005). He also

pointed out that new species suggested after DNA barcode analysis "would not be named solely with a DNA barcode: they would be given Linnaean names based on the study of curated voucher specimens, high-resolution digital images, collection locality data and other information". Several other applications for barcodes could be envisaged in forensics, animal ecology and behavior, parasitology, environment protection against exotic species etc. (Baker *et al*. 1996, Stoeckle 2003). Moreover, like any other DNA analysis, the barcode technique has the advantage of being performed using blood or small portions of animal tissue without the sacrifice of individuals. Furthermore, the barcode approach can independently assist the classic taxonomy, since it is not influenced by anatomical variations (genetic or environmental), or the individuals' age or sex. Hence, the DNA barcode initiative is a useful complementary tool to elucidate the taxonomic status of species that have no clearly distinguishable phenotypical characters to be used as taxonomic markers.

Although there is no specific reason to choose the mtDNA COI gene as the target region for DNA barcodes, this molecule show some interesting advantages to be used in these analyses. The animal mtDNA is a small circular molecule, maternally inherited without recombination, existing in several copies within the cells (Avise *et al.* 1987). The COI gene is ~1500 bp long and occurs in all eukaryotes. Although this gene possesses a slow rate of aminoacid change if compared to other mitochondrial genes (Lynch and Jarrell 1993), it exhibits an evolutionary nucleotide rate that can be three times greater than ribosomal mtDNA genes and can be successfully used to distinguish individuals between and sometimes within species (Hebert *et al.* 2003b).

The typical antbirds (Passeriformes: Suboscines: Thamnophilidae) represent a group of approximately 200 species, being one of the most numerically important families in the Neotropics (Isler *et al.* 1998, Irestedt *et al.* 2004). These birds are arboreal or undergrowth inhabitants of Neotropical forests, such as the Amazon and the Brazilian Atlantic Forest. They are known as sedentary birds, well adapted to the flight on densely vegetated areas, rarely crossing long distances through open areas (Zimmer and Isler 2003). Genetic studies suggested that their sedentary characteristics would explain the high levels of divergence observed between populations within species (Hackett 1993, Bates *et al.* 1999). These results reinforce the general view that Neotropical birds would exhibit higher levels of genetic structure if compared to temperate ones (Hackett and Rosenberg 1990, Bates 2000, Winker *et al.* 2000). Although the monophyly of Thamnophilidae is a consensus interpretation (Irestedt *et al.* 2004), many relationships between genera and species remain unclear (Zimmer and Isler 2003). To resolve these phylogenetic issues, many different markers, such as morphological, molecular and behavioral, including vocalizations or nest architecture, have been employed (Isler *et al.* 1997, 1998, Zimmer and Isler 2003). Furthermore, it is believed that some species recognized as distinct ones could be variations of a single species, while others would have to be divided in several ones after a more careful study (Zimmer and Isler, 2003). Since 1990, 19 new Thamnophilidae species have been recognized: ten of them resulted from taxonomic reviews and nine were recently recognized species (Zimmer and Isler, 2003). As depicted by the recent taxonomic changes, as well as the possibility of many undescribed taxa in Thamnophilidae, this group seems to be a good candidate for taxonomic studies in all levels, including taxonomy based on DNA sequence analysis.

In this study, we sequenced a fragment of the mtDNA COI gene of 46 individuals from 16 Thamnophilidae species that occur in the Brazilian Atlantic Forest. Our aim was to show that the COI gene can be an efficient marker for a DNA-based identification system focusing on a Neotropical bird family. In this study we do not intend to clarify relationships above the species level, which would require larger DNA sequences, a more complete sampling and other phylogenetic analyses. We aim to show that the barcode technique can be a useful tool to discriminate species within Thamnophilidae, including some recently suggested species by classic taxonomy. This is the first study using the DNA barcode methodology to identify species from an endemic Neotropical bird family.

MATERIAL AND METHODS

Forty six birds from 16 Thamnophilidae species (see Table 1) were sampled in different areas of the Brazilian Atlantic Forest in the Minas Gerais State, Southeastern Brazil. Two to four individuals of each species were selected for this study. Several individuals were also included in a previous study (Lacerda 2004) that generated Cytochrome b and control region sequences (n = 160), what allowed us to select the most divergent individuals (n = 23) within each species for the present research. For those species whose previous genetic information were not available, individuals (n = 23) from more distant sampling areas were selected.

Blood samples were obtained by venipuncture of captured birds, that were released after tissue collection. Total DNA was extracted using phenol:chloroform:isoamyl alcohol protocol (Sambrook *et al.* 1989) after digestion with proteinase K. DNA concentration was estimated through visual evaluation of the band resulting from electrophoresis of 5 µL of DNA solution in 0.8% agarose gels stained with ethidium bromide. The COI mitochondrial gene was entirely amplified using the specific primers L6615 and H8121, designed by M. D. Sorenson (http://people.bu.edu/msoren/Bird.mt.Primers.pdf). PCR reaction mixes of 12.5 µL included 2 µL of genomic DNA (~ 40 ng), 1 U of *Taq* polymerase (Phoneutria®), 200 µM of dNTPs, 1X Tris-KCl buffer with 1.5 mM $MgCl_2$ (Phoneutria®) and 0.5 µM of each primer. The amplification program consisted of 2 min at 94ºC, followed by 35 cycles of 40 s at 94ºC, 40 s at 58ºC, 2 min at 72ºC and a final extension step of 10 min at 72ºC. After amplification, PCR products were run in 0.8% agarose gels stained with ethidium bromide. Negative controls, where template DNA was omitted, were used in all am-

Table 1. Mean sequence divergence (K2P) within (bold number on diagonal) and among (below diagonal) the 16 Thamnophilidae species.

	Drf	Dro	Drs	Dsm	Dsp	Fos	Hea	Myl	Pyl	Rha	Sac	Tam	Tha	Thc	Thd	Thp
Drf	**0,0016**															
Dro	0,0739	**0,0032**														
Drs	0,1177	0,0966	**0,0000**													
Dsm	0,1331	0,1094	0,1454	**0,0073**												
Dsp	0,1001	0,1151	0,1483	0,1114	**0,0016**											
Fos	0,1270	0,1110	0,1279	0,1216	0,0989	**0,0032**										
Hea	0,1219	0,1129	0,1394	0,1210	0,1217	0,1346	**0,0097**									
Myl	0,1141	0,1101	0,1279	0,1266	0,1164	0,1211	0,1316	**0,0032**								
Pyl	0,1144	0,1167	0,1286	0,1396	0,1181	0,1319	0,1333	0,1239	**0,0040**							
Rha	0,0855	0,0792	0,1228	0,1342	0,1098	0,1067	0,1219	0,1141	0,1069	**0,0016**						
Sac	0,1307	0,1168	0,1238	0,1196	0,1378	0,1368	0,1027	0,1155	0,1268	0,1234	**0,0012**					
Tam	0,1108	0,1014	0,1340	0,1332	0,1329	0,1063	0,1306	0,1128	0,1187	0,1112	0,1230	**0,0048**				
Tha	0,1349	0,1272	0,1393	0,1355	0,1477	0,1350	0,1369	0,1345	0,1368	0,1344	0,0912	0,1279	**0,0000**			
Thc	0,1517	0,1447	0,1557	0,1256	0,1234	0,1162	0,1300	0,1393	0,1422	0,1337	0,1121	0,1393	0,0730	**0,0110**		
Thd	0,1420	0,1290	0,1321	0,1468	0,1382	0,1515	0,1202	0,1384	0,1296	0,1272	0,1132	0,1446	0,0865	0,1001	**0,0024**	
Thp	0,1369	0,1332	0,1302	0,1355	0,1383	0,1380	0,1308	0,1254	0,1368	0,1467	0,1084	0,1279	0,0479	0,0758	0,0922	**0,0000**

Drf = *Drymophila ferruginea*; Dro = *Drymophila ochropyga*; Drs = *Drymophila squamata*; Dsm = *Dysthamnus mentalis*; Dsp = *Dysthamnus plumbeus*; Fos = *Formicivora serrana*; Hea = *Hepsilochmus atricapillus*; Myl = *Myrmeciza loricata*; Pyl = *Pyriglena leucoptera*; Rha = *Rhopornis ardesiaca*; Sac = *Sakesphorus cristatus*; Tam = *Taraba major*; Tha = *Thamnophilus ambiguus*; Thc = *Thamnophilus caerulescens*; Thd = *Thamnophilus doliatus*; Thp = *Thamnophilus pelzelni*.

plification runs. Only products with a single and well-defined 1500 bp band were used in the sequencing reactions. Before sequencing, PCR products were cleaned by precipitation using 20% polyethyleneglicol + 2.5 M NaCl. Sequencing reactions were performed using only the primer L6615, and were conducted in a final volume of 10 µL containing: 2-4 µL of purified PCR product, 1-3 µL of ultrapure water, 1 µL of primer (5 µM) and 4 µL of sequencing kit (ET DYE Terminator Kit, Amersham Biosciences). The sequencing program consisted of 35 cycles of 95ºC for 25 s, 55ºC for 15 s, 60ºC for 3 min. Then, sequencing products were precipitated with ammonium acetate and ethanol, dried at room temperature, dissolved with formamide-EDTA and run in the automatic sequencer MegaBACE 1000 (Amersham Biosciences).

To avoid the amplification of nuclear sequences of mitochondrial origin, i.e. *numts* (Sorenson and Quinn 1998), the following measures were undertaken: *i*) amplification of sequences longer than 1000 bp; *ii*) amplification primers had degenerate sites and/or had annealing sites in tRNA genes; *iii*) for each individual, at least two different PCR products were used in the sequencing reactions until, at least, two high quality and independent sequences could be obtained; *iv*) for each species, at least two individuals were used in the analysis; *v*) chromatograms were carefully checked for ambiguities; *vi*) COI sequences produced in the present study were aligned and compared with others obtained from the GenBank, including a sequence of *Gallus gallus* (Desjardins and Morais 1990), to check for the presence of any start, stop or nonsense codons, as well as alignment gaps.

Consensus sequences (available upon request) were obtained and checked through the programs Phred v. 0.20425 (Ewing *et al.* 1998), Phrap v. 0.990319 (http://www.phrap.org/) and Consed 12.0 (Gordon *et al.* 1998). Alignments were done using Clustal X (Thompson *et al.* 1997), with manual edition whenever it was necessary. Sequence divergence among different haplotypes was estimated through the program MEGA 3.0 (Kumar *et al.* 2004) using Kimura 2-parameter (K2p) distance model (Nei and Kumar 2000). MEGA 3.0 was also used to construct a Neighbor Joining (NJ) tree based on the K2p model with 10,000 bootstrap replicates.

RESULTS AND DISCUSSION

A 418 bp COI fragment was analyzed in this study. The sequences obtained were aligned and compared with other avian GenBank COI sequences from passerine species and *Gallus gallus* (Desjardins and Morais 1990). These comparisons revealed neither stop or nonsense codons, nor alignment gaps. Furthermore, while analyzing the chromatograms, no high quality ambiguities that could suggest the presence of *numts* were identified. Different haplotypes were found for some species, and the maximum divergence between them was 1.1% (Table 1). Nucleotide divergence between species ranged from 4.8% to 15.6% (Table 1), with an average of 12.3%. Herbert *et al.* (2004a) found a similar value of 12.7% for mean sequence divergence within families of North American birds. Besides, levels of sequence divergence observed among the 16 analyzed species of typical antbirds were similar to those previously observed for eight of these species (5.1-14.6%, Lacerda 2004) and also for species within Cotingidae family (4.3-25.7%, Prum *et al.* 2000) using cytochrome *b* sequences.

Intrageneric sequence divergence could be estimated for three of the 10 analyzed genera (*Thamnophilus*, *Dysithamnus* and *Drymophila*) and ranged from 7.9 to 11.1%, with a mean of 9.6%. This mean value is much higher (27-fold higher) than the mean divergence observed within species (0.34%) and also the maximum within-species difference obtained in this study (1.1%). Thus, a high sequence similarity within species was observed in spite of using in this study a selected group of samples with the most divergent mtDNA (see Material and Methods).

The NJ tree (Figure 1) showed that all intraspecific haplotypes grouped together forming cohesive and strongly supported groups (99% of bootstrap support). Although this study has used only 418 bp of the COI gene, what makes difficult to infer relationships above species level, there is some phylogenetic signal. It is possible to note that the genus *Thamnophilus* formed a monophyletic and well supported group (90% of bootstrap support). Furthermore, there is a strongly supported clade (91% of bootstrap support) formed by the two closely related *T. pelzelni* and *T. ambiguus*. Indeed, until very recently those two species were part of *Thamnophilus punctatus* complex, which was divided in six different species based on morphology, geographical distribution and vocalizations (Isler *et al.* 1997). Besides, *T. pelzelni* and *T. ambiguus* appear in figure 2 as a single point detached from the others, a finding that can be easily explained by the low sequence divergence observed between them (4.8%). This value, although is the lowest one observed among the 16 species, is high enough to be considered another evidence for the species status of *T. ambiguus* and *T. pelzelni*. Other evidences include the lack of clinal intermediacy in their contact zone and some distinguishable features in their plumage patterns, in spite of the fact that the two species are very similar in morphology and exhibit overlapping but identifiable loudsongs (Naumburg 1937, Isler *et al.* 1997). Furthermore, other studies have shown good, well-defined

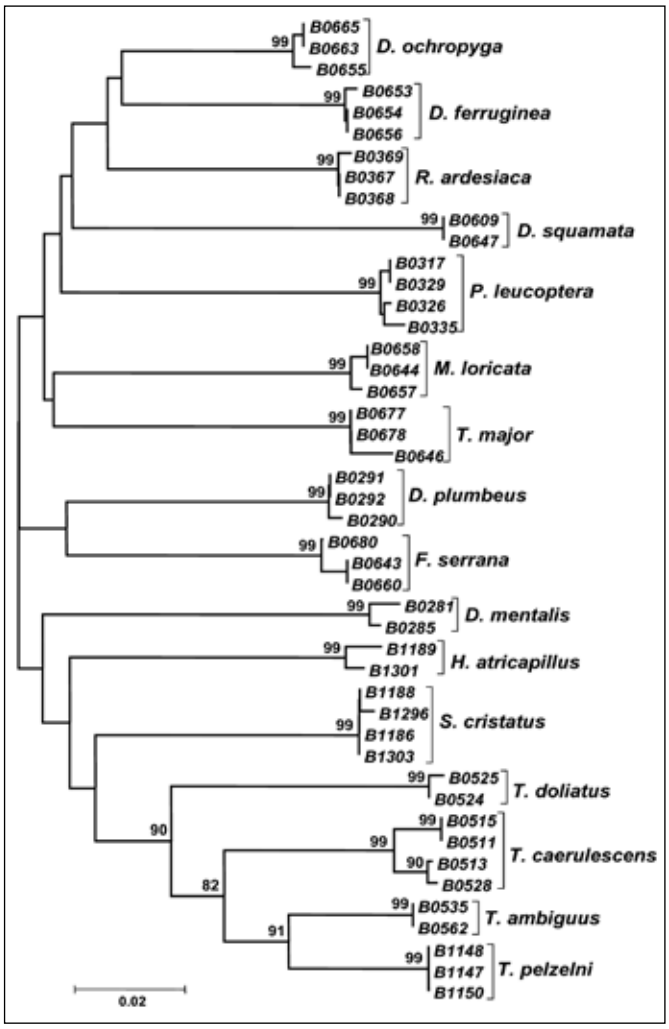

Figure 1. Unrooted NJ tree of 46 COI sequences for 16 Thamnophilidae species. Bootstrap support values are indicated on the branches, but values below 50 or within species were omitted.

Neotropical avian species exhibiting relatively low levels of mtDNA sequence divergence, *e.g.* García-Moreno and Silva (1997) for two *Lepidocolaptes* species, and Ribas and Miyaki (2004) for two *Aratinga* species.

The only intraspecific divergence higher than 1% was found for the variable antshrike (*Thamnophilus caerulescens*, (1.1%, Table 1 and Figure 2). Lacerda (2004) showed that two very divergent lineages are present within this species in the Minas Gerais State of Brazil. For this study, we selected two representatives of each lineage that probably belong to different subspecies. If we apply the threshold suggested by Herbert *et al.* (2004a) of 10X the mean intraspecific divergence for the group under study, we conclude that for the Thamnophilidae species analyzed here, any divergence below 3.4% characterize a single well-defined species. Hence, even though there is a considerable amount of divergence among *T. caerulescens* sequences, the present study made possible a consistent identification not only of the variable antshrike individuals but also of every other individuals from the 15 antbird species. Moreover, this identification method was also supported by

the presence of at least one synapomorphic character for each species (Table 2). Nevertheless, these synapomorphies should be seen with caution since they were based on few individuals from few species of the Thamnophilidae family. Some of these characters may not appear as synapomorphies in a broader sampling of individuals, species or geographical range.

The finding that each species has a characteristic COI sequence offers the prospect to identify typical antbirds using the DNA barcode technique as an auxiliary tool for taxonomy. Many Thamnophilidae species can be misidentified by non-specialists that very frequently are involved in fieldwork, when blood samples can be readily collected without collecting the individual. The perspective of using a DNA marker that can ensure the correct sample identification within a DNA bank collection is very exciting. This proposal has already been applied in our case. During the preparation of this study, one individual tagged as *Herpsilochmus atricapillus* exhibited a very unusual COI sequence if compared to the other two *H. atricapillus* analyzed (B1189 and B1301, Figure 1). When the three sequences were searched against the GenBank database, the best hits for the unusual one were COI sequences of phylogenetically distant birds, not even passerines. Based on that, only the other two individuals were used in this work and considered as *H. atricapillus*. Future studies need to be done in order to correctly identify the other individual that probably had its identification tag switched. This is just one example of the many utilities of the DNA barcode. Hopefully, more and more COI sequences of Neotropical bird species will be pro-

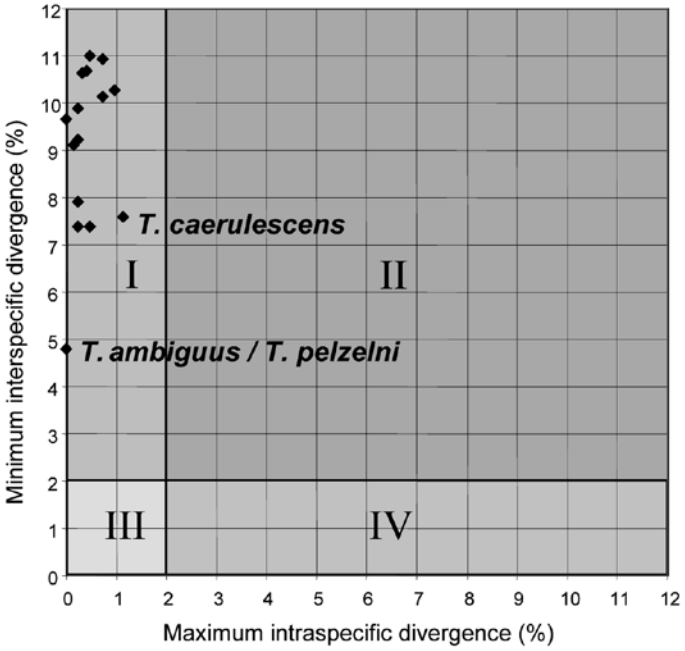

Figure 2. Maximum intraspecific sequence divergence (X axis) compared to minimum interspecific divergence (Y axis) using K2P distance for the 16 Thamnophilidae species. Following Hebert *et al*. (2004a), the cutoff of 2% of sequence divergence was used do divide the table in four quadrants, and each one represents a different category of species: I – Intraspecific distance < 2%, interspecific distance > 2%: species concordant with actual taxonomy; II – intraspecific and interspecific distance > 2%: species that require a taxonomic review; III – intraspecific and interspecific distance < 2%: recent divergence, hybridization or synonymy; IV – intraspecific distance > 2%, interspecific distance < 2%, probable misidentification of specimen.

Table 2. Synapomorphic characters (highlighted in black) for all individuals within each of the 16 analyzed Thamnophilidae species. Synapomorphic site numbers are indicated above COI variable characters.

Species	COI variable characters
	000000011111122222222223333334444
	001123500344801345566779001569011
	581495647106502451769253681928436
Thamnophilus caerulescens (n=4)	CACAAAACAACAAACCCAACAAAGCCCATAAAA
Thamnophilus ambiguus (n=2)	..................G.T...A.....
Thamnophilus pelzelni (n=2)	....G.............T...A....C....
Thamnophilus doliatus (n=2)	............G.....GT...A.T.C.....
Herpsilochmus atricapillus (n=2)	.TT............T..A.C.A.A...C...
Sakesphorus cristatus (n=4)	T..................AG..ATA.....
Dsythamnus mentalis (n=2)	.....GG............A...A.A.....G
Dsythamnus plumbeus (n=3)	.G.........G..T....A..GA.A.....
Drymophila ochropyga (n=3)	.............A....A...A.A.....
Drymophila ferruginea (n=3)	.............T..C.A...A.AA.....
Drymophila squamata (n=2)	........G..........G...A.AT.....
Myrmeciza loricata (n=3)	............C......A...A.A.....
Rhopornis ardesiaca (n=3)	.............T....A...T.AT...T..
Pyriglena leucoptera (n=4)	.........G.........A...T.A.....G.
Formicivora serrana (n=3)	.......T.....GT....A...A.AT.....
Taraba major (n=3)	...G......T....T...A...A.A.....

duced and made available in open access databases. Although the 16 species analyzed here are not well representative of the huge diversity of Thamnophilidae family, and we used only 418 bp of the COI gene, this study demonstrated that species identification through DNA sequences is readily applicable to typical antbirds and probably to other Neotropical bird families as previously showed for North American birds.

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