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INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOLOGIA GERAL
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

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# Estrutura e diversidade genética de populações de minhocuçu *Rhinodrilus alatus,* Righi 1971 (Glossoscholecidae, Clitellata) do estado de Minas Gerais, Brasil

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BELO HORIZONTE

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# Estrutura e diversidade genética de populações de minhocuçu *Rhinodrilus alatus,* Righi 1971 (Glossoscholecidae, Clitellata) do estado de Minas Gerais, Brasil

Flávia de Faria Siqueira

Dissertação submetida ao programa de Pós-graduação em Genética (Área de Concentração em Genética Evolutiva e de Populações) da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do grau de Mestre em Genética.

*Aos meus pais e ao meu companheiro Eduardo*

Charge publicada na “*Punch Magazine*” no dia 6 de dezembro de 1881, logo após o lançamento do livro de Charles Darwin, “*The formation of vegetable mould through the action of worms, with observations on their habits*”. Sua obra sobre minhocas foi a publicação de maior sucesso durante sua vida, vendendo cerca de 3500 cópias em poucos dias.

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## Sumário

**Lista de Figuras** viii

**Lista de Tabelas** viii

**Lista de Abreviaturas, Siglas e Unidades** x

**RESUMO** 12

**INTRODUÇÃO GERAL** 13

*Sobre a espécie Rhinodrilus alatus* 13

*Sobre a família Glossoscolecidae e o Gênero Rhinodrilus* 14

*Genética de Populações e da Conservação* 15

*Estudos genéticos em Annelida* 16

*Marcadores moleculares escolhidos* 17

**Manuscript for Molecular Ecology** 19

**Genetic diversity and population structure in the brazilian giant earthworm *Rhinodrilus alatus* (Annelida, Clitellata, Lumbricina, Glossoscolecidae)** *19*

**ABSTRACT** *20*

**INTRODUCTION** *21*

**MATERIALS AND METHODS** *22*

***Ecological data and distribution of species*** 22

***DNA Extraction and Polymerase Chain Reaction*** 22

***Sequencing*** 24

***Data analysis*** 24

**RESULTS** *26*

***Genetic diversity and population genetic structure with COI*** 26

***Genetic diversity and structure in the ITS1-5.8S-ITS2 segment*** 28

**DISCUSSION** *30*

**ACKNOWLEDGMENTS** *35*

**REFERENCES** *35*

**FIGURE LEGENDS** *43*

**CONCLUSÕES** 54

**REFERÊNCIAS BIBLIOGRÁFICAS** 55

**Lista de Figuras**


Figure 1 50

Figure 2 51

Figure 3 52

Figura 4 53

**Lista de Tabelas**


Table 1 45

Table 2 47

Table 3 48

Table 4 49

**Lista de Abreviaturas, Siglas e Unidades**

$\pi$ - Diversidade nucleotídica

µL - Microlitros

µM - Micromolar

AMOVA - *Analysis of Molecular Variance,* Análise de variância molecular

ºC - Graus Celsius

cm - Centímetros

COI - Subunidade I da Citocromo c Oxidase

DMSO - Dimetilsulfóxido

DNA - *Deoxyribonucleic acid,* Ácido desoxiribonucléico

dNTP - desoxinucleotídeo tri-fosfato

GPS - *Global Position System*

GTR - *General Time Reversible*

h - Diversidade haplotípica

HCl - Ácido Clorídrico

IBAMA - Instituto Brasileiro do Meio Ambiente

Ile - Isoleucina

INDEL - Inserção/Deleção

ITS - *Internal Transcribed Spacer*, Espaço Interno Transcrito

IUCN - União Internacional para a Conservação da Natureza

k - Número médio de diferenças nucleotídicas

KCl - Cloreto de Sódio

Leu - Leucina

Met - Metionina

mg/mL - Miligramas por microlitros

$MgCl_2$ - Cloreto de Magnésio

Mm - Milimolar

DNAmt – DNA mitocondrial

mv - *Median vector*

NCBI - *National Center for Biotechonology Information*

nDNA - DNA nuclear

ng - Nanogramas

NT - *Near threaned*, Quase ameaçado

Pb - Pares de Base

PCR - *Polymerase Chain Reaction*, reação em cadeia da polimerase

PEG - Polietilenoglicol

pH - Potencial Hidrogeniônico

Phe - Fenilalanina

RAPD - *Randomly Amplified Polymorphic DNA*

rRNA - Ácido ribonuclécico Ribossômico

SAMOVA – *Spatial Analysis of Molecular Variance*

SNP - *Single nucleotide polymorphism,* Polimorfismo de nucleotídeo único

SPR - *Subtree Pruning and Regrafting*

U - unidades

UFMG - Universidade Federal de Minas Gerais

**RESUMO**

O minhocuçu *Rhinodrilus alatus* (Righi 1971) é um oligoqueta gigante endêmico do cerrado central da região sudeste do Brasil, muito usado como isca no mercado da pesca. A atividade extrativista dessa espécie inclui muitas pessoas, e acarreta impactos sociais e ambientais. O objetivo desse trabalho foi caracterizar a diversidade genética e estrutura populacional genética e geográfica de *R. alatus* através do sequenciamento do gene de DNA mitocondrial (DNAmt) Subunidade I da Citocromo c Oxidase (COI) e do gene nuclear de RNAr 5.8S e os espaços internos transcritos (ITS) 1 e 2. Foram coletados 75 indivíduos *R. alatus* e 6 *R. motucu*, representando 21 pontos de coleta. A diversidade genética foi avaliada baseada na diversidade haplotípica, nucleotídica e número médio de diferenças nucleotídicas. Estrutura populacional foi caracterizada por AMOVA, Teste de Mantel, SAMOVA e análises filogenéticas (máxima verossimilhança, inferência Bayesiana e m*edian joining)*. Uma extraordinária diversidade genética foi encontrada para *R. alatus*, com 53 haplótipos para o COI, sugerindo a existência de seis linhagens, e 22 haplótipos para o segmento ITS1-5.8S-ITS2, sugerindo quatro linhagens. A linhagem mais comum para o segmento ITS1-5.8S-ITS2 coincide com três linhagens diferentes de COI e três linhagens menos freqüentes foram concordantes para ambos marcadores. Moderada estruturação genética das populações foi sugerida pela distribuição espacial das linhagens, valores de $\Phi_{ST}$ (COI = 0,65, ITS1-5.8S-ITS2 = 0,63, $p < 0,00001$) e os resultados do Teste de Mantel (COI = 0,524, ITS1-5.8S-ITS2 = 0,416, $p < 0.001$). Estes resultados podem sugerir uma baixa taxa de dispersão para essa espécie, como descrito para outros oligoquetas. Este estudo contribuirá no delineamento de ações de manejo e uso sustentável da espécie.

## INTRODUÇÃO GERAL

### *Sobre a espécie Rhinodrilus alatus*

O minhocuçu *Rhinodrilus alatus* (Righi, 1971) (Annelida, Clitellata, Glossoscolecidae), é um oligoqueta gigante endêmico do cerrado central do estado de Minas Gerais, muito apreciado como isca para pesca. Embora não incluído em listas internacionais de espécies ameaçadas, como a da IUCN, entre 1995 e 2003 o minhocuçu foi considerado ameaçado de extinção no Estado de Minas Gerais (na categoria "em perigo", por meio da publicação da Deliberação Normativa do Conselho de Política Ambiental 41/1995) e no Brasil (Instrução Normativa do Ministério do Meio Ambiente 03/2003). Atualmente, após uma revisão, a espécie será incluída sob o status de quase ameaçada (NT) (Machado *et al*. 2008).

Os animais atingem cerca de 60 centímetros de comprimento e seu ciclo anual parece ser diretamente influenciado pelas alterações climáticas, vinculado à sazonalidade dos períodos chuvosos e secos nos locais de sua ocorrência. De acordo com Drumond (2008), a estação chuvosa (setembro a março) corresponde à fase de reprodução e forrageamento, período no qual ocorre dispersão, ao passo que na estação seca (abril a agosto), os indivíduos permanecem enrolados em câmaras de quiescência, sem muito deslocamento. O minhocuçu é um animal hermafrodita que copula através de transferência mútua de espermatozóides, sendo a fecundação recíproca e cruzada. Segundo os extratores, os indivíduos reproduzem uma vez ao ano e cada ovo possui dois filhotes.

A área de ocorrência original da espécie era restrita aos municípios de Paraopeba e Sete Lagoas (Righi 1971), entretanto uma distribuição mais ampla foi confirmada por Drumond (2008), abrangendo 17 municípios da região central do estado. Esses animais ocorrem em diferentes fisionomias vegetais como cerrado, cerradão, veredas, e estão presentes também em locais modificados pelo homem, como plantações de braquiárias, canaviais e em eucaliptais.

A atividade extrativista e a comercialização ocorrem desde a década de 30 (Miranda 1987) e traz como característica conflitos legais e sociais constantes entre extratores e proprietários rurais. Além disso, impactos ambientais - como o uso de fogo, revolvimento do solo, remoção da vegetação e invasão de reservas ambientais - também contribuem para a problemática dessa atividade.

Por outro lado, a cadeia produtiva dessa atividade envolve centenas de moradores da região, que participam na extração, comercialização, e até mesmo na produção de panelas de barro onde são armazenados os animais até que sejam vendidos. Dessa forma,

a extração e a comercialização do minhocuçu representam uma alternativa importante com fonte de renda para populações humanas da região.

Nesse contexto, surgiu o Projeto Minhocuçu, que buscou elucidar aspectos da ecologia e biodiversidade desse oligoqueto e ecologia humana das comunidades da região, assim como propor estratégias de manejo e uso sustentável de *R. alatus*. O projeto envolveu diversas instituições como a Universidade Federal de Minas Gerais (UFMG), o IBAMA, o Ministério Público, Prefeituras locais, dentre outras. Na UFMG, uma parceria foi firmada entre o Laboratório de Ecologia e Comportamento de Insetos - sob a coordenação do Prof. Dr. Rogério Parentoni Martins e da Dra. Maria Auxiliadora Drumond - e o Laboratório de Genética Humana e Médica – sob coordenação da Prof. Dra. Maria Raquel Carvalho -, ambos do Departamento de Biologia Geral do Instituto de Ciências Biológicas. Além da candidata, participaram também desse projeto a Profª Dra. Cleusa Graça da Fonseca, a bióloga Sílvia Helena Campos, o biólogo Arthur Queiroz, o biólogo Javan Tarsis e o aluno de iniciação científica Sávio Henrique Cicco de Sandes. O presente trabalho foi uma das vertentes desse projeto maior e buscou caracterizar a estrutura genético-populacional e contribuir com informações importantes para futuras ações de proteção da espécie, uma vez que as populações remanescentes são reservatórios de material genético a ser iminentemente preservado.

### *Sobre a família Glossoscolecidae e o Gênero Rhinodrilus*

Os oligoquetos da família Glossoscolecidae são endêmicos na América do Sul, podendo ser encontrados em quase todos os habitats terrestres da região compreendida entre o curso do Rio Juramento – Salado na Argentina até o Paralelo de 15º Norte na Guatemala. Uma das características do solo onde se encontram as espécies dessa família é a presença de certa quantidade de umidade e de humus e que a acidez não seja excessiva (Righi 1971). A família foi inicialmente descrita por Michaelsen em 1900, e após algumas discussões, Stephenson (1930) estabeleceu a existência de cinco subfamílias: Glossoscolecinae, Sparganophilinae, Microchaetinae, Homorgastrinae e Criodrilinae.

O gênero *Rhinodrilus* (Perrier, 1872) possui 44 espécies e subespécies, sendo que a maioria desses táxons foi descrito por Gilberto Righi (Christoffersen 2007). Entretanto, são poucos os estudos relacionados à identificação e distribuição da biodiversidade desses oligoquetos.

***Genética de Populações e da Conservação***

A biodiversidade do planeta vem sofrendo uma rápida diminuição como consequência de ações antrópicas, seja de maneira direta ou indireta. Dentre essas ações, destacam-se a destruição de habitats e fragmentação, a super exploração, a poluição e a introdução de espécies exóticas. Em meio a esse declínio, os tamanhos reduzidos de populações contribuem com a perda da diversidade genética, podendo, assim, limitar a capacidade de adaptação às mudanças futuras no ambiente.

A diversidade genética, produzida ao longo dos 3,5 bilhões de anos de evolução do planeta, é um dos três níveis abordados pela Biologia da Conservação que devem ser protegidos, sendo os demais a diversidade de espécies e a de ecossistemas (Allendorf *et al* 2007). A partir daí, atua a Genética da Conservação, que faz uso de teorias e técnicas da genética para tentar minimizar o risco de extinção de espécies ameaçadas. As principais atividades propostas por essa disciplina são: o manejo genético de populações reduzidas, afim de se preservar a diversidade genética e minimizar endocruzamentos; a resolução de incertezas taxonômicas e identificação de unidades de manejo; e o uso da genética molecular na análise forense e na elucidação da biologia das espécies (Frankham *et al* 2002; O'Brien 1994). Nesse contexto, os estudos de estrutura genética revelam como a variação genética está distribuída dentro e entre populações e espécies, afim de se compreender melhor a dinâmica de populações da espécie na natureza ou monitorar populações em cativeiro (Avise 2004).

A maioria das populações é agrupada em subpopulações menores dentro das quais o cruzamento entre os indivíduos geralmente ocorre. Quando há essa estruturação, é comum encontrar diferenciação genética entre as subpopulações, muitas vezes devido aos efeitos da endogamia, deriva genética e/ou fluxo gênico (Hartl & Clark 2007; Templeton 2006). Assim, estudos com marcadores moleculares são capazes de inferir sobre tais fatores evolutivos e os processos históricos que modularam a estrutura genética da população. Desse modo, o conhecimento da variação intra-específica pode contribuir para a criação de estratégias de conservação de espécies, já que é possível a identificação de unidades de manejo (O'Brien 1994).

Outra abordagem que utiliza a variação existente dentro de espécies é a Filogeografia, que estuda os princípios e processos que geraram as distribuições geográficas de linhagens genealógicas. Essa é uma disciplina relativamente recente, que busca integrar diferentes áreas como a genética molecular, genética de populações, etologia, demografia, filogenia, paleontologia, geologia e geografia histórica (Avise 2000). O uso da informação existente em árvores filogenéticas, juntamente com dados de distribuição e ocorrência de determinadas espécies, pode fornecer importantes *insight* sobre a história

da Terra. James (2004) mostra algumas razões e exemplos para a aplicação da sistemática e da biogeografia de minhocas na compreensão da história geológica da Terra, particularmente em relação ao movimento da crosta terrestre. Por serem considerados organismos com baixa dispersão, a distribuição de minhocas pode ser justificada, muitas vezes, somente por conexões passadas entre regiões e por eventos de vicariância.

***Estudos genéticos em Annelida***

O papel fundamental das minhocas para o solo foi inicialmente descrito por Darwin, em 1881, através do livro "*The formation of vegetable mould through the action of worms, with observations on their habits*". As minhocas tem grande importância para a formação do solo através da capacidade de alterar seu habitat e criar novos ambientes para outros organismos. Dentre as modificações pode-se citar o aumento da porosidade, formação de matéria orgânica, alteração da microbiota e fornecimento de nutrientes por meio de suas fezes (Römbke *et al.* 2005). Do ponto de vista ecológico, são organismos fundamentais em algumas redes alimentares, sendo presas para vários vertebrados e invertebrados (Symondson 2000). Além disso, sua importância é ainda mais valorizada, uma vez que são bioindicadores ambientais, reforçando a necessidade de estudos de estrutura e diversidade genética, pois estes são capazes de fornecer informações valiosas sobre o táxon estudado.

Embora muito relevante, estudos moleculares com esse táxon são poucos. De modo geral, em Annelida, eles se baseiam na filogenia molecular (McHugh 2005; Erseus 2005; Struck *et al.* 2007; Huang *et al.* 2007) e em filogeografia e genética de populações (Jolly *et al.* 2005; Bastrop & Blank 2006; Jolly *et al.* 2006; King *et al.* 2008; Chang *et al.* 2008). Entretanto, a maioria desses estudos são realizados com espécies de poliquetas. Estudos moleculares em nível intraespecífico com oligoquetos já foram desenvolvidos utilizando isoenzimas (Haimi *et al.* 2007), RAPD (Kautenburger 2006; Lentzsch & Golldack 2006), microssatélites (Harper *et al.* 2006; Velavan *et al.* 2007) e haplótipos de mtDNA (Heethoff *et al.* 2004; Field *et al.* 2007 e Cameron *et al.* 2008).

O estudo da diversidade genética, estrutura de populações e filogeografia de populações de minhocuçus é inédito, não havendo seqüências disponíveis dessa espécie em banco de dados. Recentemente, foram depositadas no "*Barcode of Life Data System*" sequências do gene da Subunidade I da Citocromo c Oxidase para três indivíduos do gênero *Rhinodrilus* coletados na Amazônia (www.barcodinglife.org). Entretanto, tais sequências não estão disponíveis para a comunidade. A espécie mais próxima com sequências disponíveis é a *Lumbricus rubellus* (Hoffmeister, 1843) com mais de 20.000 sequências (NCBI 2009). Desse modo, esse é o primeiro esforço em descrever a estrutura e diversidade genética de populações de minhocuçu da região central de Minas Gerais

***Marcadores moleculares escolhidos***

O surgimento da reação em cadeia da polimerase (PCR) (Saiki et al. 1985) marcou o início da revolução na biologia molecular. Aliado a isso, o uso de iniciadores universais permitiu o sequenciamento do DNA de espécies nunca antes estudadas. O sequenciamento de algumas regiões genômicas é cada vez mais comum para estudos de diversidade genética e para inferir modelos de seleção e demográficos (Schlötterer 2004)

Um único locus ou região de DNA não é capaz de capturar totalmente a estrutura das populações de espécies e sua história evolutiva, sendo ideal o exame de mais de um loci e regiões gênicas (Templeton 2006). Estudos genético-populacionais e a reconstituição dos processos demográficos quando baseados em diferentes sistemas moleculares, trazem uma interpretação mais real dos fatos. Essa abordagem multiloci permite acessar a evolução molecular de acordo com diferentes taxas evolutivas.

O DNA mitocondrial animal (DNAmt), com poucas exceções, é um molécula circular com cerca de 15 a 20 kilobases de tamanho composto por 37 genes, sendo que 22 deles codificam RNAt, 2 RNAr e 13 codificam proteínas relacionadas no transporte de elétrons e na fosforilação oxidativa (Avise 2004). Algumas características típicas do DNAmt o tornam ferramentas valiosas para análises genéticas: ocorrência de milhares de cópias dentro das células, fácil manipulação e amplificação, herança uniparental materna, frequência mínima de recombinação, e evolução rápida a nível de sequências, devido ao ineficiente mecanismo de reparo do DNA (Simon *et al.* 1994; Avise 2004).

Em relação a outras regiões do DNAmt, postula-se que aquelas que codificam proteínas são regiões úteis para diversos estudos pois em geral não apresentam deleções/inserções. O gene da subunidade I da Citocromo c Oxidase apresenta vantagens, como o uso consolidado de primers universais testado em alguns filos de invertebrados (Folmer *et al.* 1994), e um grande alcance de sinal filogenético, sendo utilizado tanto na discriminação de espécies relacionadas, quanto na diferenciação de grupos filogeográficos dentro da mesma espécie (Hebert 2003). Assim como nos demais genes DNAmt codificantes a maior incidência de substituições nucleotídicas ocorrem na terceira posição do códon, sendo que muitas vezes as substituições são ditas silenciosas, pois não provocam mudança de aminoácido (Simon *et al.* 1994).

Os genes de RNA ribossômico no núcleo de células eucarióticas geralmente ocorrem em elementos repetidos em *tandem*. Cada unidade repetitiva é composta por sequências altamente conservadas que codificam os genes ribossomais (subunidades 18S, 5.8S e 28S) e por regiões espaçadoras menores e mais variáveis. O espaço interno transcrito 1 está localizado entre os genes de RNAr 18S e 5.8S, enquanto o espaço interno transcrito 2 está

presente entre o RNAr 5.8S e 28S. Ambos variam de tamanho entre e, às vezes, dentro de espécies (Lewin 2004). Por conseguinte, esse segmento tem como característica taxas diferentes de divergência entre as regiões codificadoras e os espaçadores, e uma evolução em conjunto (*Concerted Evolution*). Esse processo evolutivo sugere que unidades repetidas tendem ser homogeneizadas em sequências dentro das espécies ao invés de evoluir independente entre as regiões (Page e Holmes 2007).

Os espaçadores tem sido aplicados em diferentes abordagens, tanto na distinção filogenética de espécies proximamente relacionadas (Chen 2002; Lee & Foighill 2003; Meyer *et al.* 2008) quanto para comparação entre populações e estudos filogeográficos (Vogler & DeSalle 1994; Bargues *et al.* 2006) .

O presente trabalho gerou um artigo científico que será submetido à revista Molecular Ecology, conforme descrito a seguir.

**Manuscript for Molecular Ecology**

# Genetic diversity and population structure in the brazilian giant earthworm *Rhinodrilus alatus* (Annelida, Clitellata, Lumbricina, Glossoscolecidae)

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**KEYWORDS**

Earthworm, *Rhinodrilus alatus*, genetic diversity, population structure

**RUNNING TITLE**

Genetic diversity and population structure of *Rhinodrilus alatus*

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1 **ABSTRACT**

2 *Rhinodrilus alatus* (Righi 1971) is a giant worm ("minhocuçu") endemic of the central
3 savannah of Southeast Brazil, much appreciated as bait for fishing. *R. alatus*
4 extractive activity includes many people and results in social and environmental
5 impacts. The aims of this work were to characterize *R. alatus* genetic diversity and
6 population genetic and geographic structure by sequencing of the mitochondrial gene
7 cytochrome oxidase I (COI) and a nuclear segment including 5.8S rRNA gene and
8 the internal transcriber spacers 1 (ITS1) and 2 (ITS2). Sample was composed by 75
9 *R. alatus* individuals and 6 *R. motucu* ones collected at 21 sampling sites. Genetic
10 diversity was evaluated based on haplotype diversity, nucleotide diversity and
11 average number of nucleotides differences. Population structure was characterized
12 with AMOVA, Mantel tests, SAMOVA, and phylogenetic analysis (maximum
13 likelihood, Bayesian inference and median joining). Extraordinary genetic diversity
14 was found for this species, with 53 haplotypes for the COI, suggesting the existence
15 of six lineages, and 22 haplotypes for the ITS1-5.8S-ITS2 segment, suggesting four
16 lineages. Most common ITS1-5.8S-ITS2 lineage segregates with three different COI
17 lineages and the three less frequent lineages were coincident for both markers.
18 Moderate population structure was suggested by the spatial distribution of lineages,
19 $\Phi_{ST}$ values (COI = 0.65, ITS1-5.8S-ITS2 = 0.63, $P < 0.00001$), and Mantel tests
20 results (COI = 0.524, ITS1-5.8S-ITS2 = 0.416, $P < 0.001$). These results can be
21 taken as suggestive of low dispersal rate for the species as described for other
22 earthworms. This study will contribute in the delineation of actions for management
23 and sustainable use for the species.

24

1 **INTRODUCTION**

2 Earthworms are important for soil formation because of its ability to change 3 characteristics such as porosity, amount of organic matter, composition of the 4 microbiota, providing nutrients through their faeces (Römbke *et al.* 2005). This way, 5 they modify their environments and create new habitats for other organisms. Also, 6 they are involved in some fundamental ecological interactions and are prey for many 7 vertebrates and invertebrates (Symondson 2000). They can also be directly applied 8 in bioremediation strategies to promote biodegradation of organic contaminants 9 (Ceccanti *et al.* 2006; Hickman & Reid 2008). Earthworms had also great prominence 10 in Charles Darwin studies (1881), who regarded them as one of the most important 11 groups of animals in the planet.

12 Brazil is among the countries with greatest earthworm biodiversity in the world, 13 with about 305 species and subspecies described (James & Brown 2007). 14 Earthworm populations have been found in various ecosystems of the country, both 15 in native environments as well as in those modified by man. Giant earthworms longer 16 than 30 cm and with a diameter larger than 1 cm are collectively called “minhocuçus”. 17 It was estimated that more than 50 species of “minhocuçus” inhabit the country 18 (Brown & James 2007; Christoffersen 2007a,b).

19 The “minhocuçu” *Rhinodrilus alatus* (Righi 1971; Clitellata: Glossoscolecidae) 20 is a terrestrial giant oligochaete endemic to the savannah (*cerrado*) of Southeast 21 Brazil. The genus *Rhinodrilus* (Perrier, 1872) has at least 44 species and subspecies, 22 most of them described by Gilberto Righi (Christoffersen 2007b). The family 23 Glossoscolecidae has a geographic distribution restricted to South America.

24 *R. alatus* has been highly exploited as bait for fishing since the 1930s and 25 therefore, was included in local lists of threatened species (Machado *et al.* 2008). In 26 addition to the presumed impact of the high extraction on the genetic diversity of the

1 species, there are constant legal and social conflicts between extractors and
2 landowners. Moreover, environmental impacts such as the use of fire, soil
3 disturbance, removal of vegetation, and invasion of environmental reserves are
4 additional problems related to minhocuçu collection activity. On the other hand,
5 collecting and marketing the animals is an important alternative source of income for
6 human populations in the region.

7 Worldwide, few studies on earthworm diversity or population genetic structure
8 have been carried out (Kautenburger 2004; King *et al.* 2008; Cameron *et al.* 2008),
9 none of them in Brazil. The aim of this study was to characterize the genetic structure
10 and phylogeography of the populations of the giant worm *R. alatus*.

11 **MATERIALS AND METHODS**

12 ***Ecological data and distribution of species***

13 The collection of individuals was made with the help of the locals. For the
14 population genetic studies, 75 *R. alatus* individual were sampled in Southeast Brazil.
15 Additionally, 6 *R. motucu* individuals have been collected from swamps in Midwest
16 Brazil. Sampling points were geocoding (Table 1). In the field, animals were
17 anesthetized with 10% ethanol and thereafter set on 70% ethanol. Species
18 identification was based on the criteria proposed by Righi (1971).

19 ***DNA Extraction and Polymerase Chain Reaction***

20 In the laboratory, muscle fragments from the posterior region of the body wall
21 were dissected for use in molecular analysis and then the specimens were fixed in
22 10% formaldehyde. Muscle fragments were kept in 70% ethanol at 4 °C until DNA
23 extraction, which was performed following a salting out protocol (Miller *et al.* 1988),
24 adapted according to the following description: 0.4 X 0.4 cm fragments of muscle
25 tissue were transferred to 1.5 mL tubes containing 500 μL of SE (75 mM NaCl, 25
26 mM EDTA, pH 8.0) and 25 μL 20 % sodium dodecyl sulfate, and 20 μL proteinase K

1 (20 mg/mL). After an overnight incubation at 56 °C, 50 μL 5M NaCl was added.
2 Tubes were vortexed and centrifuged for 10 minutes at 13000 rpm. Then, the
3 supernatant was transferred to another 1.5 mL tube and precipitated with 2 volumes
4 100% ethanol at -20 °C. Tubes were incubated for 20 minutes at -20 °C and
5 centrifuged for 15 minutes at 13000 rpm. The supernatant was discarded and the
6 pellet was washed twice with 70% ethanol at -20 °C. After removing the 70% ethanol
7 of the second wash, tubes remained open at room temperature for 15 minutes in
8 order to let evaporate ethanol rests. DNA was resuspended in 300 μL of TE (10 mM
9 TRIS, 1 mM EDTA, pH 7.4).

10 A 1050 bp fragment containing the internal transcribed spacer region 1 (ITS1),
11 the 5.8S rDNA gene, and the internal transcribed spacer region 2 (ITS2) was
12 amplified with primers: ETTS1: 5' TGCTTAAGTTCAGCGGGT 3', ETTS2: 5'
13 TAACAAGGTTTCCGTAGGTGAA 3' (Kane & Rollinson 1994). ETTS1 primer was
14 anchored at the 3' end of 18S rRNA gene and the ETTS2 primer at the 5' region of
15 28S rRNA. PCR reactions were performed in a 50 μL final volume with 10 mM Tris-
16 HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP,
17 5% DMSO, 1 U Taq DNA polymerase, 0.3 μM of each primer, and approximately 100
18 ng of genomic DNA. Amplification process consisted of an initial denaturation step at
19 95 ºC for 5 minutes followed by 32 cycles consisting of denaturation at 94 ºC for 45
20 seconds, annealing at 60 ºC for 1 minute and elongation at 72 ºC for 2 minutes,
21 followed by a last extension step at 72 ºC for 2 minutes.

22 A 650 bp fragment of COI gene was amplified with universal primers
23 HCO2198: 5 'TAAACTTCAGGGTGACCAAAAAATCA 3' and LCO1490: 5
24 'GGTCAACAAATCATAAAGATATTGG 3' (Folmer *et al.* 1994). PCR reactions were
25 set in the same concentrations described above except for primers (1 μM each). The

PCR program consisted of an initial denaturation step at 94 ºC for 4 minutes, 25 cycles with denaturation at 94 ºC for 1 minute, annealing at 50 ºC for 1 minute and extension at 72 ºC for 90 seconds, followed by a 5 minutes last extension step at 72 °C.

The same individuals were used for both ITS1-5.8S-ITS2 and COI PCR amplification. However, some individuals amplified for only one marker, even after multiple attempts, so the sample size differs between the molecular markers used.

***Sequencing***

Amplicons were purified with PEG 8000 (Sambrook & Russel 2001). For each individual, two to four sequences were produced, from independent PCR reactions, so that at least one complete sequence was obtained from each direction for all the individuals. DYEnamic ET Dye Terminator Sequencing (GE HealthCare, Little Chalfont, UK) and ABI PRISM ® BigDye ® Terminator v.3.1 Cycle Sequencing (Applied Biosystems, Foster City, USA) kits were used to produce sequences in the MegaBACE ® 1000 (GE Healthcare, USA) or ABI 3130 (Applied Biosystems, USA) automatic sequencer. In the sequencing, the same primers were used as in the PCR reactions.

***Data analysis***

Sequences were analyzed with Polyphred software of the Phred-Phrap-Consed package (Ewin *et al.* 1998, Gordon *et al.* 1998, Nickerson *et al.* 1997). In order to confirm the polymorphisms, all chromatogram peaks were conferred by visual inspection and compared to a reference sequence, which was the first one obtained for the species by us.

Identification of 5.8S rRNA gene was performed by alignment with the *Eisenia fetida* sequence (accession number EF534709) available in the database of NCBI, using the program ClustalW implemented in MEGA software version 4.0 (Tamura *et*

1 *al.* 2007). After confirmation of the SNPs, data were subjected to a pipeline to
2 generate input files (W. Magalhães & E. Tarazona-Santos, personal communication)
3 for PHASE v.2.1 software (Stephens *et al.* 2001) and DnaSP v.4.50.3 package
4 (Rozas *et al.* 2003). With the PHASE v.2.1 software, phase and haplotypes were
5 inferred with 10,000 numbers of iterations, 100 thinning interval and 1,000 burn-in.

6 Haplotype diversity (h), nucleotide diversity ($\pi$) and average number of
7 nucleotide differences (k) were calculated for the total sample and for each lineage
8 inferred by phylogenetic analysis (see below) using DnaSP 4.50.3 (Rozas *et al.*
9 2003). Analysis of molecular variance (AMOVA) was based on the index $\Phi_{ST}$ and
10 was implemented by the program Arlequin v.3.0, using 1,000 permutations (Excoffier
11 *et al.* 2005).

12 Pairwise comparison with the evolutionary model of Tamura and Nei (1993)
13 was used to establish genetic distances matrixes between all haplotypes (MEGA 4.0
14 software) and between the lineages identified in phylogenetic studies (Arlequin 3.0
15 software). Other evolutionary models were also tested with similar results.

16 Phylogenetic relationships between haplotypes were reconstructed with three
17 different methods: maximum likelihood (Felsenstein 1981) using PhyML v.3.0
18 software (Guindon & Gascuel 2003), Bayesian inference using MrBayes 3.0
19 (Ronquist & Huelsenbeck 2003), and median joining (Bandelt *et al.* 1999) with
20 Network v.4.5.1.0 software (Available at: http://www.fluxus-technology.com). Best
21 evolutionary model was selected with MrModelTest V.2.3 software (Nylander 2004)
22 based on Akaike Information Criterion. Maximum likelihood trees were constructed by
23 subtree pruning and regrafting (SPR) methods and the branches were supported by
24 approximate likelihood ratios. Parameters for Bayesian inference were based on two
25 independent runs and four Markov chains with $1x10^6$ generations for COI and 1.5

1 x10$^{6}$ generations for the segment ITS1-5.8S-ITS2, with sampling every 300
2 generations. The number of generations was set corresponding to $P < 0.01$. The first
3 170 trees of COI and the first 250 trees of the segment ITS1-5.8S-ITS2 were
4 discarded as burn-in, retaining for analysis only those trees present in the stationary
5 phase of the runs. Trees were rooted with *R. motucu* as out group.

6 Spatial analysis of molecular variance (SAMOVA) was used in order to
7 ascertain population genetic structure and identification of potential barriers to
8 dispersal with SAMOVA v.1.0 software (Dupanloup *et al.* 2002). This software
9 defines groups of geographically homogeneous populations and maximize the
10 proportion of genetic variance due to differences between populations ($F_{CT}$), based
11 on the number of groups (K) defined *a priori* through simulated annealing
12 procedures. SAMOVA was performed using 100 simulated annealing procedures for
13 K values from 2 to 9. Correlation between genetic and geographic distances was
14 accessed with Mantel test (Mantel 1967), using 1,000 permutations and logarithmic
15 transformations for both genetic and geographical distances with Alleles in Space
16 software (Miller 2005).

17 **RESULTS**

18 ***Genetic diversity and population genetic structure with COI***

19 Good quality, partial sequences of COI gene (593 bp) were obtained for 69
20 individuals *R. alatus* and 6 *R. motucu*. There were 203 nucleotide substitutions and
21 no insertions or deletions.

22 Considering the 203 (34.2%) polymorphic positions found in both species, 183
23 were parsimoniously informative and 59 haplotypes were identified. Most haplotypes
24 had low frequencies and occurred only in one collection point (Tab.1). Through
25 phylogenetic analysis we identified six distinct lineages of *R. alatus*. Genetic diversity
26 levels for each lineage and for the whole sample are shown in table 2. Estimates for

1 h varied from 0.933 (lineage 4) to 1.00 (lineage 2 and 6), for $\pi$ from 0.003 (lineage 6)
2 to 0.040 (lineage 2) and for k from 2 (lineage 6) to 24 (lineage 2). The most frequent
3 amino acid substitution observed was a methionine to isoleucine, which was present
4 in some individuals in all lineages.

5 Three lineages were the most divergent: lineages 4, 5 and 6. The greatest
6 number of amino acid substitutions per lineage was observed in lineage 5 (six
7 substitutions), and 3 of them were unique to this lineage (2 Ile → Met; 1 Leu → Phe).
8 Lineage 6 has 3 non-synonymous substitutions, the same number as the lineage 4.
9 However, an asparagine to lysine substitution was found only in the last lineage.
10 Moreover, substitutions were also observed in other lineages, for example, in the
11 lineage 1 there was an individual with a replacement of a tryptophan by a serine and
12 in the lineage 2 an individual had a substitution of an isoleucine for a tyrosine.

13 AMOVA test showed a fixation index $\Phi_{ST}$ equal to 0.65 ($P$ < 0.00001).
14 Difference between the 53 haplotypes according to the model of Tamura and Nei
15 (1993) ranged from 0.17% to 23.08%, with an average of 8.82%. Pairwise distance
16 matrix for the lineages (Table 3) showed that most of the observed distances are
17 significant ($P$ < 0.01), while the smallest difference was 59.3% between lineages 1
18 and 2 and the highest was 87.6% between lineages 2 and 4, considering only the
19 significant values.

20 Phylogenetic trees generated with both the maximum likelihood analysis and
21 Bayesian inference showed similar topologies, with few branches not being
22 recovered by both methods (Fig. 1). Approximate likelihood ratios and *a posteriori*
23 Bayesian probabilities were higher than 0.5 in all branches and showed close
24 similarity between the two methods of phylogenetic reconstruction. The lineages

1 identified in the phylogenetic trees were also supported by median joining haplotype
2 network (Fig. 2).

3 It was possible to identify some patterns of geographic distribution of lineages
4 (Fig. 3). Lineage 1 is widely distributed, occurring throughout the center of the
5 sampled area, from north to south and limited by Três Marias Reservoir/Pará River
6 and Das Velhas River. Lineage 2 was present at three points close to each other on
7 both riversides of Das Velhas River. More evolutionarily divergent lineages, such as
8 3, 4, 5, and 6, had restricted distributions, respectively, to points 20, 1, 13, and 11/13.

9 The highest $F_{CT}$ value identified with SAMOVA was observed under K = 2
10 model, separating individuals from point 1 (UTM1 470914, UTM2 7831039) from the
11 other ($F_{CT}$ = 0.715, $P$ < 0.05). $F_{CT}$ values diminished as K values increased (Table 4)
12 and the proposed groupings were not congruent with the lineages identified. Thus, a
13 possible genetic or geographical barrier should be considered separating point 1
14 from the other sampling sites. A significant and positive correlation was detected
15 between genetic and geographic distances by Mantel test with $r$ equal to 0.524 ($P$ <
16 0.001).

17 ***Genetic diversity and structure in the ITS1-5.8S-ITS2 segment***

18 In order to investigate a nuclear DNA segment, a 935 bp fragment was PCR
19 amplified, which included 492 bp of the ITS1, 157 bp of the 5.8S rRNA gene, and 286
20 bp of ITS2. In this segment, nucleotide substitution and insertion/deletion (INDEL)
21 polymorphisms were identified. For example, ITS1 size ranged from 447 to 492 bp
22 (INDEL blocks varied from 1 to 8 nucleotides) and ITS2 size went from 282 to 286 bp
23 (with only one, 4 nucleotides INDEL block). There were no INDELS in 5.8 rRNA
24 gene.

1 Nucleotide substitutions had the following distribution: in ITS1 there were 48
2 transitions and 31 transversions, in ITS2 there were 19 transitions and 18
3 transversions and in 5.8S rRNA there was only one transition.
4 The most common ITS1-5.8S-ITS2 haplotype (H11) is distributed over 7
5 sampling points, while the other ones had restricted distributions or were unique to a
6 sampling point (Table 1). Based on the genetic diversity present in this gene segment
7 and phylogenetic analysis, *R. alatus* was subdivided into four lineages. Lineage 1
8 showed a greater number of sequences (126), but has only 18 haplotypes. h
9 remained between 0.0005 (Lineage 3) and 0.818 (Lineage 1), $\pi$ ranged from 0.0005
10 (Lineage 3) to 0.003 (Lineage 1) and k was 0.5 (Lineage 3) to 3.470 (Lineage 1)
11 (Table 2).
12 With AMOVA, most of the variance was located between populations with $\Phi_{ST}$
13 equal to 0.637 ($P$ < 0.05). Genetic divergence between the 22 ITS1-5.8S-ITS2
14 haplotypes observed in *R. alatus* ranged from $1x10^{-6}$% and 6.94%, with an average
15 of 2.4%. Most of the distances observed in the pairwise distance matrix between the
16 lineages were significant ($P$ <0.01) (Table 3). The smallest genetic divergence
17 occurred between lineages 1 and 2 (91.7%) and highest between 2 and 4 (100%),
18 considering only the significant values.
19 Phylogenetic reconstructions with both maximum likelihood and Bayesian
20 inference, produced highly similar results for ITS1-5.8S-ITS2 considering topologies,
21 with few branches not being recovered by one of the methodologies used (Fig. 1).
22 Approximate likelihood ratios and *a posteriori* Bayesian probabilities rendered similar
23 values (greater than 0.5) by phylogenetic reconstruction with the ITS1-5.8S-ITS2.
24 Five lineages were obtained in the phylogenetic reconstructions as well as in the
25 median joining networking of the ITS1-5.8S-ITS2 haplotypes (Fig. 4).

1 Lineage 1 showed a very wide distribution, being absent only on the left
2 riverside of the Pará River (Fig. 3). Other lineages had more restricted spatial
3 distribution, e. g., lineage 2 present only at sampling site 1.

4 Similarly to COI results, SAMOVA indicated a higher value of $F_{CT}$ (= 0.821, $P <$
5 $0.05$) to K equal to 2, separating site 1 from the others. Lower but still significant $F_{CT}$
6 values were obtained for K values 3 to 9 (Table 4). Thus, the same putative genetic
7 and/or geographical barrier was observed with both COI and ITS1-5.8S-ITS2. A
8 significant and positive correlation between genetic and geographic distances was
9 identified by Mantel test ($r = 0.416$, $P <0.001$).

10 Besides, the 6 COI and the 4 ITS1-5.8S-ITS2 lineages were compared in
11 terms of intra-individual and spatial distribution (for a better understanding the
12 nomenclature correlating lineages of both markers will be: lineage-marker/lineage-
13 marker, for example 6-COI/3-ITS). At intra-individual level and spatial distribution
14 there was a perfect coincidence between 4-COI/2-ITS (sampling site 1), 5-COI/4-ITS
15 (sampling site 13), and 6-COI/3-ITS (sampling sites 11 and 13). ITS lineage 1 comes
16 together in individuals with COI lineages 1, 2, and 3 (Fig. 3).

17 **DISCUSSION**

18 This work represents the first effort to describe relevant aspects about the
19 biology of a giant oligochaete with great local ecological, economic and social
20 importance. There are reports of the use of “minhocuçu” *R. alatus* as bait for fishing
21 since the 1930s. Consequently, it was included in regional and national lists of
22 endangered species. A better understanding of the role of these invertebrates in
23 ecosystem processes will help delineating conservation strategies for the species
24 (Lewinsohn *et al* 2005; Dupont 2009).

25 Currently, just few studies have investigated genetic/geographic population
26 structure based on sequencing results in oligochaete (Heethoff *et al.* 2004, Field *et*

1 *al.* 2007, Cameron *et al.* 2008, King *et al.* 2008, review by Dupont 2009). In the
2 present study, molecular analysis revealed a high intra-specific genetic diversity in *R.*
3 *alatus* with 31.87% polymorphic sites in COI and 15.4% in the segment ITS1-5.8S-
4 ITS2. Similar values were described for COI gene in *Allolobophora chlorotica* with
5 30.2% of polymorphic sites (King *et al.* 2008). Lower diversity was observed for the
6 partenogenetic species *Dendrobaena octahedron*, with 10.7% of polymorphic
7 positions in COI (Cameron *et al.* 2008). In *Octolasion tyrtaeum,* 32.53% variable sites
8 were identified in cytochrome oxidase II gene (Heethoff *et al.* 2004) and in *Lumbricus*
9 *terrestris,* 12.44% polymorphic sites were found in subunits 2 and 4 of the NADH
10 gene (Field *et al.* 2007). This is the first work describing genetic diversity in the whole
11 ITS1-5.8S-ITS2 segment for an oligochaete species. This region has already been
12 investigated in polychaetes of the genus *Perinereis*, including four species, in which
13 27.6% of variation was found (Chen *et al.* 2002).

14 The identification of cryptic lineages within populations of *R. alatus* is
15 consistent with results found in phylogenetic studies of other earthworms. Six
16 different lineages were determined in *Tubifex tubifex*, by the sequencing of 16S
17 mitochondrial ribosomal gene (Beauchamp 2001) and 5 lineages for *A. chlorotica*
18 with COI (King 2008). Heethoff (2004) found evidence for two genetic and
19 morphologically distinct lineages of *O. tyrtaeum.* One cryptic species was identified in
20 a species complex *Metaphire formosae* in Taiwan (Chang *et al.* 2008). In other
21 groups of Annelida, two and three lineages cryptic were identified in populations of
22 *Pectinaria koreni* and *Owenia fusiformis*, respectively, in the Northeast Atlantic (Jolly
23 *et al.* 2005; Jolly *et al.* 2006). Accordingly, the existence of lineages in *R. alatus* was
24 supported by the following evidences: a) the high genetic divergence observed in the
25 matrices of pairwise distance (Table 3), b) pattern of haplotypes distribution in the

1 phylogenetic reconstructions and their branch sizes (Fig. 1) and, c) the large number
2 of mutational steps between the lineages in the haplotype networks (Figs. 2 and 4).
3 We observed six lineages with COI and four with ITS1-5.8S-ITS2. The most
4 frequent lineage was ITS1-5.8S-ITS2 lineage 1, which occurred in individuals bearing
5 COI lineages 1-COI, 2-COI, or 3-COI. Moreover, there were three less frequent
6 lineages for both systems that always coincided at intra-individual level (5-COI/4-ITS,
7 4-COI/2-ITS, and 6-COI/3-ITS). Two of them (5-COI/4-ITS and 6-COI/3-ITS) were
8 identified at collection points where the most common ones were also detected.
9 These two lineages should be treated with caution because they contain only,
10 respectively, one and two individuals each.
11 At AMOVA, significant and high values (63% of variance between populations)
12 were obtained for both markers, suggesting a genetic structure. Higher
13 interpopulational variation was also described for *D. octahedron* with COI ($F_{ST}$=
14 0.717; Cameron *et al.* 2008), for *L. terrestris* based on RAPD ($\Phi_{ST}$ = 0.424;
15 Kautenburger *et al.* 2006); and *Sabella spallanzanii* an invader polichaete with ITS2
16 ($F_{ST}$ values from 0.533 to 0.838; Patti & Gambi 2001). It has been suggested that
17 FST values higher than 0.15 evidence significant differentiation between fragments
18 (Frankham *et al.* 2002). High values of divergence among fragments or populations
19 suggest low dispersal ability.
20 Generally, earthworms are considered low dispersion and highly localized
21 reproduction animals (James, 2004). However, dispersal rates may differ among
22 earthworm species due to many reasons, such as reproduction systems and
23 ecological niches. The results reported here suggest that *R. alatus* most frequent
24 lineages extend throughout the species geographical distribution area, while those
25 with higher genetic divergence are restricted and peripheral (Fig.3). Highly positive

1 and significant correlation values between genetic and geographic distances
2 obtained for both COI and ITS1-5.8S-ITS2 markers at Mantel tests suggest distance
3 as an important factor for genetic differentiation of *R. alatus* populations. The
4 restricted distribution of genetic diversity observed in some lineages reinforces the
5 idea of low dispersion for this species. These findings contradict the opinions the
6 locals, who report that the “minhocuçu” *R. alatus* is able to travel long distances over
7 the soil surface. According to Brown and James (2007), except for some species with
8 wide distribution (*Pontoscolex corethrurus* and *Urobenus brasiliensis*), about 80% of
9 Brazilian species of earthworms have restricted dispersals, occurring in only a few,
10 nearby locations. Similarly, King *et al.* (2008) showed that the relatively sedentary
11 behavior of *A. chlorotica* contributed to genetic differentiation of lineages and
12 subsequent sympatric speciation.

13 In addition to the dispersion data, the inference of possible barriers to gene
14 flow may also be useful in understanding the distribution patterns of the species. The
15 results of SAMOVA (Table 4) for both markers showed the existence of a potential
16 barrier between the Point 1 and other sampling points. Other simulated values for K
17 were also significant, however presented lower $F_{CT}$ values than those found for K=2.
18 Plotting the sampling points over different maps of the region (vegetation,
19 topography, hydrology, and geological formation), points to the Pará River as a
20 possible geographical barrier, that contributed to the genetic differentiation of this
21 population (Fig. 3). Therefore, SAMOVA with K=2 reinforces the identification of the
22 lineage 4-COI/2-ITS.

23 In this study, a multiloci approach was adopted, using nuclear and
24 mitochondrial markers, for addressing different evolutionary rates. Representing the
25 nuclear genome, it was used a segment including the coding region of the 5.8S rRNA

gene and parts of the internal transcribed spacers 1 and 2 (ITS1 and ITS2). RNA ribosomal gene complex (rDNA) is a nuclear unit of tandem repeats, with one to many hundreds of copies (Hillis & Dixon 1991). Due to its moderate rate of molecular evolution, ITS sequences are important tools for population genetics and phylogenetic studies (Hillis & Davis 1986, Vogler & DeSalle 1994, Reed *et al.* 2000, Chen *et al* 2002). In order to ascertain the mitochondrial genome mutation rate, the region chosen was the gene for subunit I of cytochrome C oxidase (COI). Mitochondrial DNA has proven useful in the reconstruction of historical models of population demography, migration, biogeography and speciation (Brown *et al.* 1979, Moore 1995, Hebert *et al* 2003, Hurst & Jiggins 2005).

Higher genetic diversity was detected with COI (6 lineages), when compared to ITS1-5.8S-ITS2 segment (4 lineages). COI location in the mtDNA explains its high mutation rate, in spite of being a protein coding gene (Simon *et al.* 1994). ITS1-5.8S-ITS2 segment, due to its large number of copies in tandem, is subject to gene conversion mechanisms typical of such sequences, which reduce their genetic diversity. Furthermore, these sequences are subject to greater selective pressure because they contain signals required for the processing of RNA transcribed (Gerbi 1985, Page & Holmes 1998). The results reported here reinforce the usefulness of associating genetic markers with different evolutionary dynamics.

An amazing aspect of this study is the high amount of genetic diversity observed in individuals sampled over a relatively small geographic area, supporting the current idea of large genetic diversity among earthworms. Our study intended to elucidate important features of life history and conservation of a species with high local importance. As recommended by Machado *et al.* (2008), basic research is essential for conservation biology of *R. alatus*, and to encourage management

1 projects for its population. High genetic diversity values observed for this species in
2 such a small area reiterate the necessity of protection of the species. The present
3 results will help delineating conservation. The initial characterization of genetic
4 diversity and geographical distribution of species that will be useful in the follow up of
5 projects for the conservation and sustainable use *R. alatus*.

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**FIGURE LEGENDS**

**FIGURE 1** Phylogenetic tree of 53 haplotypes of COI and the 22 haplotypes of ITS1-5.8S-ITS2. Topologies shown were based on the maximum likelihood method and the branches not reconstituted by Bayesian inference have an asterisk. Respectively, the values of Bayesian posterior probabilities and the approximate likelihood ratios are indicated next to branch. The scale represents 0.1 substitutions per site for COI tree and 0.02 for segment ITS1-5.8S-ITS2 tree.

**FIGURE 2** Haplotype network of COI. Colored ellipses emphasize the lineages identified and their numbers are next to them. The white circles represent 53 haplotypes found and their size is proportional to the frequency of them. Small gray circles (mv = mediam vector) represent hypothetical haplotypes. The size of the branches is not proportional to genetic divergence and the numbers above them indicate the number of mutational steps between the different lineages.

**FIGURE 3** Map of Brazil showing the location of Southeast region. *R. alatus* sampling points are shown in the rectangles. COI lineages are shown in the top and ITS1-5.8S-ITS2 lineages in botton rectangle. Numbers refer to sampling sites (Table 1). The colors used to represent the lineages in both cases are the same used in figure 1. Observe that ITS1-5.8S-ITS2 lineage 1-ITS (in violet) occurs over the central geographic distribution area and coincided at intra-individual level with COI lineages 1-COI (orange), 2-COI (grey), and 3-COI (pink). Less frequent lineages are represented by colors blue (5-COI/4-ITS), green (4-COI/2-ITS), and yellow (6-COI/3-ITS) for both markers. Note the most periphery distribution of these lineages.

1 **FIGURE 4** Haplotype network of ITS1-5.8S-ITS2. Colored ellipses illustrate the
2 lineages identified and their numbers next to them. Lineages 2, 3 and 4 have the
3 same color of the lineages 4, 6 and 5 of the COI, respectively, because they
4 represent the same individuals. The white circles represent the 22 haplotypes found
5 and their size is proportional to the frequency of them. Small gray circles (mv =
6 mediam vector) represent hypothetical haplotypes. The size of the branches is not
7 proportional to genetic divergence and the numbers above them represent the
8 number of mutational steps between the different lineages.

**TABLE 1**

Characterization of the sampling sites, haplotypes and lineages present. Part a represents COI and b ITS1-5.8S-ITS2 segment. Ncr = number of chromosomes, NH = number of haplotypes, Lin. Pres. = Lineages present.

a) COI

| Sampling Site | UTM1 | UTM2 | Ncr | NH | Lin. Pres. | Lin. 1 | Lin. 2 | Lin. 3 | Lin. 4 | Lin. 5 | Lin. 6 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 470914 | 7831039 | 6 | 5 | 4 | | | | H49,50,51,52,53 | | |
| 2 | 585530 | 7848179 | 4 | 4 | 1,2 | H40,21 | H35,36 | | | | |
| 3 | 590033 | 7847313 | 1 | 1 | 2 | | H34 | | | | |
| 4 | 535291 | 7849567 | 5 | 3 | 1 | H42,43,44 | | | | | |
| 5 | 539341 | 7857588 | 5 | 4 | 1 | H9,10,41,46 | | | | | |
| 6 | 554312 | 7856446 | 1 | 1 | 1 | H39 | | | | | |
| 7 | 551789 | 7877140 | 4 | 4 | 1 | H2,3,11,16 | | | | | |
| 8 | 559229 | 7870722 | 6 | 5 | 1 | H7,8,13,14,15 | | | | | |
| 9 | 562251 | 7877894 | 1 | 1 | 1 | H12 | | | | | |
| 10 | 522661 | 7878691 | 4 | 2 | 1 | H5,38 | | | | | |
| 11 | 569464 | 7899894 | 11 | 8 | 1,6 | H22,23,24,25,26,28,30 | | | | | H48 |
| 12 | 535526 | 7887775 | 4 | 4 | 1 | H17,31,32,33 | | | | | |
| 13 | 563591 | 7905440 | 4 | 3 | 1,5,6 | H28 | | | | H1 | H47 |
| 14 | 546328 | 7910163 | 1 | 1 | 1 | H29 | | | | | |
| 15 | 540239 | 7940493 | 1 | 1 | 1 | H4 | | | | | |
| 16 | 525726 | 7986906 | 3 | 2 | 1 | H20,28 | | | | | |
| 17 | 515194 | 7977096 | 3 | 2 | 1 | H18,19 | | | | | |
| 18 | 489648 | 7962718 | 3 | 2 | 1 | H27,45 | | | | | |
| 19 | 617279 | 7858220 | 1 | 1 | 2 | | H6 | | | | |
| 20 | 750849 | 7810197 | 1 | 1 | 3 | | | H37 | | | |
| 21* | 700813 | 8514271 | 6 | 6 | - | - | - | - | - | - | - |

b) ITS1-5.8S-ITS2

| Sampling site | UTM1 | UTM2 | Ncr | NH | Lin. Pres. | Lin. 1 | Lin. 2 | Lin. 3 | Lin. 4 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 470914 | 7831039 | 12 | 1 | 2 | | H20 | | |
| 2 | 585530 | 7848179 | 8 | 2 | 1 | H10, 12 | | | |
| 3 | 590033 | 7847313 | 4 | 1 | 1 | H12 | | | |
| 4 | 535291 | 7849567 | 10 | 2 | 1 | H6,8 | | | |
| 5 | 539341 | 7857588 | 10 | 5 | 1 | H6,7,9,10,11 | | | |
| 6 | 554312 | 7856446 | 2 | 1 | 1 | H10 | | | |
| 7 | 551789 | 7877140 | 8 | 1 | 1 | H11 | | | |
| 8 | 559229 | 7870722 | 14 | 5 | 1 | H10,11,14,17,18 | | | |
| 9 | 562251 | 7877894 | - | - | - | | | | |
| 10 | 522661 | 7878691 | 8 | 1 | 1 | H19 | | | |
| 11 | 569464 | 7899894 | 26 | 4 | 1,4 | H11,15 | | | H21,22 |
| 12 | 535526 | 7887775 | 8 | 2 | 1 | H11,19 | | | |
| 13 | 563591 | 7905440 | 8 | 4 | 1,3,4 | H11,13 | | H22 | H1 |
| 14 | 546328 | 7910163 | - | - | - | | | | |
| 15 | 540239 | 7940493 | 4 | 1 | 1 | H11 | | | |
| 16 | 525726 | 7986906 | 6 | 3 | 1 | H3,4,5 | | | |
| 17 | 515194 | 7977096 | 6 | 1 | 1 | H3 | | | |
| 18 | 489648 | 7962718 | 6 | 1 | 1 | H2 | | | |
| 19 | 617279 | 7858220 | 2 | 1 | 1 | H12 | | | |
| 20 | 750849 | 7810197 | 2 | 1 | 1 | H16 | | | |
| 21* | 700813 | 8514271 | 10 | 1 | - | - | - | - | - |

**TABLE 2**

Summary details of the number of sequences (Nseq), haplotypes (Nhap), segregating sites (S), and values of haplotype (h) and nucleotide (π) diversity, and average number of nucleotide differences (k). At the top (a), data of 6 COI lineages and bottom (b) data of the 4 ITS1-5.8S-ITS2 lineages. Standard deviation is in parentheses.

a) COI

| | **Total *R. alatus*** | **Total *R. motucu*** | **Lin. 1** | **Lin. 2** | **Lin. 3** | **Lin. 4** | **Lin. 5** | **Lin. 6** |
|---|---|---|---|---|---|---|---|---|
| Nseq | 69 | 6 | 55 | 4 | 1 | 6 | 1 | 2 |
| Nhap | 53 | 6 | 40 | 4 | 1 | 5 | 1 | 2 |
| S | 189 | 42 | 67 | 47 | - | 12 | - | 2 |
| h | 0.988(±0.005) | 1.000(±0.092) | 0.983(±0.008) | 1.000(±0.176) | - | 0.933(±0.121) | - | 1.000(±0.500) |
| $\pi$ | 0.064(±0.031) | 0.026(±0.016) | 0.025(±0.012) | 0.040(±0.027) | - | 0.007(±0.004) | - | 0.003(±0.004) |
| k | 38.403(±16.894) | 15.933(±8.309) | 14.923(±6.777) | 24.00(±13.468) | - | 4.200(±2.424) | - | 2.000(±1.732) |

b) ITS1-5.8S-ITS2

| | **Total *R. alatus*** | **Total *R. motucu*** | **Lin. 1** | **Lin. 2** | **Lin. 3** | **Lin. 4** |
|---|---|---|---|---|---|---|
| Nseq | 144 | 10 | 126 | 12 | 4 | 2 |
| Nhap | 19 | 1 | 18 | 1 | 2 | 1 |
| S | 84 | - | 20 | - | 1 | - |
| h | 0.854(±0.023) | - | 0.818(±0.029) | - | 0.5(±0.265) | - |
| $\pi$ | 0.019(±0.009) | - | 0.003(±0.002) | - | 0.0005(±0.0006) | - |
| k | 17.779(±7.939) | - | 3.470(±1.782) | - | 0.5(±0.519) | - |

**TABLE 3**

Matrixes of pairwise differences between lineages, according to model of Tamura and Nei. At the top (a), distance between the 6 COI lineages and bottom (b) distance between of 4 ITS1-5.8S-ITS2. The significance level adopted was P < 0.01. NS: not significant.

a) COI

| | **Lin. 1** | **Lin. 2** | **Lin. 3** | **Lin. 4** | **Lin. 5** | **Lin. 6** |
|---|---|---|---|---|---|---|
| **Lin. 1** | - | | | | | |
| **Lin. 2** | 0.693 | - | | | | |
| **Lin. 3** | 0.738(NS) | 0.586(NS) | - | | | |
| **Lin. 4** | 0.856 | 0.876 | 0.950(NS) | - | | |
| **Lin. 5** | 0.846(NS) | 0.765(NS) | 1.000(NS) | 0.953(NS) | - | |
| **Lin. 6** | 0.847 | 0.803(NS) | 0.978(NS) | 0.953(NS) | 0.976(NS) | - |

b) ITS1-5.8S-ITS2

| | **Lin. 1** | **Lin. 2** | **Lin. 3** | **Lin. 4** |
|---|---|---|---|---|
| **Lin. 1** | - | | | |
| **Lin. 2** | 0.917 | - | | |
| **Lin. 3** | 0.952 | 0.997 | - | |
| **Lin. 4** | 0.956 | 1.000 | 0.994 (NS) | - |

**TABLE 4**

Simulations of SAMOVA. Respectively, values of K, FSC (variation among populations within groups), FST (variation within populations), and FCT (variation between groups), are showed. The significance level adopted was P < 0.05 and are in parentheses. At the top (a), values of COI and bottom (b) values of ITS1-5.8S-ITS2.

a) COI

| K | FSC (*P*) | FST (*P*) | FCT (*P*) |
|---|---|---|---|
| 2 | 0.497(0.000) | 0.856(0.000) | 0.715(0.041) |
| 3 | 0.477(0.000) | 0.848(0.000) | 0.709(0.001) |
| 4 | 0.458(0.000) | 0.840(0.000) | 0.705(0.000) |
| 5 | 0.435(0.000) | 0.833(0.000) | 0.705(0.000) |
| 6 | 0.436(0.000) | 0.821(0.000) | 0.683(0.000) |
| 7 | 0.440(0.000) | 0.809(0.000) | 0.659(0.000) |
| 8 | 0.096(0.000) | 0.674(0.000) | 0.640(0.000) |
| 9 | 0.040(0.000) | 0.675(0.000) | 0.661(0.000) |

b) ITS1-5.8S-ITS2

| K | FSC (*P*) | FST (*P*) | FCT (*P*) |
|---|---|---|---|
| 2 | 0.372(0.000) | 0.887(0.000) | 0.821(0.030) |
| 3 | 0.370(0.000) | 0.873(0.000) | 0.798(0.001) |
| 4 | 0.269(0.000) | 0.84(0.000) | 0.781(0.016) |
| 5 | 0.267(0.000) | 0.829(0.000) | 0.767(0.000) |
| 6 | 0.271(0.000) | 0.819(0.000) | 0.752(0.000) |
| 7 | 0.027(0.003) | 0.742(0.000) | 0.735(0.000) |
| 8 | 0.025(0.000) | 0.74(0.000) | 0.733(0.000) |
| 9 | 0.028(0.000) | 0.739(0.000) | 0.731(0.000) |

**Figure 1**

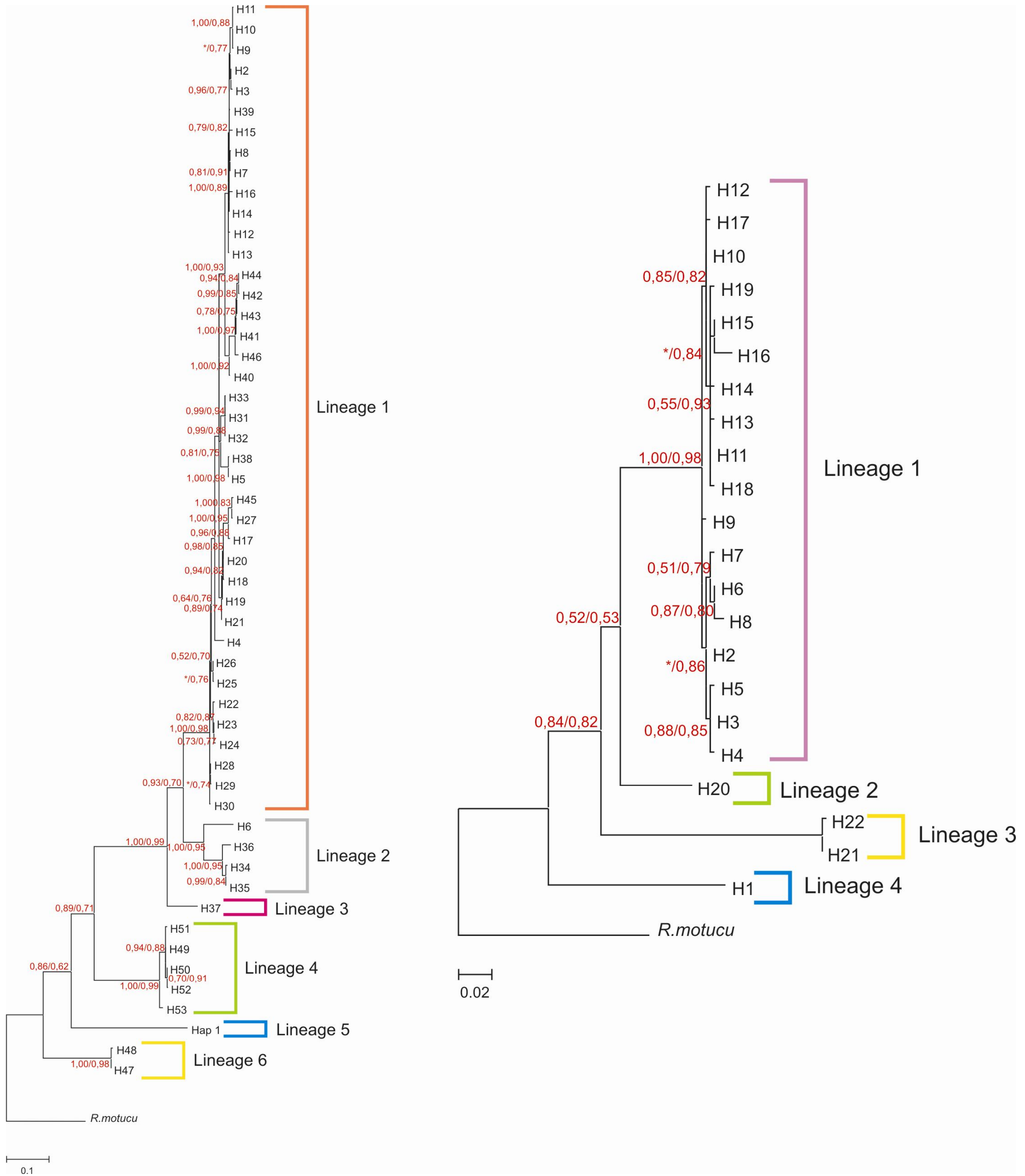


**Figure 2**

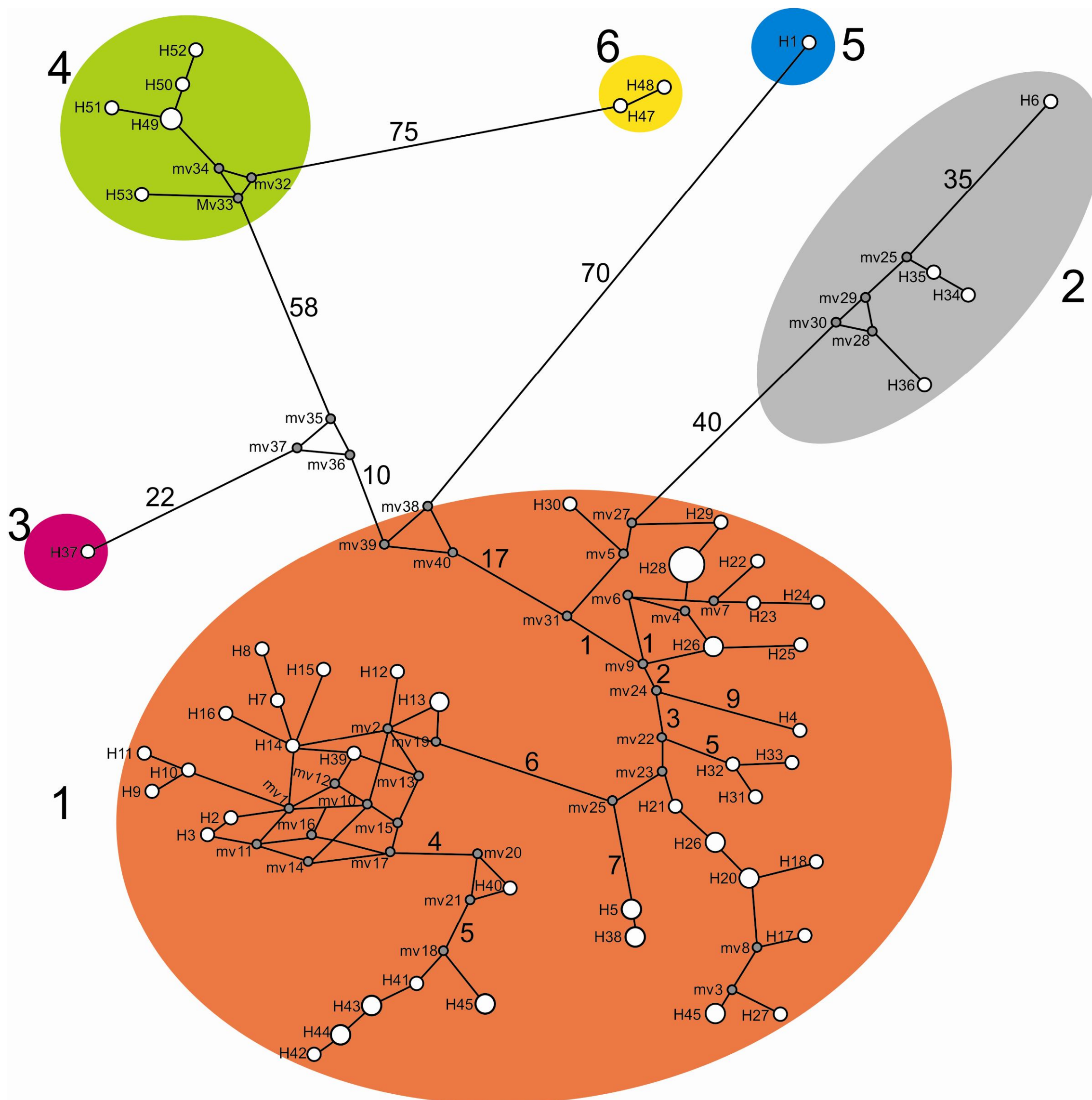


**Figure 3**

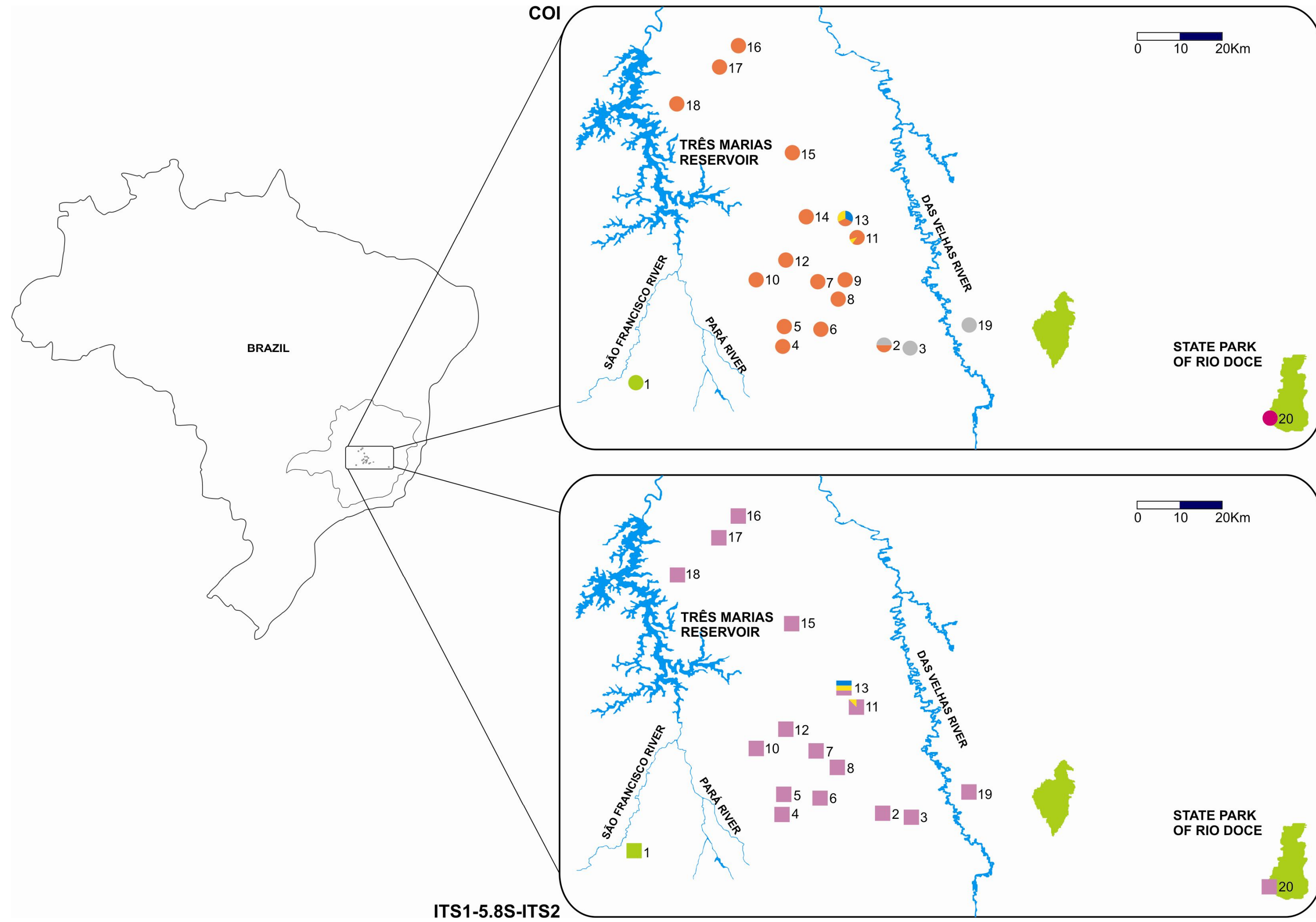
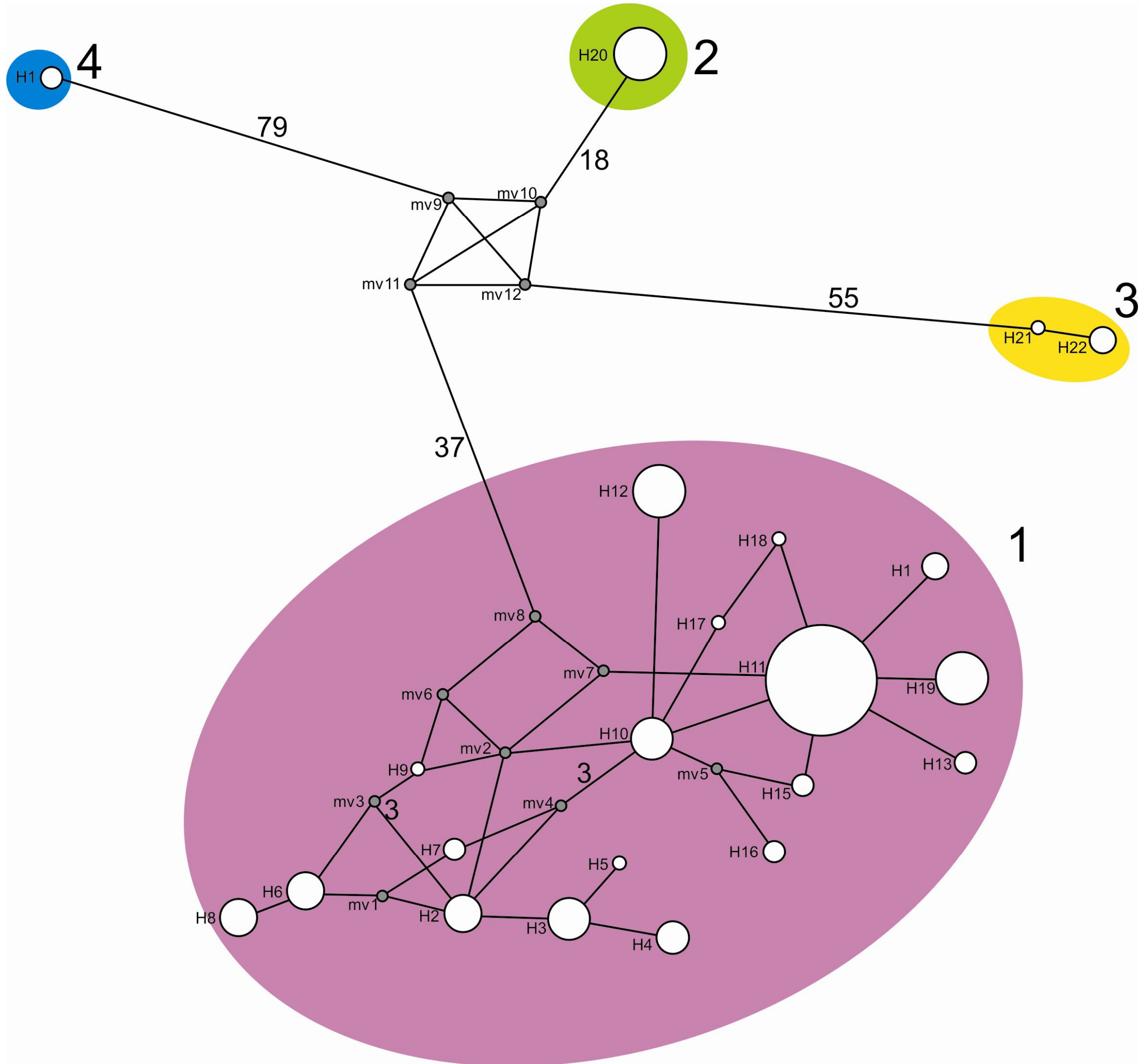


**Figura 4**



**CONCLUSÕES**

As populações do minhocuçu *R. alatus* revelaram uma alta diversidade genética, sendo possível a identificação de linhagens distintas para ambos marcadores.

Houve concordância de indivíduos para ambos marcadores em três linhagens, as quais foram menos freqüentes. Por outro lado, a linhagem mais comum para o segmento ITS1-5.8S-ITS2 coincidiu com três linhagens diferentes de COI.

A distribuição espacial da diversidade genética da espécie no estado de Minas Gerais se mostrou moderada, o que pode ser justificada pela baixa capacidade de dispersão, comum entre minhocas.

O presente estudo elucidou características relevantes para o conhecimento da história de vida e para a conservação de uma espécie com grande importância local. Pesquisas básicas em qualquer âmbito da biologia da conservação de *R. alatus* são úteis para o delineamento de medidas de manejo adequado. Desse modo, entende-se que esse seja apenas o primeiro passo como contribuição para a preservação e uso sustentável do minhocuçu.

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