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Análise Genética de Populações Fragmentadas de *Conopophaga lineata* (Passeriformes) da Mata Atlântica de Minas Gerais

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Ao Juninho e ao Giovanni,
que iluminaram minha vida por um
tempo efêmero mas deixaram uma
infinita saudade.

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"Toda la gloria es nada, toda vida es sagrada (...)
No dejaremos huella, sólo polvo de estrellas.
Vale una vida lo que un sol vale"
(Jorge Drexler)

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RESUMO

A Mata Atlântica é um bioma majoritariamente brasileiro que apresenta altos níveis de biodiversidade e avançada degradação. Assim estudos visando a sua conservação são urgentes. A filogeografia é uma abordagem genética que permite o delineamento de estratégias para a preservação de espécies, associando a distribuição espacial da variabilidade genética com aspectos históricos (distribuição temporal). No presente trabalho relatamos o uso de seqüências da região controle do mtDNA (1074 pb) para analisar aspectos históricos que influenciaram a distribuição das linhagens maternas (haplótipos) em populações de *Conopophaga lineata* em fragmentos da Mata Atlântica de Minas Gerais (MG). Foram coletadas amostras de sangue de 104 indivíduos de *C. lineata* em 12 fragmentos florestais de diferentes tamanhos. As análises sugeriram uma diversidade genética específica alta em comparação às de outros passeriformes neotropicais. As populações só se mostraram fortemente estruturadas entre regiões geográficas muito distantes (nordeste e sudeste de MG – 500 km). Também foi observada a existência de dois clados de haplótipos ocorrendo em simpatria no sudeste de MG. A divergência entre estes dois clados foi de 5,92%, comparável ao normalmente encontrado para comparações entre espécies congenéricas de passeriformes neotropicais. O clado menos freqüente mostou-se geneticamente mais parecido com indivíduos do estado de São Paulo do que com os de MG. Esta região poderia representar um contato secundário entre duas linhagens genéticas que se divergiram em alopatria há, no mínimo, 180 mil anos. Não foi observada correlação direta entre o nível de diversidade nucleotídica e o tamanho do fragmento. Entretanto, a estruturação da diversidade genética encontrada em fragmentos pequenos e em fragmentos grandes foi diferente.

INTRODUÇÃO

A destruição ambiental e a perda de hábitats, causadas principalmente pela expansão das atividades do homem, como agricultura e pecuária, são graves ameaças à biodiversidade e principais causas da atual onda de extinção (Scharlemann *et al.* 2004). Nos últimos 300 anos as áreas de aves endêmicas ("Endemic Bird Areas" - EBAs) foram as mais afetadas pela transformação de áreas de florestas tropicais em plantações comerciais (Scharlemann *et al.* 2004). O Brasil destaca-se entre os países que apresentam mais alta biodiversidade e dois de seus biomas – a Mata Atlântica e o Cerrado – são considerados pontos mundialmente prioritários para conservação ("hotspots") devido ao seu elevado grau de endemismo e avançada degradação (Myers *et al*. 2000). A Mata Atlântica passou por um forte processo de fragmentação florestal e ocupa hoje o equivalente a apenas 7,5% de seu território original (Myers *et al*. 2000).

A variabilidade genética tem um importante papel na sobrevivência de populações e espécies a médio e longo prazo, pois se acredita que uma baixa variação possa restringir o surgimento de futuras adaptações evolutivas (Milligan *et al*. 1994). Isto aumentaria o risco de extinção da espécie, que ficaria mais susceptível aos efeitos da estocasticidade ambiental, demográfica ou genética (Frankham 1995, Turner, 1996). A fragmentação ambiental pode ser determinante na perda de variabilidade intra-populacional, reduzindo os tamanhos populacionais e favorecendo processos como endogamia e deriva genética. Além disso, a fragmentação leva a uma diminuição do fluxo gênico, decorrente do resultante isolamento geográfico e, portanto, reprodutivo (Frankham *et al.* 2002).

Assim, o conhecimento dos efeitos da fragmentação florestal sobre as populações é de grande importância para a conservação da biodiversidade. Entretanto, um pequeno número de estudos analisando os efeitos da fragmentação tem sido realizado em florestas tropicais e estes são principalmente com aves (Turner, 1996).

Os Suboscines são pássaros que ocorrem majoritariamente na região Neotropical. Dentre as 938 espécies de Passeriformes que ocorrem no Brasil, 609 pertencem à Subordem Suboscines, sendo 102 espécies endêmicas no país (Sick 1997). Entretanto, este é um grupo pouco conhecido do ponto de vista genético. A maioria dos trabalhos abordando estrutura genética populacional em aves foi realizada no hemisfério Norte, com Passeriformes da Subordem Oscines. Desta forma, a maioria das seqüências nucleotídicas e dos marcadores genéticos de passeriformes publicados até o momento é de Oscines, o que dificulta a padronização de técnicas moleculares para analisar espécies de Suboscines.

Conopophaga lineata, conhecida popularmente como chupa-dente ou cuspidor, é uma espécie de pássaro que ocorre na Mata Atlântica e pertence à família Conopophagidae, da Subordem Suboscines (Passeriformes). Ela se distribui do Ceará ao Rio Grande do Sul (no

Brasil) e no nordeste da Argentina (Misiones), leste do Uruguai e leste do Paraguai (Whitney 2003; Sick 1997). Por ocupar uma extensa área de distribuição, *C. lineata* reconhecidamente apresenta variações no canto e plumagem, mas que ainda não foram delimitadas geograficamente (Pinto 1978; Whitney 2003). Esta espécie é dependente florestal (Andrade e Marini 2001), possui hábitos insetívoros e generalistas e consegue utilizar hábitat de bordas e florestas secundárias, o que facilita a sua vida em ambientes perturbados (Ribon 1998; Whitney 2003). Por estar presente em vários fragmentos de mata de distintos tamanhos, *C. lineata* pode ser um bom modelo para o conhecimento do efeito da fragmentação sobre a diversidade genética das populações.

Dados obtidos através do uso de marcadores genéticos podem revelar muitas informações acerca da subdivisão das populações (Haig 1998; Templeton 1998), favorecendo estudos sobre fragmentação florestal antrópica, vicariância e outros eventos históricos antigos. Além disso, se forem utilizados marcadores que possibilitem a contextualização da distribuição da variabilidade não só em âmbito geográfico, mas também em âmbito temporal, estudos filogeográficos também podem ser realizados (Avise *et al*. 1987). A filogeografia lida com a filogenia de linhagens ou haplótipos e sua distribuição espacial e pode ser feita baseada em análises de seqüências de DNA mitocondrial (Avise *et al*. 1987).

O DNA mitocondrial (DNAmt) de vertebrados, por sua vez, é uma molécula circular simples (15-20 Kb), que apresenta alta taxa evolutiva se comparada ao DNA nuclear e herança materna, sem recombinação, favorecendo seu uso em estudos microevolutivos (Avise *et al*. 1987). A região controle ou hipervariável do DNAmt é uma região não codificadora que apresenta taxa evolutiva maior que o restante da mitocôndria. Como conseqüência, esta região apresenta uma maior quantidade de variações nucleotídicas e é muito utilizada em estudos de variabilidade intra-específica (Baker e Marshall 1997; Milot *et al.* 2000), que podem ser utilizados em projetos para conservação da biodiversidade.

Neste trabalho, o seqüenciamento da região controle do mtDNA foi utilizado para analisar a diversidade genética de *C. lineata* em populações de fragmentos da Mata Atlântica de Minas Gerais. Este é um dos três estados brasileiros que possuem menor proporção de áreas protegidas de Mata Atlântica; apenas 3,37 % do total da área ocupada por Mata Atlântica no estado encontram-se dentro de parques e reservas (Ayres *et al.* 1997). Além disto, o que resta é principalmente composto por pequenos fragmentos florestais isolados, muitas vezes de matas secundárias. Assim, estratégias para conservação deste bioma em Minas Gerais são necessárias.

Considerando-se estes fatos, surgiu a idéia do estudo aqui relatado. Este projeto começou a partir de capturas de aves em fragmentos grandes (maiores que 1000 ha) e pequenos (menores que 50 ha) de Mata Atlântica em Minas Gerais, isolados entre si e de

outras matas por monoculturas ou pastagens por uma distância mínima de um quilômetro. As capturas foram realizadas entre os anos de 2000 e 2003 pela equipe do Prof. Miguel Â. Marini e em 2004, por alunos do Prof. Marcos Rodrigues. *Conopophaga lineata* foi escolhida como a espécie a ser utilizada para as análises genéticas por ter sido abundantemente capturada em muitos dos fragmentos amostrados. Além disto, nenhum trabalho populacional tinha sido antes realizado com seqüenciamento de DNAmt desta espécie. Outras características da espécie, como sua dependência florestal e a variação geográfica de seu canto e plumagem, também foram importantes para sua escolha.

Os objetivos deste trabalho foram:

1) padronizar a amplificação e o seqüenciamento da região controle do mtDNA de *C. lineata*;

2) analisar a variabilidade genética e a distribuição hierárquica desta nas populações de *C. lineata* amostradas em fragmentos de Mata Atlântica de Minas Gerais;

3) inferir a distribuição geográfica das linhagens de haplótipos traçando a filogeografia da espécie;

4) verificar se a diversidade genética e a sua distribuição são significativamente diferentes em fragmentos grandes e pequenos;

Para isto foram analisados 104 indivíduos de *C. lineata* coletados em 12 fragmentos florestais de nove localidades de Minas Gerais (Fig. 1). Todas as amostras de DNA extraídas destes espécimes encontram-se depositadas no banco de DNA do Laboratório de Biodiversidade e Evolução Molecular do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (fiel depositário de amostras de componentes do patrimônio genético através da deliberação nº 46, de 18 de dezembro de 2003, do CGEN/MMA). As análises genéticas foram feitas sob autorização especial do IBAMA/MMA de acesso e remessa de amostra de componente do patrimônio genético nº 03/2004 e as capturas realizadas com as seguintes licenças concedidas pelo IBAMA/MMA: 011/2000, processo nº 1835/2000; 053/2001, processo nº 1835/00-07; 070/2002, processo nº 02015.001835/00-07; 207/2003, processo nº 02015.023482/98-38.

Este estudo visa ainda obter resultados que apóiem estratégias de conservação da espécie estudada e, conseqüentemente, da Mata Atlântica. Áreas prioritárias para conservação podem ser propostas por possuírem populações com uma identidade genética única, altos índices de diversidade ou por representarem focos de manutenção da variabilidade genética de *C. lineata*.

ARTIGO:

Historical and Anthropic Events Affecting Genetic Structure in Populations of the Atlantic Forest Bird *Conopophaga lineata*

Manuscript for Molecular Ecology

Historical and Anthropic Events Affecting Population Genetic Structure of the Rufous Gnateater, *Conopophaga lineata*, in the Brazilian Atlantic Forest

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Abstract

The Brazilian Atlantic forest is one of the five leading world *Hotspots for Conservation Priorities* that presents high biodiversity levels and has been strongly destroyed. Then researches concerning its conservation are urgently required. Phylogeographic surveys can give support to species' preservation policies, associating the spatial distribution of genetic variability with historical events, like vicariance, and anthropic ones, as habitat fragmentation. This study investigated *Conopophaga lineata* phylogeography in recently fragmented populations in the Atlantic forest. Mitochondrial control region sequences of 1074 bp were used to analyse 104 individual from 12 forest fragments, differently sized, of Minas Gerais State (MG), Brazil. The species presented genetic diversity much higher than other Neotropical passerine species. Strong genetic variability structure was found only between distant geographic regions (southeastern and northeastern, 500 km apart). In southeastern region, two clades, occurring simpatrically in three populations, were identified. Divergence between clades was 5.92%, comparable to values found between Neotropical congeneric bird species. The less common clade (EN) was more similar to individuals from São Paulo State than to those from MG. This finding might indicates that southeastern MG is a region of secondary contact between two populations that were separated until 180,000 years ago, when the clades evolved allopatrically. No direct correlation was found between nucleotide diversities and fragment/population sizes. However, genetic structure and isolation by distance were found to be different among large fragments and among small fragments.

Introduction

In recent years, the contribution of genetics to conservation biology has become widespread, since molecular markers are now able to precisely reveal many features about interactions within and among populations (O´Brien 1994; Haig 1998). Therefore some genetic markers indirectly inform about historical events that has lead populations to the current distribution and its outcomes (Haig & Avise 1996, Haig 1998). Ancient events, as vicariance and dispersal, and more recent ones, like anthropic habitat fragmentation can be verified by analysing a specific distribution of the genetic variability (Avise *et al.* 1987; Haig & Avise 1996; Cunningham & Moritz, 1998; Segelbacher *et al.* 2003; Brown *et al.* 2004; Cabanne 2004).

Phylogeographical surveys allow evaluating a species genetic diversity distribution in its geographical range and also in an evolutionary time scale, *i.e.* they incorporate intraspecific phylogenies into species geographical distribution (Avise *et al.* 1987). Mitochondrial DNA (mtDNA) is a molecular marker that has been successfully used to infer the phylogeography of many vertebrate species, correlating the findings to historical events (Cunningham & Moritz, 1998; Milá *et al.* 2000; Milot *et al.* 2000; Pope *et al.* 2000; Zink & Blackwell-Rago 2000). Mitochondrial sequences can be used to establish association between microevolutionary processes (*e.g.* mutation, genetic drift, populational genetic structure) and phylogenetics and macroevolution, making this genetic marker suitable for phylogeographical studies (Avise *et al.* 1987).

Habitat destruction can lead populations to fragmentation, reducing their effective size and usually the genetic flow among them, due to geographic isolation (Frankham 1995; Turner 1996). As a consequence, fragmented populations become more susceptible to genetic drift and inbreeding, frequently resulting in a reduction of genetic variability within populations and a marked structure among populations. Nevertheless consequences of habitat fragmentation depend on the previous population structure and sometimes it can be inferred by phylogeography (Turner 1996; Frankham *et al.* 2002;). It is known that genetic diversity is very important to populations and species persistence in nature, since low genetic variation could restrict the emergence of future evolutionary adaptations (Milligan *et al.* 1994). As a result, species and populations with low variability levels may not be able to cope with stochastic environmental changes and could go extinct (Frankham 1995).

A striking example of habitat destruction is the Brazilian Atlantic Forest, which has been strongly devastated particularly in the last 50 years (Hirota 2003). Today it occupies approximately only 7.5% of its original area (Myers *et al.* 2000) and the majority of the forest remnants are isolated and smaller than 100 ha (Chiarello 2000). This Neotropical rainforest also presents high concentration of endemic species, and therefore is considered

as one of the five leading world hotspots and should be a priority for conservation, management and scientific research (Myers *et al.* 2000). This biome has 1020 bird species, from which 188 are endemic and 104 are threatened (Conservation International *et al.* 2000), making it the most endangered group of the Neotropical bird assemblage (Stotz *et al.* 1996). Habitat destruction is recognised as a major cause of biodiversity loss, notwithstanding there are few studies on tropical rainforests analysing fragmentation effects, and most of them are focused in the Amazon Forest (Turner, 1996). There are even fewer considering genetics of bird species in fragmented populations (Dantas 2003; Cabanne 2004; Brown *et al.* 2004). Therefore, it is a critical priority to carry out surveys on genetic effects of anthropic fragmentation for Atlantic Forest birds.

Conopophaga lineata (Passeriformes: Conopophagidae), the Rufous Gnateater, is a small (12cm length; 20-25g) Suboscine bird with generalist insectivorous habits (Ribon 1998; Whitney 2003). It is widely distributed in Brazilian Atlantic Forest, also occurring in northeastern Argentina (Misiones), eastern Paraguay and eastern Uruguay (Sick 1997; Whitney 2003). This is an understory species that lives in areas with dense herbaceous and woody plants, in secondary growth forest and in fragment borders (Ribon 1998; Sick 1997; Whitney 2003). Besides, it can be found even in small forest patches (Ribon 1998; Willis *et al.* 1983), making *C. lineata* capture accessible in many locations along its distribution range.

Conopophagidae is one of the least known Neotropical bird families; published information is vague and relationships between species and subspecies are undefined (Whitney 2003). *Conopophaga lineata* presents remarkable geographical variation in song and plumage (Pinto 1978; Whitney 2003), but the "distributional limits of these vocalizations, and the extent to which they correspond to named populations, requires much additional research" (Whitney 2003).

Currently three subspecies are officially recognised, but with largely indefensible range limits along Brazilian States: *C. l. cearae* Cory (NE Brazil, from Ceará to Pernambuco and northeastern Bahia, where it requires confirmation), *C. l. lineata* Wied (NE Brazil, from Pernambuco to southern Bahia; Central Brazil, in the south of Goiás and northern Mato Grosso do Sul), *C. l. vulgaris* Ménétriès (south-centre and SE Brazil, from southern Mato Grosso do Sul and southern Espírito Santo to Rio Grande do Sul; eastern Paraguay, Misiones and eastern Uruguay) (Whitney 2003). However, there are many regions inside *C. lineata* distribution where the subspecies to be found is not predictable. The southeastern Brazilian State, Minas Gerais, is one of them, with a large area and situated in the middle of *C. l. lineata* and *C. l. vulgaris* geographical distributions, according to Whitney (2003). Minas Gerais is also included in the distributional range of three biomes: Atlantic Forest, Caatinga and Brazilian Cerrado (another world hotspot for conservation priorities) (Myers *et al.* 2000), consequently presenting interesting transition areas between them.

Here we report the use of mtDNA control region sequences to assess genetic variability in 12 populations of *C. lineata* from Minas Gerais State. We tested if populations' genetic lineages are associated to its geographical distribution and if recent habitat fragmentation has affected the distribution of this genetic diversity.

Materials and Methods

Field sampling

All *Conopophaga lineata* specimens (n = 104) were captured with mist nets in 12 Atlantic Forest fragments (in nine different localities) of the Minas Gerais State, southeast Brazil (Fig.1). A single individual was sampled into the locality of Ouro Preto and its sequence was used only for phylogeographical analysis. Large and small fragments were sampled (Table 1). At three localities (Araponga, Simonésia and Nova Lima) birds were captured in two differently sized fragments (one larger than 999 ha and the other smaller than 30 ha), separated by at least five kilometres. Birds were numbered with aluminium leg bands, punctured on the brachial or tarsal vein to get blood samples, morphologically measured and released. Blood samples were stored in microtubes with absolute or 70% ethanol and kept at 4°C until DNA isolation. Some specimens were collected and vouchers are deposited at the zoological colections of Universidade Federal de Minas Gerais and Universidade de Brasília.

Molecular methods

Total genomic DNA extraction was made following standard phenol-chloroform method (Sambrook *et al.* 1989). A fragment of approximately 1200 bp of the mitochondrial control region (CR) was amplified by Polymerase Chain Reaction (PCR) using primers LEC1 (5' TCCACACTCGACATCTCATT 3') and H16137 (5' AAAATRYCAGCTTTGGGAGTTG 3'). LEC1 was designed in this study for *C. lineata* and it hybridizes in the beginning of CR, approximately 80 bp downstream of the $tRNA^{Thr}$ gene. H16137, located at $tRNA^{Pro}$ gene, was modified for Thamnophilidae (Lacerda 2004, based on primers designed by M. D. Sorenson). PCR reaction mixtures were performed in 12,5 µl with 1 U Taq Polymerase (Phoneutria), 1X Taq Buffer (Phoneutria), 200 µM dNTPs, 0.4 µM of each primer and approximately 10-50 ng of genomic DNA. Amplification cycling protocol was: initial denaturation at 94°C for 2 min; 36 cycles at 94°C for 30 seg, 57°C for 40 seg, 72°C for 2 min and a final extension step at 72°C for 10 min. Products concentration were verified on an ethidium bromide-stained 0.8% agarose gel. PCR products were purified with 20% PEG (polyethylene glycol)/ 2.5 M NaCl precipitation. Both strands of purified DNA were sequenced using the primers above and also internal primers: LEC2 (5' TGTACCTGTTGTGGTCGGCGTA 3'), designed specifically for *C. lineata* and H4 (5' TTGACGAGGTAAAAATATGTCT 3'), made for Thamnophilidade (Lacerda 2004). Purified PCR products were cycle-sequenced with ET DYE Terminator Kit (Amersham Biosciences) according to manufacturer's instructions with 35 cycles at 95°C for 25 s, 50°C for 15 s and 60°C for 3 min. The runs were done in MegaBACE (Amersham Biosciences) automatic sequencer. In order to produce unambiguous sequences, two independent PCR reactions

were made for each individual and both products were sequenced. A mtDNA CR sequence of *Conopophaga melanops* was produced to be used as outgroup in phylogenetic analysis. The aligned sequences are deposited in GenBank under Acession number XXX.

Analyses of Sequences, Intraspecific Phylogenies and Population Structure

Consensus sequences (of the two PCR products, each sequenced with four primers) were made for every individual using computer programs: Phred v. 0.20425 (Ewing & Green 1998; Ewing *et al.* 1998), Phrap v. 0.990319 (www.phrap.org) and Consed 12.0 (Gordon *et al.* 1998). Clustal W was used for sequence alignment of different consensus from distinct individuals using *default* parameters. Model testing analysis (Posada & Crandall 1998) was made with Hyphy program (Pond *et al.* 2004) to determine the evolutionary mutation model of these sequences. Based on this analysis' results, the Tamura-Nei model with gamma distribution (α parameter = 0.786272) was chosen to estimate genetic distances in further procedures.

Nucleotide diversity (π), haplotype diversity (h), average number of nucleotide differences (k) and transition/transversion rates (R) were calculated with programs DnaSP (version 4.0; Rozas *et al.* 2003), MEGA (version 3.1; Kumar *et al.* 2004) and Arlequin (version 2.000; Schneider *et al.* 2000). Relationships among unique haplotypes were assessed by building a network using the median-joining algorithm (Bandelt *et al.* 1999) with the program NETWORK (version 4.1.0.8; www.fluxus-engineering.com). Additional phylogenies were constructed with both Maximum Parsimony (MP) and Neighbor Joining (NJ) algorithms using MEGA (version 3.1; Kumar *et al.* 2004). Both trees were tested with 1000 bootstrap replicates. MP bootstrap consensus tree was produced by heuristic search using CNI (Close Neighbor Interchange) with 10 repetitions of random additions of sequences (complete deletion of gaps/ missing data). NJ tree was made with Tamura-Nei model with gamma distribution (α parameter = 0.786272). Sequences used in all phylogenetic analysis were uncorrected.

Divergence time of 17.44% was used to estimate separation between phylogroups, as employed by Milot *et al*. (2000) for the first domain of mitochondrial control region of the Oscine passerine *Dendroica petechia*.

Hierarchical distribution of genetic diversity was verified through Analysis of Molecular Variance (AMOVA; Excoffier *et al.* 1992) with Arlequin (version 2.000; Schneider *et al.* 2000). Each fragment sampled was considered as a population. In order to test the phylogeographical structure, two different tests were made: (1) three-level AMOVA, considering **fragments** (n = 11) as **populations** and **localities** (n = 8) as **groups**; and (2) three-level AMOVA, also considering **fragments** (n = 11) as **populations** but **groups**

represented by **geographical regions** (n = 2: northeast and southeast). More details are described in table 2. Significance of Φ statistics, which estimates the percentage of genetic variation within populations, among populations within groups and among groups, was tested with 1000 nonparametric permutations.

To test whether geography define population genetic structure, simple linear regressions were made to correlate geographical distances with two genetic distance indexes: Φst between pairs of populations, calculated with Arlequin, and log of Nei distance (average number of nucleotide substitutions per site between populations; Nei 1987), calculated with DnaSP. Log of Nei distance was used because neither Nei values nor their residuals from regressions presented a normal distribution. Great circle geographical distances between populations were calculated with the program Earth (Jonh A. Byers – www.wcrl.ars.usda.gov/cec/moregen.htm) using coordinates for each fragment.

Analyses of habitat fragmentation effects

In order to verify whether effects of anthropic fragmentation are expressed over genetic diversity, a simple linear regression between population nucleotide diversity (π) and log of fragment size was made. Also, ANOVA was used to compare average π in populations among fragment size classes. Three different arrangements of fragment classes were tested: (1) large ($\geq$ 1000 ha; n = 4) *versus* small ($\leq$ 100 ha; n = 5); (2) large ($\geq$ 1000 ha; n = 4) *versus* medium (between 1000 ha and 30 ha; n = 3) *versus* small ($\leq$ 30 ha; n = 4); and (3) considering only large and small fragments sampled on same localities, *i.e.* large ($\geq$ 1000 ha; n = 3) *versus* small ($\leq$ 30 ha; n = 3).

Then, populations from small and large fragments were considered separately to perform two kinds of analyses: simple linear regressions between Φst and geographical distances separating pairs of populations and two-level AMOVA (Excoffier *et al.* 1992; performed with Arlequin by Schneider *et al.* 2000) (Table 2). In these analyses, fragments considered were those from the following localities: Araponga, Nova Lima, Simonésia, Bocaiúva and Jequitinhonha (four small and four large fragments). AMOVA tests also were performed without northeastern populations (Bocaiúva – BO – and Jequitinhonha –JE), only with populations from the three localities where both one small and one large fragment were sampled.

Results

Mitochondrial Control Region Sequences

The complete sequence alignment of 104 individuals resulted in 1074 bp of the mitochondrial control region, with the beginning approximately 150 bp downstream $tRNA^{Thr}$ and the end 30 bp upstream $tRNA^{Pro}$. Fifty-one haplotypes were found, with 106 mutations in 98 variable sites, from which 81 were parsimony informative. Gaps were present at 13 sites. As expected, the light (L) strand presented low Guanine content (G = 14.4%) and transitions were higher than transversions (R = 3.0). As described by Baker and Marshall (1997), most differences between sequences were observed in domains I and III, but mainly into third domain. The preserved boxes F, D, C (Baker & Marshall 1997) and B, and the bird similarity box (Ruokonen & Kvist 2002) could be identified. Total nucleotide diversity (π) was estimated as 0.0128 $\pm$ 0.0021, haplotype diversity (h) as 0.973 $\pm$ 0.006 and average number of nucleotide differences (*k*) as 12.6250 $\pm$ 5.7396. Values of π, h and *k* for each population are depicted in table 1. It is noticeable the high π and *k* values in populations AJ, NLJ, NLP and BO.

Intraspecific Phylogenies

When median-joining network was drawn, two very different clusters were identified (Fig. 2). Most specimens (n = 97) belonged to one cluster and seven to a different one. However these two clusters are not geographically correlated and individuals from both clusters occur sympatrically in three populations (AJ, NLJ and NLP). Trees constructed with both Maximum Parsimony (MP) and Neighbor Joining (NJ) algorithms strongly supported the existence of two clades (Fig 3). Average percentage of nucleotide differences between them was 5.92% (uncorrected sequences). In order to clarify results explanation, clades were named as TY, for the bigger clade (with 97 individuals) and as EN, for the smaller (with 7 individuals).

Afterwards, all sequences were compared to the mtDNA CR sequences of two individuals from northeast of São Paulo State (SP; unpublished sequences produced by R. O. Pessoa), located at the southern boundary of Minas Gerais State (MG). SP sequences were shorter than the obtained in this study. Then, nucleotide alignment of all *C. lineata* plus *C. melanops* sequences resulted in 1055 bp. Using this new alignment, 49 haplotypes were identified. In NJ and MP trees, two main branches were identified: one with all individuals from TY clade and another with EN clade and SP individuals; this last branch was strongly supported by bootstrap replicates (100% for NJ – data not shown – and 99% for MP – Fig. 3). Hence, all populational analysis done considering data as a whole (n = 103) were also done excluding individuals from EN clade (n = 7; more details on the discussion).

Besides that, within the TY clade, two haplotype lineages were identified on MJ network and NJ and MP trees (Figs. 2 and 3): one from northeastern Minas Gerais State (BO and JE) and the other from southeastern regions (mainly composed of haplotypes from southeastern populations). JE population presented unique haplotypes, but BO population presented one haplotype from the same lineage of JE and two belonging to southeastern haplotype lineage. Average percentage of nucleotide differences between those two haplotype lineages was 1.3%.

AMOVA and regressions between genetic and geographical distances

AMOVA revealed no geographic structure of genetic diversity among localities because the majority of the variation, 74.20%, was found within populations/fragments and the remaining was detected almost entirely among populations within localities (25.54%; $\Phi st = 0.258$, $p = 0.000$). When comparison was made between northeastern and southeastern regions, a stronger geographic structure was found ($\Phi st = 0.422$, $p = 0.000$), but most variation was still within populations/fragments (57.8%). Considering only the TY clade, higher Φst values were obtained: for localities comparison, Φst was 0.360 ($p = 0.000$), with most percentage of variation within populations/fragments (64.0%); for regions contrast, Φst was 0.613 ($p = 0.000$) and most part of diversity (55.24%) was between northeastern and southeastern regions and not within populations (Table 2).

In Φst pairwise comparisons, JE population presented highest significant values of Φst against all other populations (from 0.394, against BO, to 0.681, against SS), considering all individuals or just TY clade. For pairwise percentage sequence divergences, considering TY and EN, values ranged from 0.39% (CA x SB) to 4.4% (NLJ x JE) and highest values were those from all comparisons with NLJ population; excluding EN clade, values ranged from 0.33% (NLP x SB) to 1.44% (JE x AB), and highest values were those from comparisons with JE population.

Determination coefficients from all simple linear regressions between geographical and genetic distances were significant. Considering all individuals, geographical distance could explain 26.26% of genetic differences measured as Φst values ($R^2 = 0.263$, $p = 0.000$) and 10.52 % of log of Nei distance ($R^2 = 0.105$, $p<0.016$). Excluding individuals from the EN clade, geographical distances explained a greater part of genetic distances: 45.10% of Φst values ($R^2 = 0.451$, $p = 0.000$) and 73.24% of log of Nei distance ($R^2 = 0.732$, $p = 0.000$). Genetic distances' residuals from all regressions presented normal distribution.

Habitat fragmentation effects

Simple linear regression between population nucleotide diversity (π) and log of fragment size provided small and non-significant correlation coefficients (R). All ANOVA tests also presented non-significant results.

AMOVA revealed that diversity among large fragments was geographically structured, considering individuals from both clades (Φst = 0.535, p = 0.000) or excluding the EN clade (Φst = 0.573, p = 0.000). On the other hand, diversity structure among small fragments was noticeably weaker, considering all individuals (Φst = 0.011, $p \geq 0.326$; not significant) or not (Φst = 0.132, p = 0.000).

Contrasting to the results with four fragments, AMOVA tests (without EN clade) considering only three localities (Nova Lima, Araponga and Simonésia) revealed a stronger structure among small fragments (Φst = 0.148, p = 0.002) than among large fragments (Φst = 0.079, p = 0.085, marginally significant).

Simple linear regressions between genetic and geographic distances showed that in large fragments, geographic distances can explain a high percentage of genetic divergence between pairs of populations: 72.49% (R^2 = 0.725, p = 0.031) and 82.48% (R^2 = 0.825, p = 0.012) excluding EN clade. For small fragments, there was no association of genetic and geographic distances between pairs of populations, since regressions presented small and not significant correlation coefficients.

Discussion

Intraspecific Diversity and Phylogenies

Nucleotide diversity found for *C. lineata* (1.28%) was similar to diversity values obtained using mitochondrial control region (CR) of passerine species sampled along larger areas in temperate regions (Milot *et al.* 2000; Kvist *et al.* 2001). Nevertheless, *C. lineata* diversity is higher than some values recently obtained for Neotropical passerines with mitochondrial assays. Using restriction enzymes over 2800 bp of mitochondrial DNA (control region, cytochrome *b* and ND6). Brown *et al.* (2004) registered diversity values of 0.52% for *Henicorhina leucosticta* and 0.45% for *Eucometis penicillata*. Lacerda (2004) estimated that intraspecific diversity for three Thamnophilidae species varied from 0.43% to 0.78% and Cabanne (2004) found 0.82% (with great SD: 0.45) for *Xiphorhynchus fuscus fuscus*. In these two latter studies, nearly 600 bp of the control region were used to investigate Atlantic Forest populations with geographical distributions compatible to the one considered in this work. Therefore, the higher value found for *C. lineata* may be related to the use of longer sequences of mitochondrial control region. This region is divided into three domains: a central and more conserved one, flanked by two variable domains (Baker & Marshall 1997). Sequences obtained for this work included all three domains and, for *C. lineata*, the third domain was noticeable more variable than the first one. The two latter studies mentioned before included only first domain and part of the central domain. Hence, more variation was already expected to be found within the 1074 bp analysed for *C. lineata*. When only the first 600 bp were considered for *C. lineata*, a nucleotide diversity of 0.88% was found.

Furthermore, high total diversity can be justified by the existence of two very divergent clades: EN and TY. Divergence between these clades (5.92%) was similar to divergence among some different neotropical passerine species within *genera*, as described for *Xiphorhyncus* (3.4% - 10.0%; Aleixo 2002), *Cranioleuca* (0.5% - 4.7%; García-Moreno *et al.* 1999), *Thamnophilus* (2.7% - 7.4%; Lacerda 2004), *Ramphocelus* (0.1% - 9.5%; Hackett 1996) and two *Drymophila* species (7.2%; Bates *et al.* 1999). Once more, the divergence value between clades could be explained by the size of *C. lineata* sequences, although Aleixo (2002) analysed sequences with 2430 bp length (from cytochrome *b*, ND2 and ND3) and Hackett (1996) analysed sequences with 1412 bp length (cyt *b* and ND2) on their surveys and found comparable divergence values between species. Also, Zink and Blackwell-Rago (2000) investigated 1115 bp (CR, cyt *b* and ND2) of the thrasher *Toxostoma curvirostre* and found specific nucleotide diversity of 1.1%. In this latter example, since the percentage of sequence divergence between two *T. curvirostre* subspecies' groups diverge in 2% and there were morphological differences between them, the authors suggested that both groups warrant a species status.

Then what could explain the existence of so divergent clades within *C. lineata*, not geographically separated? One explanation proposed for this phylogeographic pattern would be that southeastern Minas Gerais State (MG) is a region of secondary contact between populations that expanded after being isolated for a long time, when clades have evolved allopatrically (Avise *et al.* 1987). The grouping of EN clade with São Paulo State (SP) sequences supports this model. Also, TY clade was much more frequent, indicating that southeastern MG is closer to the region where this clade evolved. In contrast, EN clade seems to have evolved more to the south of Brazil, because SP location is under the south boundary of MG.

The separation time between clades TY and EN was estimated as approximately 180,000 years, meaning that the populations where they evolved were unconnected at least for this period. This time is coincident with recent Pleistocene, when repeated glaciations affected neotropical forests' distribution, resulting on vicariant effects over species diversity (Ab'Saber 1979). It is suggested that the mountain chain called Serra do Mar was an Atlantic Forest refugee during last glacial periods (Ab'Saber 1979). The Serra do Mar is situated in northeastern SP and southern Rio de Janeiro, near to the coast. The analysed samples from São Paulo State were collected very close to this mountain region (in the city of Bananal) and might indicate that this area could have been where EN clade evolved. Nevertheless, this divergence time estimative might be heedfully interpreted, since the mutation rate used was calculated for the first domain of control region and *C. lineata* sequences presented almost no variation in central domain, but most part of variation was into third domain.

Furthermore, specimens from EN clade were observed only in three populations (AJ, NLJ and NLP) within two localities, but also these localities presented the largest population sizes. So, this could have been affected by unequal sampling, suggesting that more EN individuals could be found in distinct populations if more birds were captured. Even though, if this clade really evolved allopatrically in the south of Brazil, the proportion of EN clade is expected to diminish towards northern MG down to null in JE locality, as it was detected. A similar phylogeographical pattern was previously described for *Thamnophilus caerulescens* on a study that explored the same geographic range area (Lacerda 2004). In this study, Investigating 721 bp of mitochondrial control region the existence of two simpatric haplotype lineages (presenting divergence of 1.38%) was identified on the same localities (Araponga and Nova Lima). Coincidentally, these places also had bigger sample sizes. On the other hand, Zink (1997) explains that compatible phylogeographies can point towards the same historical event.

An alternative explanation presented by Avise *et al.* (1987) for the phylogenetic discontinuity without spatial separation, is that reproductively isolated sibling species were

misinterpreted as belonging to a single species. This is a reasonable possibility for *C. lineata* taking into account its unknown variations in song and plumage. There are three recognized subspecies, but their geographical ranges are not clearly defined. The subspecies *C. l. lineata* was described in Bahia State, which is at the northern boundary of MG; *C. l. vulgaris* was described in Rio de Janeiro State, which makes southeastern frontier of MG (Pinto 1978).

Hence, MG is close to both places where these two subspecies were described and it would not be unforeseen the presence of both subspecies in this State. SP sequences certainly belong to *C. l. vulgaris* even though, as it was explained above, the divergence between two clades is not only compatible with two subspecies, but with species divergence. Unfortunately, only four vouchers were collected, because we did not have a license to collect all specimens, and they all belong to TY clade. However, if these clades representing subspecies prove to be reproductively isolated, they will deserve a species' status. Future fieldwork, to compare songs and collect vouchers, and investigation of morphology, plumages and DNA from museum samples are essentially required.

All of the analyses were also made without the EN clade to allow an independent estimate excluding species ascertainment biases. If the existence of two species is to be confirmed, then our population comparisons would be ineffective.

Nucleotide diversity without EN clade was 0.6%, a value comparable to other Neotropical passerines (Bates *et al*. 2003; Brown *et al.* 2004; Cabanne 2004; Lacerda 2004) and lower than diversity levels calculated for temperate birds (Milot *et al.* 2000; Kvist *et al.* 2001).

Particularly considering TY clade, it is clear the existence of a lineage with individuals only from northeastern MG (JE and BO populations). All samples from JE belong to this lineage, but only one from BO belongs to it. This could be a consequence of low gene flow resulting from the long distance between southeastern and northeastern populations (until 520 km), as suggested by Avise *et al.* (1987) to explain this kind of phylogeographical structure. It seems that JE might be isolated from southeastern populations due to an ancient extrinsic barrier to gene flow (Avise *et al.* 1987). Although, TY clade is a polyphyletic group, what makes it inaccurate to estimate the divergence time between lineages.

A similar phylogeographical pattern was also described for *Xiphorhynchus fuscus fuscus* (Cabanne 2004), *Thamnophilus ambiguus* and *Pyriglena leucoptera* (Lacerda 2004) in works that surveyed some of the localities considered here, within the same geographic range. However, none of these studies analysed populations on east-central MG, and the two lineages could be explained simply by geographical distance (correlation between genetic and geographic distances will be discussed afterwards). Besides, occurrence of both

lineages in BO could indicates gene flow between this population and the others. Another explanation by Avise *et al.* (1987) would be the extinction of intermediate haplotypes, what is congruous with the wide distribution and the habitat destruction of all these species.

Population's Diversity, AMOVA and Geographical Correlations

Not surprisingly, populations that revealed higher diversity values (π and *k*) were those with individuals from TY and EN clades (AJ, NLJ and NLP) and BO, which had haplotypes from the two TY lineages. In addition, highest values of pairwise percent sequence divergence between populations were those that included populations NLJ. High values were also found in all pairwise divergences including JE population, due to its unique haplotype lineage. Bates *et al.* (2003) found for some populations of Cerrado birds, separated by 1800 km, comparable genetic distances: 0.29% (in *Lepidocolaptes angustirostris*) and 1.76% (in *Formicivora rufa*), using 379 bp of cyt *b*. Lacerda (2004) also found similar values for *T. ambiguus*, varying from 0.23% (between two northeastern MG populations) to 1.2% (between populations from two MG regions). However, all these values were smaller than the divergence found between NLJ and other populations considering both clades.

Hierarchical structure among populations showed that genetic variation was not arranged among localities, but between geographical regions. Although Φst values were high in all AMOVA tests, a great part of the diversity was usually found within populations. Considering only the TY clade, higher Φst values were obtained and it was expected ever since populations with individuals from both clades present high diversity values, increasing variation within populations and among populations within groups. In addition, lower pairwise differences between southeastern populations might have been caused by one or more characteristics, such as lack of obvious geographical barriers, increased gene flow into this region or recent population range expansion (as we suggested above).

Opposing to the results found here for mitochondrial control region sequences, 10.77% of genetic diversity was observed among these *C. lineata* populations (except SB and BO) with RAPD markers, showing that variation was mostly within rather than among populations or regions (Dantas 2003). Although, strong structure between northeastern and southeastern MG was also described for *T. ambiguus* (75.67%) but it was not that strong for *P. leucoptera* (16.96%; Lacerda 2004). Furthermore, four populations of *Gymnopithys leucapsis* and *Henicorhina leucosticta* separated by at most 110 km in Costa Rica showed lower Φst values (25.6% and 25.1%, respectively). All these species inhabit understorey humid forests and have insectivorous habits and this might indicate that population genetic structure is more related to a species origin and expansion history than to its ecological features.

In agreement with hierarchical structure of genetic diversity, regressions between genetic and spatial distances were significant, showing higher values without individuals from EN clade (until 73.24%). The strong effect of geography over genetic distances indicates isolation by distance, corroborating that reduced gene flow between regions was important to the diversification of the northeastern lineage.

Analyses of Anthropic Fragmentation Effects

Genetic diversity structure and association between genetic and geographic distances are correlated to equilibrium between gene flow and genetic drift in populations (Hutchinson & Templeton 1999). Habitat fragmentation limits gene flow and reduces population size, increasing genetic drift. Hence, expected genetic consequences of fragmentation are: loss of genetic variability within populations, stronger population structure (Φst) and weaker correlation between geographic and genetic distances (Cunningham & Moritz 1998).

There are very few studies exploring effects of anthropic fragmentation over genetic diversity, especially on tropical forests. In Costa Rica, three passerine species presented significantly decrease of mtDNA restriction polymorphisms in smaller forest fragments, isolated for only 50 years (Brown *et al.* 2004). Also, an European Cappercaillie (*Tetrao urugallus*) presented more allelic variability (measured with microsatellites) in larger forest areas compared to smaller ones (Segelbacher *et al.* 2003). Moreover, in continuous areas *T. urugallus* presented significant correlation between geographic and genetic distance opposing to smaller fragments, where no pattern of isolation by distance was identified.

On the other hand, as described here, some surveys could not find a direct correlation between fragment size or connectivity and diversity (ANOVA and regressions) but also used analyses of genetic structure and isolation by distance to contrast small and large fragments (or continuous areas). Two different works (studies) analysed the genetic diversity of an Australian rain forest lizard in fragmented populations. In the first work, Cunningham & Moritz (1998) did not find relationship between diversity and fragment size using mtDNA restrictions, and they found a higher Gst value in some continuous areas than in fragments. Otherwise, they registered that diversity distribution in this species was more affected by older historical processes. Then, Sumner *et al.* (2004) compared differently sized fragments and continuous areas within a smaller region where the lizard presented a uniform history, as described by the former study. They also did not find association between number of microsatellite alleles or heterozigosity and size of forest area. In addition, Fst value was slightly smaller in fragments and the effect of geography on genetic distances was stronger in continuous forest.

This same pattern was described here for *C. lineata* when northeastern populations were considered in fragmentation analysis: although there was no predictable stronger diversity structure (Φst) in small fragments, the effects of their isolation were detectable by the disruption of the strong correlation between geographical and genetic distances. This could be explained by increased gene flow among southeastern fragments and between these and BO population, as proposed before because of low pairwise Φst values identified for these comparisons. It is relevant to mention that JE, considered in AMOVA of large fragments, was the only population that presented significant Φst against all other populations. The corresponding population used in AMOVA for small fragments was BO, which has only three individuals – contrasting to the big sample size of populations from other small fragments – and presents only one haplotype from the northeastern lineage.

When AMOVA was performed considering only populations from localities where both one small and one large fragment were sampled, small fragments presented slightly stronger genetic structure (Φst = 0.148, p = 0.002) than large did (Φst = 0.079, p = 0.085), as an expected outcome of fragmentation. It supports the idea that stronger genetic structure found among large fragments when JE and BO were considered might represent a result of an asymmetrical test for different sizes of fragments, due to higher genetic distance of JE to the other three populations. This shows that even not so conspicuous historical events like a possible natural extrinsic barrier to gene flow can interfere on analyses of genetic effects of habitat fragmentation.

It was expected that both small and large fragments compared, presented the same amount of gene flow, as they are much similarly distributed (on the same locality, small and large fragment are separated by five to 20 km). The difference between them would indicate that smaller fragments were under stronger genetic drift. Although, this could not be assured from the present data because of the small number of populations analysed. Also, there was a great difference in number of individuals analysed in large and small fragments.

Nevertheless, habitat fragmentation might be too recent a process so that its effects can be assessed, because it takes time for the genetic structure to return to equilibrium after changes in landscape. Thus, populations that have declined rapidly due to habitat loss could have normal levels of genetic variation while low genetic variability could be found in populations with long histories of small population sizes and inbreeding (Frankham 1995). Fragmentation puts animals into a drastically new situation, and how they will cope with the fragmented landscape depends not only on inherent characteristics, such as whether they are territorial within the fragments and whether they are able to move among different fragments (Pires & Fernandez, 1999) but also on the distance and kind of matrix between forest patches. Then, another drawback in estimating effects of recent fragmentation habitat is that previous genetic diversity distribution is not known.

Other explanation for the absence of direct association between diversity and fragment size would be that small fragments might not contain smaller populations since more birds were captured in them. Nonetheless, the minor relation between sizes of a small and a large fragment was 1/10 (BO, with 100 ha and SS and NLJ, with 1000 ha) and this would be not so a plausible suggestion. Even so, *C. lineata* is a generalist insectivorous and could be more frequent in small fragments due to specialists (potential competitors) decreasing in these areas (Kattan 1994). Besides, both small and large fragments did not present biased sexual rate, indicating that the species is reproductively successful, independently of the forest patch size (Dantas 2003).

In addition, loss of diversity could have been imperceptible because of the molecular marker used. The control region has a higher evolution rate if compared to the rest of mitochondrial DNA, but random nuclear markers like short tandem repeats (STRs) or RAPD usually gauge higher levels of variability within populations. However, RAPD markers were used to analyse most of these *C. lineata* populations but also could not reveal diversity loss in small fragments (Dantas 2003).

Implications for Conservation

Diversity distribution patterns are determined not only by recent events, like anthropic fragmentation, but also by long term processes, like vicariance, dispersal and population expansions. This can potentially confound the understanding of populations' genetic dynamic but can be more clearly investigated by using haplotype trees (Templeton 1998). In this study an obvious historical event determined the variability distribution of *C. lineata* populations in Minas Gerais State. Because it was possible to identify the results of this event (existence of two clades) analyses of fragmentation effects were not blurred. Also, the existence of a less striking event, like the slightly divergent northeastern lineage could have affected fragmentation analysis.

As no remarkable anthropic effect was identified, conservation of this bird should focus on populations with high diversity or with singular identity, which might represent evolutionary significant units (Moritz 1994). In this sense, localities where EN clade was identified can be considered important for conservation of *C. lineata* genetic variability for their high diversity and for the preservation of the rarer clade. Likewise, JE population represent another significant unit because of its particular identity, a recurrent singularity observed in other suboscines species (Cabanne 2004; Lacerda 2004)

C. lineata is not a threatened species, but it is a dependant species from the endangered Atlantic forest. This bird could be useful as a model to estimate effect of fragmentation over more rare species living in the same area with similar habits and to

identify regional historical events. Furthermore, the identification of evolutionary significant units of this species and others may enforce the need for preservation of important areas for the Atlantic forest conservation.

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Figure Legends

Fig. 1: Map of Minas Gerais State (Brazil) with the nine localities where individuals were sampled.

Fig. 2: Median-joining network of *Conopophaga lineata* in Minas Gerais drew using control region haplotypes (1074 bp). Circle size represents haplotype frequency and different colors symbolize different populations. Lines between haplotypes are proportional to number of mutations between them. Black points represent hypothetical haplotypes.

Fig. 3: Haplotypes Maximum Parsimony consensus tree. Haplotypes are identified by clade name (Ty or En) and the haplotype identification number. N correspond to haplotypes from northeastern lineage. In parenthesis, number of individuals of each population that show the corresponding haplotype.

Table 1: *Conopophaga lineata* populations (fragments) and individuals sampled in Atlantic forest fragments and genetic variation estimations: N, number of sampled individuals; h, haplotype diversity; π, nucleotide diversity; k, average number of nucleotide differences. Fragments' names are localities' names; when two fragments were sampled on the same locality, name is followed by the fragment identification.

Fragments	Area (ha)	N	Haplotypes	h ± SD	π ± SD	*k* ± SD
Northeastern						
Jequitinhonha (JE)	5000	10	5	0.84 ± 0.08	0.0052 ± 0.0031	5.60 ± 2.94
Bocaiúva (BO)	100	3	3	1.00 ± 0.27	0.0102 ± 0.0080	10.94 ± 6.89
Southeastern						
Araponga_Brig (AB)	13000	8	8	1.00 ± 0.06	0.0052 ± 0.0032	5.55 ± 2.99
Araponga_JoRi [a] (AJ)	20	17	8	0.85 ± 0.07 [a]	0.0107 ± 0.0057 [a]	11.51 ± 5.49 [a]
Viçosa (VI)	120	5	4	0.90 ± 0.16	0.0038 ± 0.0026	4.04 ± 2.42
Simonésia_Sose (SS)	1000	6	4	0.80 ± 0.17	0.0035 ± 0.0023	3.71 ± 2.18
Simonésia_Lula (SL)	30	8	7	0.96 ±0.08	0.0038 ± 0.0024	4.09 ± 2.28
Caratinga (CA)	30	11	6	0.91 ± 0.05	0.0043 ± 0.0026	4.60 ± 2.45
Santa Bárbara (SB)	605	8	6	0.89 ± 0.11	0.0037 ± 0.0024	3.97 ± 2.22
Nova Lima_Jamb [b] (NLJ)	1000	5	5	1.00 ± 0.13 [b]	0.0391 ± 0.0240 [b]	41.94 ± 22.07 [b]
Nova Lima_Prim [c] (NLP)	27	22	8	0.86 ± 0.05 [c]	0.0161 ± 0.0083 [c]	17.32 ± 8.00 [c]
Ouro Preto (OP)	3000	1	1	-	-	-
All		104	51	0.97 ± 0.01	0.0128 ± 0.0021	12.63 ± 5.74
All without individuals from EN clade		97	45	0.97 ± 0.01	0.0060 ± 0.0005	6.29 ± 3.01

[a] diversity values for Araponga_JoRi population without the individual that belong to the EN clade: samples = 16; h = 0.8333 ± 0.072 ; π = 0.0044 ± 0.0026; *k* = 4.72 ± 2.44; [b] diversity values without individuals (n = 3) from the EN clade were not calculated for Nova Lima _Jamb population due to its small size (samples = 2); [c] diversity values for Nova Lima_Prim population without individuals (n = 3) from the EN clade: samples = 19; h = 0.8129 ± 0.0600; π = 0.0024 ± 0.0015; *k* = 2.59 ± 1.45.

Table 2: Hierarchical analyses of molecular variance (AMOVA) of the *Conopophaga lineata* populations. Four sets of comparisons were calculated considering populations from large and small fragments. In order to test anthropic fragmentation effects six other sets were calculated considering either large or small fragments in 5 localities (Araponga, Nova Lima, Simonésia, Bocaiúva and Jequitinhonha).

Analysis– Hierarchy (N)	Φst	Φct	% molecular variance (df)		
			Among groups	Among populations within groups	Within populations
Populations and localities (103)	0.2580**	0.0026^{NS}	0.26 (7)	25.54 (3)	74.20 (92)
Populations and localities without EN clade (96)	0.3596**	0.2939 ^{NS}	29.39 (7)	6.57 (3)	64.04 (85)
Populations and geographic regions (103)	0.4219**	0.2949*	29.49 (1)	12.70 (9)	57.81 (92)
Populations and geographic regions without EN clade (96)	0.6133**	0.5525*	55.24 (1)	6.08 (9)	38.67 (85)
Among large fragments (29)	0.5351**	-	-	53.51 (3)	46.49 (25)
Among large fragments without EN clade (26)	0.5731**	-	-	57.31 (3)	42.69 (22)
Among small fragments (50)	0.0113 ^{NS}	-	-	1.13 (3)	98.87 (46)
Among small fragments without EN clade (46)	0.1316**	-	-	13.16 (3)	86.84 (42)
Among three southeastern large fragments without EN clade (16)	0.0786 ^{NS}	-	-	7.86 (2)	92.14 (13)
Among three southeastern small fragments without EN clade (43)	0.1484*	-	-	14.84 (2)	85.16 (40)

** = p < 0.01; *= p < 0.05; NS = not significant; significance of Φ statistics was tested with 1000 nonparametric permutations; N = number of individuals analysed; df = degrees of freedom.

Fig. 1:

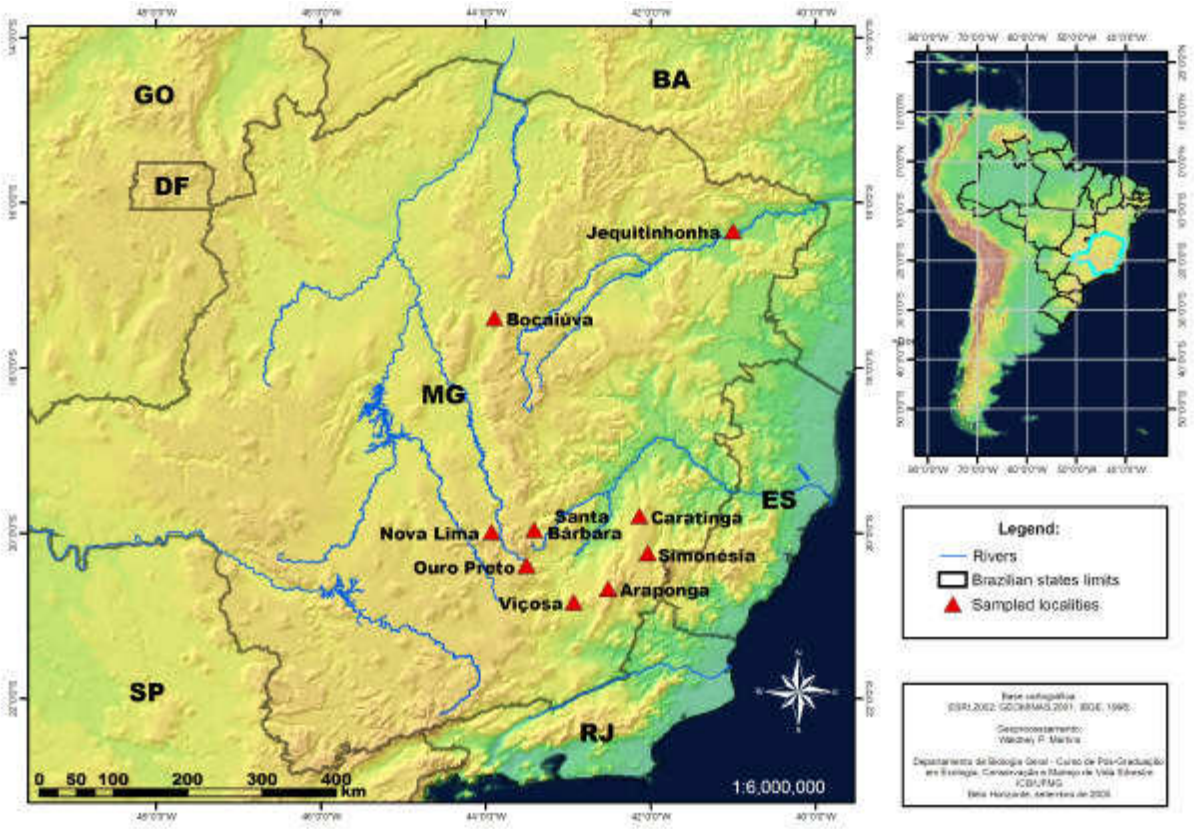

GO
BA
DF
Jequitinhonha
Bocaiúva
MG
ES
Santa Bárbara
Caratinga
Nova Lima
Simonésia
Ouro Preto
Araponga
Viçosa
SP
RJ
0 50 100 200 300 400 km
1:6,000,000
Legend:
Rivers
Brazilian states limits
Sampled localities

Fig. 2:

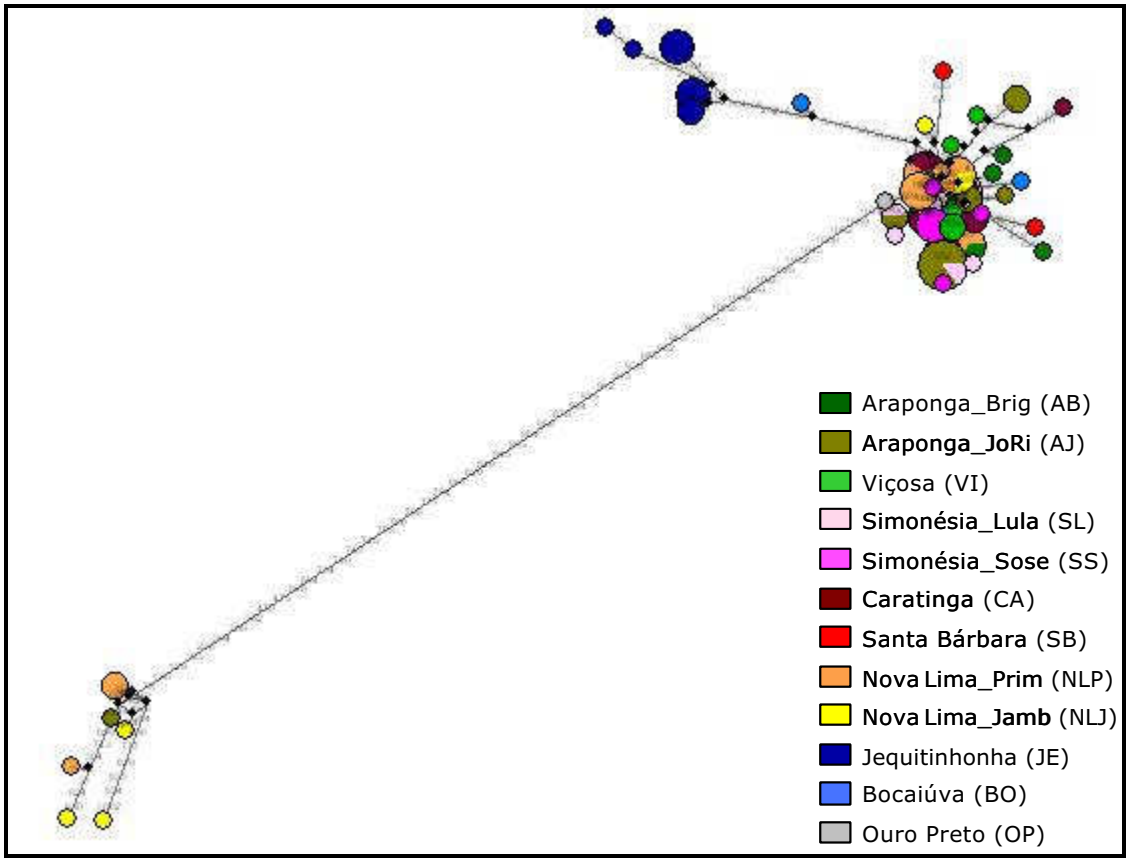
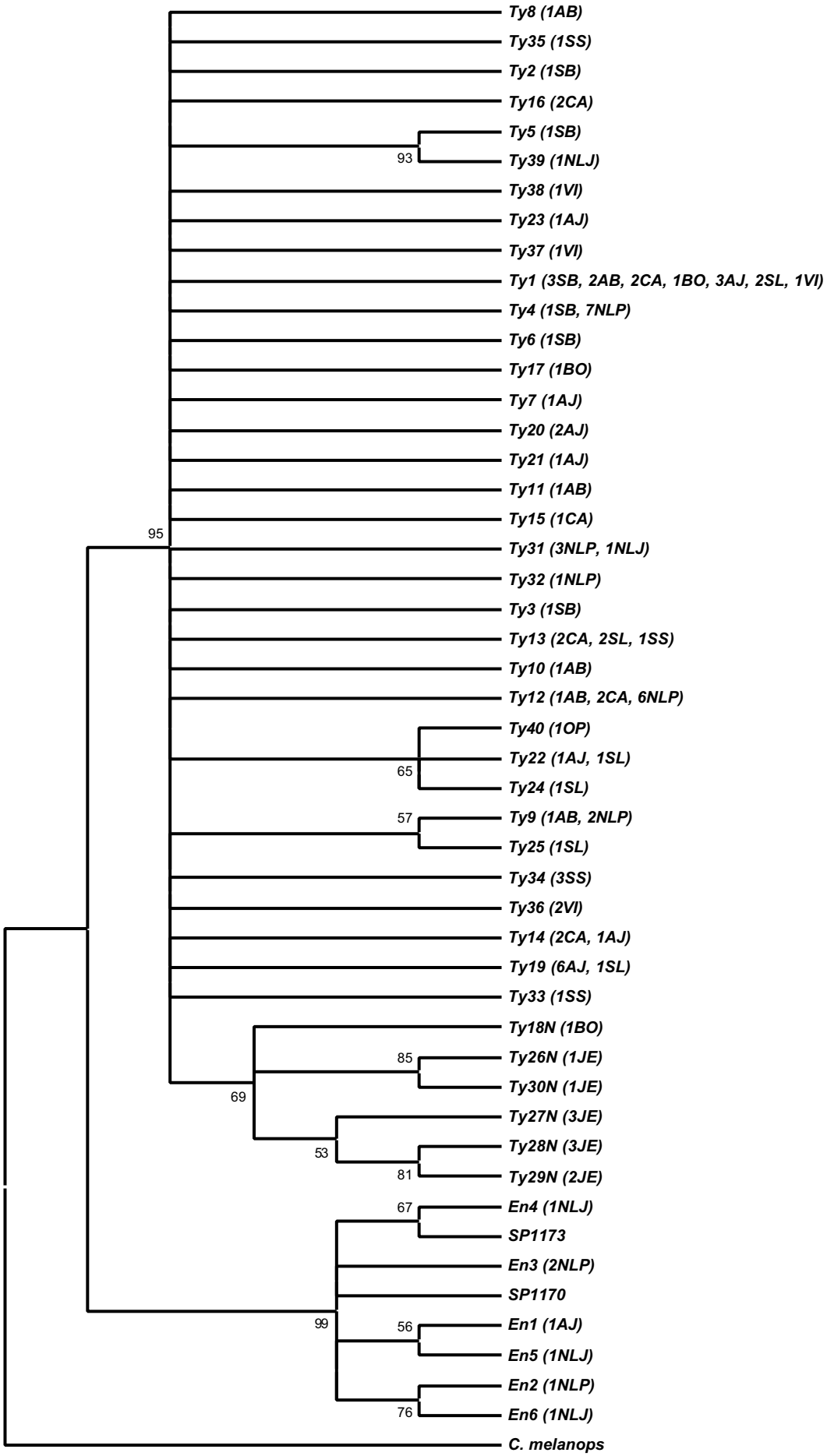

Araponga_Brig (AB)
Araponga_JoRi (AJ)
Viçosa (VI)
Simonésia_Lula (SL)
Simonésia_Sose (SS)
Caratinga (CA)
Santa Bárbara (SB)
Nova Lima_Prim (NLP)
Nova Lima_Jamb (NLJ)
Jequitinhonha (JE)
Bocaiúva (BO)
Ouro Preto (OP)

Fig. 3:


Ty8 (1AB)
Ty35 (1SS)
Ty2 (1SB)
Ty16 (2CA)
Ty5 (1SB)
93
Ty39 (1NLJ)
Ty38 (1VI)
Ty23 (1AJ)
Ty37 (1VI)
Ty1 (3SB, 2AB, 2CA, 1BO, 3AJ, 2SL, 1VI)
Ty4 (1SB, 7NLP)
Ty6 (1SB)
Ty17 (1BO)
Ty7 (1AJ)
Ty20 (2AJ)
Ty21 (1AJ)
Ty11 (1AB)
Ty15 (1CA)
95
Ty31 (3NLP, 1NLJ)
Ty32 (1NLP)
Ty3 (1SB)
Ty13 (2CA, 2SL, 1SS)
Ty10 (1AB)
Ty12 (1AB, 2CA, 6NLP)
Ty40 (1OP)
Ty22 (1AJ, 1SL)
65
Ty24 (1SL)
57
Ty9 (1AB, 2NLP)
Ty25 (1SL)
Ty34 (3SS)
Ty36 (2VI)
Ty14 (2CA, 1AJ)
Ty19 (6AJ, 1SL)
Ty33 (1SS)
Ty18N (1BO)
85
Ty26N (1JE)
Ty30N (1JE)
69
Ty27N (3JE)
53
Ty28N (3JE)
81
Ty29N (2JE)
67
En4 (1NLJ)
SP1173
En3 (2NLP)
SP1170
99
56
En1 (1AJ)
En5 (1NLJ)
En2 (1NLP)
76
En6 (1NLJ)
C. melanops

CONCLUSÕES

• Foram eficientemente padronizados os protocolos de amplificação e seqüenciamento de região controle do DNA mitocondrial de *Conopophaga lineata*, o que possibilitou a análise de 1074 pb dos 104 indivíduos amostrados.

• A espécie *Conopophaga lineata* apresentou um alto índice de diversidade se comparada com outras espécies de passeriformes neotropicais.

• A alta diversidade da espécie justificou-se pela coexistência de dois clados em algumas populações do sudeste de Minas Gerais (MG), que apresentaram divergência compatível com a de duas espécies congenéricas. Um destes clados formou um grupo monofilético com indivíduos coletados em São Paulo, na região da Serra do Mar.

• A coexistência dos dois clados pode ser explicada pela identificação errônea de duas espécies crípticas isoladas reprodutivamente, com uma só espécie. Outra explicação proposta foi que o sudeste de MG seria uma zona de contato secundário entre populações que se expandiram após um isolamento de aproximadamente 170 mil anos (época coincidente com a do Pleistoceno). Durante esta separação os dois clados teriam evoluído em diferentes refúgios pleistocênicos da Mata Atlântica, sendo um deles proposto como a Serra do Mar.

• Encontrou-se ainda uma forte estrutura filogeográfica entre as populações do sudeste e do nordeste de MG, que pode ser explicada pela grande distância geográfica e conseqüente baixo fluxo gênico. A estrutura hierárquica da diversidade genética também foi encontrada somente entre estas regiões (com um alto valor de Φst) e não entre todas as localidades ou populações.

• A diversidade nas populações, excluindo-se o clado EN também foi comparável ao encontrado na literatura para outros passeriformes neotropicais.

• Foi encontrado forte efeito de isolamento por distância para a espécie, observando-se uma alta correlação entre distâncias genéticas e geográficas entre populações.

• Ao contrário do que se esperava decorrente da fragmentação antrópica, não se verificou menor variabilidade nos fragmentos pequenos e a estrutura da diversidade genética entre fragmentos grandes apresentou-se mais forte do que entre os pequenos. Entretanto, o isolamento por distância só foi observado entre fragmentos grandes.

• Unidades evolutivamente significativas, importantes para a conservação de *C. lineata* e de outras espécies de suboscines da Mata Atlântica foram identificadas nas localidades de Jequitinhonha, Nova Lima e Araponga.

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ANEXOS

ANEXO 1: Lista de indivíduos utilizados, por população, com números das anilhas e do Banco de DNA do LBEM

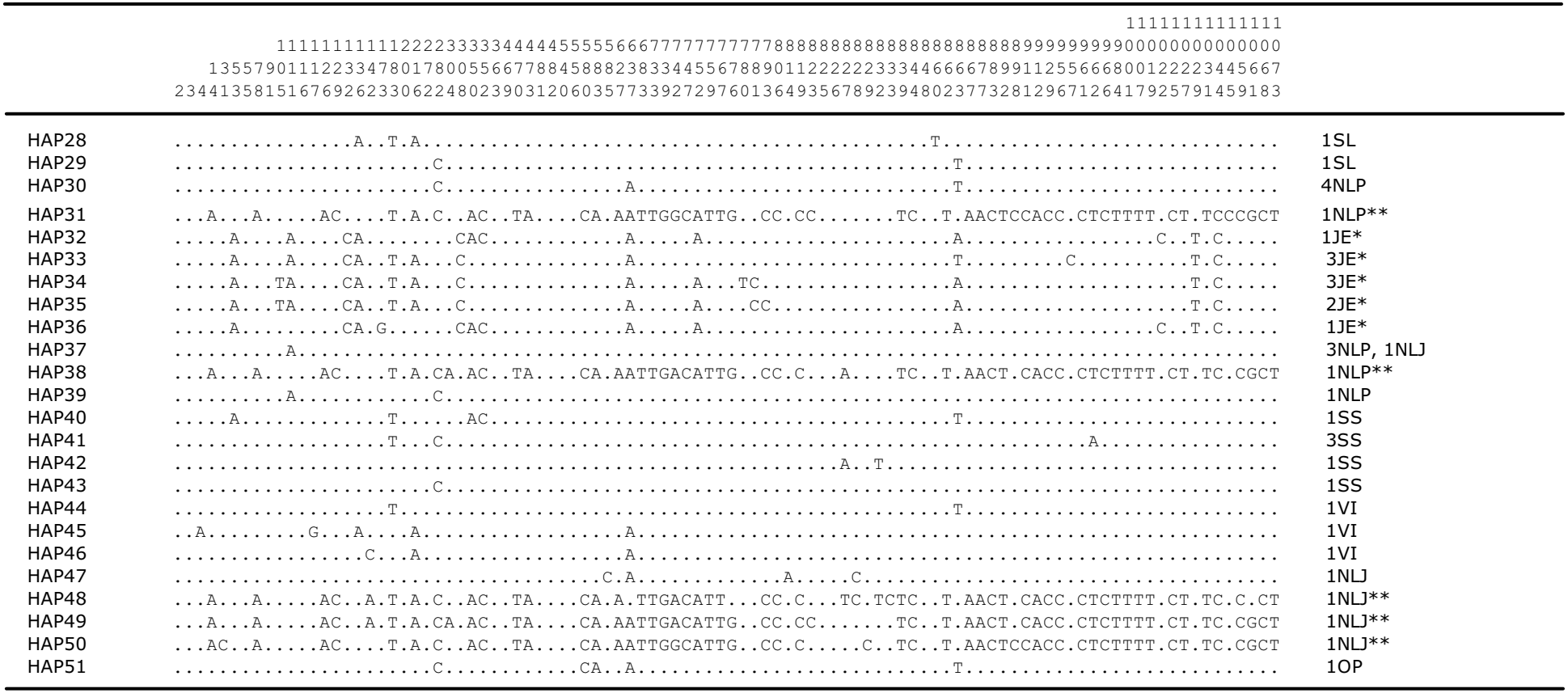
População (Localidade)	Sigla	Anilha	Número no Banco de DNA
Fazenda da Mata Escura (Jequitinhonha)	JE	E40616	B0267
Fazenda da Mata Escura (Jequitinhonha)	JE	E40609	B0417
Fazenda da Mata Escura (Jequitinhonha)	JE	E40624	B0418
Fazenda da Mata Escura (Jequitinhonha)	JE	E40625	B0419
Fazenda da Mata Escura (Jequitinhonha)	JE	E40627	B0420
Fazenda da Mata Escura (Jequitinhonha)	JE	E40629	B0421
Fazenda da Mata Escura (Jequitinhonha)	JE	E40630	B0422
Fazenda da Mata Escura (Jequitinhonha)	JE	E40632	B0266
Fazenda da Mata Escura (Jequitinhonha)	JE	E40639	B0423
Fazenda da Mata Escura (Jequitinhonha)	JE	E40610	B0424
Fazenda Corredor (Bocaiúva)	BO	coletado	B0840
Fazenda Corredor (Bocaiúva)	BO	coletado	B0841
Fazenda Corredor (Bocaiúva)	BO	E62358	B1063
PE da Serra do Brigadeiro (Araponga)	AB	D39677	B0313
PE da Serra do Brigadeiro (Araponga)	AB	D39680	B0314
PE da Serra do Brigadeiro (Araponga)	AB	D39685	B0315
PE da Serra do Brigadeiro (Araponga)	AB	E41163	B0316
PE da Serra do Brigadeiro (Araponga)	AB	E41160	B0372
PE da Serra do Brigadeiro (Araponga)	AB	E41166	B0373
PE da Serra do Brigadeiro (Araponga)	AB	E41173	B0374
PE da Serra do Brigadeiro (Araponga)	AB	E41182	B0375
Mata do João Ribas (Araponga)	AJ	D39664	B0262
Mata do João Ribas (Araponga)	AJ	coletado	B0263
Mata do João Ribas (Araponga)	AJ	E41115	B0259
Mata do João Ribas (Araponga)	AJ	coletado	B0299
Mata do João Ribas (Araponga)	AJ	D39665	B0300
Mata do João Ribas (Araponga)	AJ	D39666	B0302
Mata do João Ribas (Araponga)	AJ	D39669	B0301
Mata do João Ribas (Araponga)	AJ	D39671	B0303
Mata do João Ribas (Araponga)	AJ	D39672	B0304
Mata do João Ribas (Araponga)	AJ	D39673	B0305
Mata do João Ribas (Araponga)	AJ	E41102	B0306
Mata do João Ribas (Araponga)	AJ	E41110	B0307
Mata do João Ribas (Araponga)	AJ	E41128	B0308

Mata do João Ribas (Araponga)	AJ	E41107	B0309
Mata do João Ribas (Araponga)	AJ	E51967	B0310
Mata do João Ribas (Araponga)	AJ	E51970	B0311
Mata do João Ribas (Araponga)	AJ	E51971	B0312
Viçosa - Mata 36	VI	E32952	B0376
Viçosa - Mata 36	VI	E32954	B0377
Viçosa - Mata 36	VI	E32955	B0378
Viçosa - Mata 36	VI	E32956	B0379
Viçosa - Mata 36	VI	E32961	B0380
RPPN do Sossego (Simonésia)	SS	E40584	B0394
RPPN do Sossego (Simonésia)	SS	E40572	B0247
RPPN do Sossego (Simonésia)	SS	E40575	B0258
RPPN do Sossego (Simonésia)	SS	E40587	B0248
RPPN do Sossego (Simonésia)	SS	E40591	B0251
RPPN do Sossego (Simonésia)	SS	E40651	B0252
Mata do Lula (Simonésia)	SL	B0391	B0391
Mata do Lula (Simonésia)	SL	B0392	B0392
Mata do Lula (Simonésia)	SL	B0393	B0393
Mata do Lula (Simonésia)	SL	E40659	B0253
Mata do Lula (Simonésia)	SL	E40660	B0254
Mata do Lula (Simonésia)	SL	E40661	B0255
Mata do Lula (Simonésia)	SL	E40675	B0257
Mata do Lula (Simonésia)	SL	E40677	B0256
EB de Caratinga (Caratinga)	CA	E52006	B0264
EB de Caratinga (Caratinga)	CA	E52017	B0265
EB de Caratinga (Caratinga)	CA	E52001	B0382
EB de Caratinga (Caratinga)	CA	E52002	B0383
EB de Caratinga (Caratinga)	CA	E52003	B0384
EB de Caratinga (Caratinga)	CA	E52005	B0385
EB de Caratinga (Caratinga)	CA	E52007	B0386
EB de Caratinga (Caratinga)	CA	E52014	B0387
EB de Caratinga (Caratinga)	CA	E52009	B0388
EB de Caratinga (Caratinga)	CA	E52015	B0389
EB de Caratinga (Caratinga)	CA	E52016	B0390
EPDA-PETI (Santa Bárbara)	SB	E60011	B0958
EPDA-PETI (Santa Bárbara)	SB	E60043	B0959
EPDA-PETI (Santa Bárbara)	SB	E60051	B0961
EPDA-PETI (Santa Bárbara)	SB	E60060	B0960

EPDA-PETI (Santa Bárbara)	SB	D34605	B0962
EPDA-PETI (Santa Bárbara)	SB	D34606	B0963
EPDA-PETI (Santa Bárbara)	SB	D34609	B1395
EPDA-PETI (Santa Bárbara)	SB	D34611	B1396
RPPN do Jambreiro (Nova Lima)	NLJ	D39691	B0413
RPPN do Jambreiro (Nova Lima)	NLJ	D39692	B0260
RPPN do Jambreiro (Nova Lima)	NLJ	D39693	B0414
RPPN do Jambreiro (Nova Lima)	NLJ	D39700	B0415
RPPN do Jambreiro (Nova Lima)	NLJ	D39696	B0416
Mata da Piedade (Nova Lima)	NLP	E32918	B0268
Mata da Piedade (Nova Lima)	NLP	E32906	B0381
Mata da Piedade (Nova Lima)	NLP	E32907	B0395
Mata da Piedade (Nova Lima)	NLP	E32908	B0396
Mata da Piedade (Nova Lima)	NLP	E32909	B0397
Mata da Piedade (Nova Lima)	NLP	E32910	B0398
Mata da Piedade (Nova Lima)	NLP	E32911	B0399
Mata da Piedade (Nova Lima)	NLP	E32912	B0400
Mata da Piedade (Nova Lima)	NLP	E32917	B0401
Mata da Piedade (Nova Lima)	NLP	E32978	B0402
Mata da Piedade (Nova Lima)	NLP	E32979	B0403
Mata da Piedade (Nova Lima)	NLP	E32980	B0412
Mata dos Primos (Nova Lima)	NLP	E32986	B0269
Mata dos Primos (Nova Lima)	NLP	E32982	B0404
Mata dos Primos (Nova Lima)	NLP	E32983	B0405
Mata dos Primos (Nova Lima)	NLP	E32985	B0406
Mata dos Primos (Nova Lima)	NLP	E32990	B0407
Mata dos Primos (Nova Lima)	NLP	E32991	B0408
Mata dos Primos (Nova Lima)	NLP	E32993	B0409
Mata dos Primos (Nova Lima)	NLP	E32994	B0410
Mata dos Primos (Nova Lima)	NLP	D50660	B0411
Mata dos Primos (Nova Lima)	NLP	D50642	B0425
Grande Mata (São Bartolomeu)	OP	SB-14 Preta dir	B0426

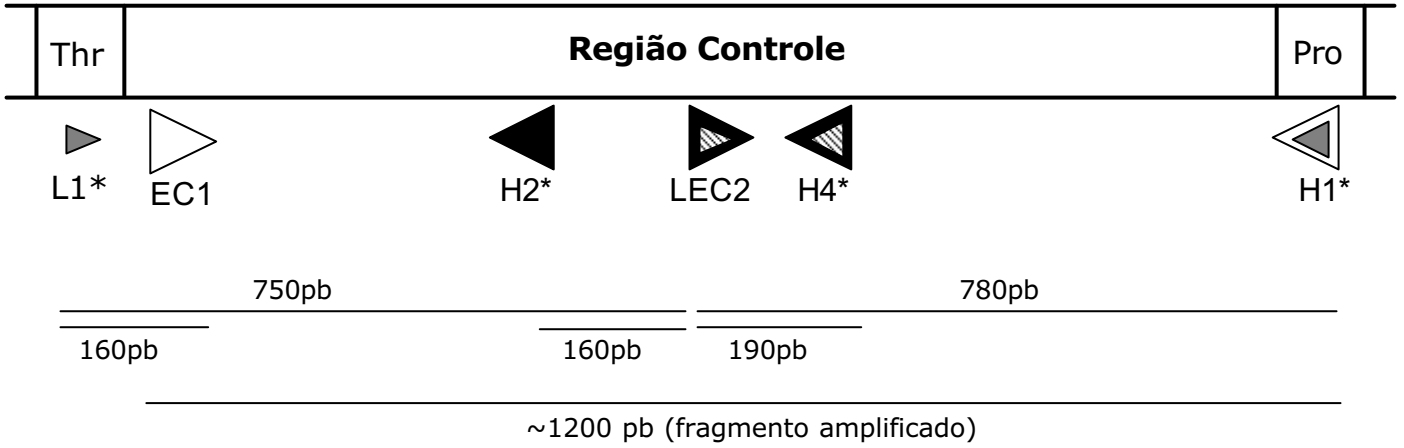
ANEXO 2: Tabela de haplótipos de região controle do DNA mitocondrial (1174 pb analisados) encontrados para *Conopophaga lineata* (excluindo-se os gaps de alinhamento) e número de indivíduos em cada população que os apresentam.

	Sítios Polimórficos	
	11111111111111 1111111111122223333344444555556667777777777788888888888888888888889999999990000000000000 1355790111223347801780055667788458882383344556788901122222233344666678991125566680012222344566 7	**Nº de haplótipos em cada população**
Haplótipo	2344135815167692623306224802390312060357733927297601364935678923948023773281296712641792579145918 3	
HAP1	TACTTGCGACGCAGTTGGCCCGGTTTCTGGACCTCATGTCTGAACTGCCACTTTTTTTTGTTAAATACCCCGTCTTGTTTTCTCCACTGCCCTTTATC	3SB, 1AB, 2CA, 3AJ, 1SL
HAP2	.....T.....A.....A..T.....T.....	1SB
HAP3	.....C....A.....	1SB
HAP4	.....A.....	1SB, 7NLP
HAP5	.CA...G.....A.....A.....C.T.....	1SB
HAP6	.....C.....	1SB
HAP7	.....A.....T.....A.....T.....	1AB
HAP8	..........T.....A.....A.....C...A..T.....	1AB
HAP9	.....A..T.....T.....	1AB, 2NLP
HAP10	.....C.....T.....A.....	1AB
HAP11	.....T.....	1AB, 1BO, 1VI
HAP12	.CA.....A.....A.....	1AB
HAP13	.....C.....A.....	1AB, 2CA, 2NLP
HAP14	..A.....C.....T.....	2CA
HAP15	.....A.....A.....T.....	2CA, 1AB
HAP16	ACA.....A.....A....A.....T....T.....	1CA
HAP17	.....G....T.....	2CA
HAP18	.....G.....CAC.....	1BO
HAP19	.....A...TA....CA.....C.....A.....A.....A.....T.C.....	1BO*
HAP20	.....A.....AC.....T.....	6AJ, 1SL
HAP21	.....T.....	2AJ
HAP22	.....TC.....T.....	1AJ
HAP23	...A...A.....AC....T.A.CA.AC..TA....CA.AATTGACATTG..CC.CC.....TC..T.AACT.CACC.CTCTTTT.CT.TC.CGCT	1AJ**
HAP24	.....C.....CA.....T.....	1AJ, 1SL
HAP25	.CA.....A....A.....A.....C....T.....	2AJ
HAP26	.....A.....	1SL
HAP27	.....C.....GCA.....T.....	1SL



* Haplótipos pertencentes à linhagem do nordeste de Minas Gerais, dentro do clado TY. **Haplótipos pertencentes ao clado EN.

ANEXO 3: Primers utilizados neste estudo para amplificação e seqüenciamento da região controle do DNA mitocondrial de *Conopophaga lineata* e *Conopophaga melanops*



Amplificação de *C. lineata*

Amplificação de *C. melanops*

Seqüenciamento de *C. lineata*

Seqüenciamento de *C. melanops*

Seqüências dos Primers:

L1(L16087)*: 5′ tggtcttgtaaaccararactgaag 3′

EC1: 5′ tccacactcgacatctcatt 3′

LEC2: 5′ tgtacctgttgtggtcggcgta 3′

H1(H16137)*: 5′ aaaatrycagctttgggagttg 3′

H2(H'537')*: 5′ ctgaccgaggaaccagaggcgca 3′

H4(HFII-left)*: 5′ ttgacgaggtaaaaatatgtct 3′

*Lacerda (2004)

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