**RESEARCH ARTICLE**

# Intense genomic reorganization in the genus *Oecomys* (Rodentia, Sigmodontinae): comparison between DNA barcoding and mapping of repetitive elements in three species of the Brazilian Amazon

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## Abstract

*Oecomys* Thomas, 1906 is one of the most diverse and widely distributed genera within the tribe Oryzomyini. At least sixteen species in this genus have been described to date, but it is believed this genus contains undescribed species. Morphological, molecular and cytogenetic study has revealed an uncertain taxonomic status for several *Oecomys* species, suggesting the presence of a complex of species. The present work had the goal of contributing to the genetic characterization of the genus *Oecomys* in the Brazilian Amazon. Thirty specimens were collected from four locations in the Brazilian Amazon and three nominal species recognized: *Oecomys auyantepui* (Tate, 1939), *O. bicolor* (Tomes, 1860) and *O. rutilus* (Anthony, 1921). COI sequence analysis grouped *O. auyantepui*, *O. bicolor* and *O. rutilus* specimens into one, three and two clades, respectively, which is consistent with their geographic distribution. Cytogenetic data for *O. auyantepui* revealed the sympatric occurrence of two different diploid numbers, 2n=64/NFa=110 and

2n=66/NFa=114, suggesting polymorphism while *O. bicolor* exhibited 2n=80/NFa=142 and *O. rutilus* 2n=54/NFa=90. The distribution of constitutive heterochromatin followed a species-specific pattern. Interspecific variation was evident in the chromosomal location and number of 18S rDNA loci. However, not all loci showed signs of activity. All three species displayed a similar pattern for 5S rDNA, with only one pair carrying this locus. Interstitial telomeric sites were found only in *O. auyantepui*. The data presented in this work reinforce intra- and interspecific variations observed in the diploid number of *Oecomys* species and indicate that chromosomal rearrangements have led to the appearance of different diploid numbers and karyotypic formulas.

**Keywords**

Oryzomyini, FISH, telomere, rDNA, heterochromatin, COI

## Introduction

The order Rodentia is divided into nine taxonomic families in Brazil. The family Cricetidae contains the most members, among which the subfamily Sigmodontinae includes 86 genera and 395 species (*sensu* Reig 1980) according to Prado and Percequillo (2013). Oryzomyini is the most diverse tribe of the Sigmondontinae, and the genus *Oecomys* Thomas, 1906 is one of the most diverse of the tribe Oryzomyini (Prado and Percequillo 2013). However, its morphological and karyological distinction and generic status were only recognized relatively recently (Andrades-Miranda et al. 2001, Carleton and Musser 1984, Gardner and Patton 1976, Reig 1984, 1986 as cited in Musser and Carleton 2005). Similarity among species and the limited understanding of morphological variations in *Oecomys* (including interspecific, intraspecific, geographic, and specimen age-inherent variations) have rendered species identification difficult.

Currently, 16 species are recognized within this genus (Musser and Carleton 2005, Carleton et al. 2009), but only nine species have been studied for karyotypes, showing 11 different diploid numbers, varying between 54 and 86 chromosomes (Table 1). In Brazil 12 species were registered and 9 of which can be found in Amazon biome; *O. auyantepui* Tate, 1939, *O. bicolor* (Tomes, 1860), *O. concolor* (Wagner, 1845), *O. paricola* (Thomas, 1904), *O. rex* Thomas, 1910, *O. roberti* (Thomas, 1904), *O. rutilus* Anthony, 1921, *O. superans* Thomas, 1911 and *O. trinitatis* (J. A. Allen & Chapman, 1893) (Bonvicino et al. 2008; Flores 2010). Variations in fundamental number have also been reported in species with the same diploid number, which is an indicator of chromosomal rearrangements within the group (Rosa et al. 2012). However, morphological and morphometric analysis in conjunction with molecular and cytogenetic approaches revealed uncertainty in the delimitation and distribution of *Oecomys* species, suggesting the presence of a complex of species (Patton and Sherwood 1983, Emmons and Feer 1997, Patton et al. 2000, Musser and Carleton 2005, Carleton et al. 2009, Flores 2010, Rosa et al. 2012).

Hence, in the present study, we used classic and molecular cytogenetics approaches in order to enable the genetic characterization of three species of the genus *Oecomys* from the Brazilian Amazon. Further, we used DNA barcoding to evaluate the intra- and interspecific distances, and infer the utility in species identification by combining this dataset with sequences deposited in GenBank.

**Table 1.** Karyotypes recorded for species of the genus *Oecomys*. Diploid Number (2n), fundamental number (FN) and location are listed.

| Species | Location | 2n | FN | Reference |
|---|---|---|---|---|
| *O. auyantepui* | Jari river – PA | 72 | 80 | Lira (2012) |
| *O. auyantepui* | Jatapu river – AM | 64 | 110 | Present paper |
| | | 66 | 114 | Present paper |
| *O. bahienses*** | São Lourenço da Mata – PE | 60 | 62 | Langguth et al. (2005) |
| *O. bicolor* | Jari river – PA | 54 | 82 | Lira (2012) |
| *O. bicolor* | SUR | 80 | – | Baker et al. (1983) |
| *O. bicolor* | RR<br>Ipameri and Serra da mesa– GO | 80 | 124 | Andrades-Miranda et al. (2000)<br>Andrades-Miranda et al. (2001) |
| *O. bicolor* | Curanja river – PER | 80 | 134 | Gardner and Patton (1976) |
| *O. bicolor* | Curanja river – PER | 80 | 136 | Gardner and Patton (1976) |
| *O. bicolor* | Juruá river – AM | 80 | 140 | Patton et al. (2000) |
| *O. bicolor* | Purus and Jatapu river – AM | 80 | 142 | Present paper |
| *O. bicolor* | ?<br>Hydropower plant UEH Samuel – GO | 82 | 110 | Andrades-Miranda et al. (2000)<br>Andrades-Miranda et al. (2001) |
| *O. bicolor* | Jari river – PA | 82 | 116 | Lira (2012) |
| *O. bicolor* | Jurua river – AM | 86 | 98 | Patton et al. (2000) |
| *O. catherinae* | GO,<br>São Lourenço da Mata – PE<br>Ubatuba – SP, Cruz do Espírito Santo – PB,<br>Igarassú, Jaqueira and Paudalho – PE<br>RJ | 60 | 62 | Andrades-Miranda et al. (2001)<br>Andrade and Bonvicino (2003)<br>Langguth et al. (2005)<br>Pinheiro and Geise (2008)<br>Asfora et al. (2011) |
| *O. catherinae* | Ubatuba – SP<br>RJ | 60 | 64 | Pinheiro e Geise (2008)<br>Asfora et al. (2011) |
| *O. catherinae* | RJ, SP | 86 | 98 | Patton et al. (2000) |
| *O. concolor* | PAN | 58 | – | Baker et al. (1983) |
| *O. concolor* | SUR | 60 | – | Baker et al. (1983) |
| *O. concolor* | Villavicencio – COL | 60 | 62 | Gardner and Patton (1976) |
| *O. concolor* | MEX | 60 | – | Andrade and Bonvicino (2003) |
| *O. concolor* | MEX | 61 | – | Andrade and Bonvicino (2003) |
| *O. concolor* | Curanja River – PER | 80 | 112 | Gardner and Patton (1976) |
| *O. concolor* | DF, RJ, GO, SP, RO | 60 | 62 | Gardner and Patton (1976)<br>Svartman (1989)<br>Andrades-Miranda et al. (2000)<br>Andrades-Miranda et al. (2001)<br>Andrade and Bonvicino (2003) |
| *O. paricola* | Environment Park – PA | 68 | 72 | Rosa et al. (2012) |
| *O. paricola* | Marajó island – PA | 70 | 72 | Rosa et al. (2012) |
| *O. paricola* | Environment Park – PA | 70 | 76 | Rosa et al. (2012) |
| *O. rex* | Jari river – PA | 62 | 80 | Lira (2012) |
| *O. roberti* | AM | 80 | 114 | Patton et al. (2000) |
| *O. roberti* | Juruá river – AM<br>Jamari river – RO | 82 | 106 | Langguth et al. (2005) |
| *O. rutilus* | Negro river – AM | 54 | 90 | Present paper |
| *O. superans* | PER<br>Jurua river – AM | 80 | 108 | Gardner and Patton (1976)<br>Andrade and Bonvicino (2003)<br>Patton et al. (2000) |
| *O. trinitatis* | Jurua river – AM | 58 | 96 | Patton et al. (2000) |
| *Oecomys* sp. | Cuieiras river – AM | 54 | 84 | Lira (2012) |
| *Oecomys* sp. | Jatapu – AM | 54 | 86 | Lira (2012) |
| *Oecomys* sp. | MS | 72 | 90 | Andrade and Bonvicino (2003) |

*The location indicates the sampled countries or Brazilian states. AM = Amazonas, GO = Goiás, MS = Mato Grosso do Sul, PA = Pará, PB = Paraíba, PE = Pernambuco, RJ = Rio de Janeiro, RO = Rondônia, RR = Roraima, SP = São Paulo, COL= Colombia, MEX = Mexico, PAN = Panama, PER = Peru, SUR = Suriname.
**Synonym of *O. catherinae*.

## Materials and methods

### Samples

Thirty specimens were collected from five locations in the Brazilian Amazon (Fig. 1, Table 2) and euthanized according to the recommendations of Resolution CFBIO N. 301 from December 8th, 2012. Voucher specimens were prepared or fixed, and stored in 70% ethanol; the specimens are currently stored in the mammal collection of the National Institute of Amazonian Research [Instituto Nacional de Pesquisas da Amazônia – INPA] (Table 2). The methods for the collection, maintenance and processing of the material complied with the guidelines of the Brazilian College of Animal Experimentation [Colégio Brasileiro de Experimentação Animal – COBEA] and were approved by the Ethics Committee On Animal Use of the Federal University of Amazonas [Comissão de Ética no Uso de Animais da Universidade Federal do Amazonas] (043/2013-CEUA/UFAM). Individuals were collected with the permission of the Chico Mendes Institute for Biodiversity Conservation [(Instituto Chico Mendes de Conservação da Biodiversidade – ICMBIO), License No. 10832-1 /35513-1). It must be noted that the collections took place outside of conservation units and that these species are not threatened with extinction. Samples were collected from the hematopoietic organ of each individual following euthanasia to obtain chromosome preparations and muscle tissue for DNA extraction.

### Chromosome analysis

Mitotic chromosomal preparations were obtained using the protocol described by Ford and Harmerton (1956), with some modifications. Nucleolus organizing regions (NORs), heterochromatin and G-banding were identified through silver nitrate staining (Howell and Black 1980), the C-banding technique (Sumner 1972) and trypsin solution (Seabright 1971), respectively. 5S and 18S rDNA probes were obtained after PCR amplification using the following primers: 5Sf (5'-CAG GGT CGG GCC TGG TTA GTA-3') and 5Sr (5'-CTT CYG AGA TCA GAC GAG ATC-3'); 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGG ACC TCA CTA AAC CA-3') (Gross et al. 2010). For telomere sequences, DNA-free amplifications were performed using the primers (TTAGGG)$_5$ and (CCCTAA)$_5$ (Ijdo et al. 1991). Amplification reactions were conducted in a total volume of 25 μl (~100 ng of genomic DNA), containing 10x reaction buffer (final concentration: 10 mM Tris-HCl; 1.5 mM MgCl$_2$; 50 mM KCl; pH 8.3), 0.3 units of Taq DNA polymerase, 0.2 mM each dNTP, 0.2 μl of each primer and Milli-Q water to the final volume; the annealing temperature was 56 ºC for 18S rDNA and 59 °C for 5S rDNA, and the final volume was 25 μl. The 5S gene PCR product was labeled with Biotin (Biotin Nick translations mix, Roch) and the 18S gene and telomere sequences with digoxigenin (Dig-Nick Translation mix, Roche), following the manufacturer's instructions. Alexa Fluor 488-conjugated
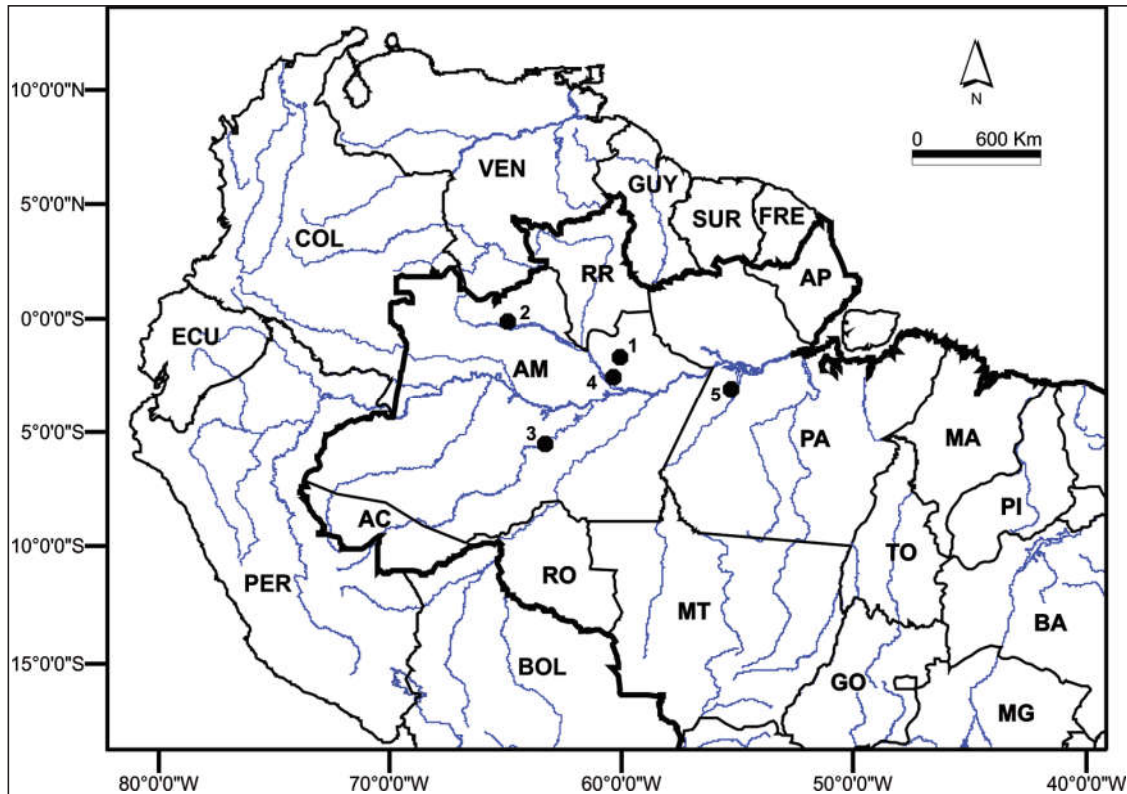
**Figure I.** Map of the Brazilian Amazon, indicating the collection sites. The left and right banks of the following Amazonas state rivers were sampled: **1** Jatapú (near the city of São Sebastião do Uatumã - 0°50' to 1°55'S; 58°50' to 60°10'W) **2** Negro (near the city of Santa Isabel do Rio Negro - 0°24.4'N; 65°1.017'W) **3** Purus (near the city of Tapauá - 05°42.183'S, 63°13.967'W) **4** Cuieiras (02°47'S, 60°27'W) **5** Tapajós (03°21.283'S, 55°11.733'W). BOL = Bolivia, PER = Peru, ECU = Ecuador, COL = Colombia, VEN = Venezuela, GUY = Guyana, SUR = Suriname, FRE = French Guyana, RR = Roraima, AP = Amapa, AM = Amazonas, PA = Para, RO = Rondonia, AC = Acre, MA = Maranhão, PI = Piaui, TO = Tocantins, BA = Bahia, MT = Mato Grosso, GO = Goias, MG = Minas Gerais.

streptavidin (Life technologies) and anti-digoxigenin rhodamine (Roche) antibodies were used to detect the probe signal. Fluorescent *in situ* hybridization was carried out based on the protocols described by Pinkel et al. (1986).

Slides were screened for metaphases, at least 30 for each technique were analyzed and the best metaphases were photo-documented using an Olympus BX-51 epifluorescence microscope. Chromosomes were organized by decreasing size, and their morphology was determined based on the centromere position, being classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a) (Levan et al. 1964).

## Mitochondrial DNA analysis

DNA was extracted according to the protocol described by Sambrook and Russel (2001). The cytochrome oxidase subunit I (COI) gene sequence was obtained through

**Table 2.** Species of *Oecomys* collected in present work: The voucher, collection sites, sex, diploid number (2n), fundamental number (FN), karyotype formula, Nucleolus organizer region (NOR), rDNA 18S (18S), rDNA 5S (5s) are listed; M = male; F = female; m = metacentric; sm = submetacentric; st = sub-telocentric; a = acrocentric; X = Sexual chromosome X; Y = Sexual chromosome Y. Bold voucher were karyotyped in the present work.

| Species | Voucher | Sex | Collection sites | 2n | FN | Karyotype formula | NOR | 18S | 5S |
|---|---|---|---|---|---|---|---|---|---|
| *O. auyantepui* | **INPA 6754** | M | Brazil, AM – Jatapú River | 64 | 110 | 12m+10sm+26st +16a+XY | 10p and 14p | 10p and 14p | 5p |
| | **INPA 6751** | M | | 66 | 112 | 16m+6sm+26st+ 14a+XY | | | |
| | INPA 6753 | M | | – | – | – | – | – | – |
| | INPA 6747 | M | | – | – | – | – | – | – |
| *O. bicolor* | **INPA 6772** | M | Brazil, AM – Purus River | 80 | 142 | 18m+10sm+36st+ 14a+XY | 15p, 18p, 21p, 22p and 26p | 2p, 3p, 13p, 15p, 16p, 18p, 19p, 21p, 22p, 25p 26p and 30p | 7p |
| | **INPA 6749** | M | Brazil, AM – Jatapú River | | | | | | |
| | **INPA 6756** | M | | | | | | | |
| | **INPA 6758** | M | | | | | | | |
| | **INPA 6757** | F | | | | | | | |
| | INPA 6752 | M | Brazil, AM – Jatapú River | – | – | – | – | – | – |
| | INPA 6773 | F | Brazil, AM – Purus River | – | – | – | – | – | – |
| | INPA 6770 | M | Brazil, AM – Negro River | – | – | – | – | – | – |
| | INPA 6775 | M | Brazil, PA – Tapajós River | – | – | – | – | – | – |
| *O. rutilus* | **INPA 6760** | F | Brazil, AM – Negro River | 54 | 90 | 24m+6sm+8st+ 14a+XX | 4p and 23p | 4p and 23p | 1p |
| | **INPA 6761** | F | | | | | | | |
| | **INPA 6762** | F | | | | | | | |
| | **INPA 6768** | M | | | | | | | |
| | INPA 6767 | F | | – | – | – | – | – | – |
| | INPA 6769 | M | | – | – | – | – | – | – |
| | INPA 6766 | F | | – | – | – | – | – | – |
| | INPA 6763 | F | | – | – | – | – | – | – |
| | INPA 6764 | F | | – | – | – | – | – | – |
| | INPA 6765 | F | | – | – | – | – | – | – |
| | INPA 6774 | F | | – | – | – | – | – | – |
| | INPA 6759 | F | | – | – | – | – | – | – |
| | INPA 6745 | F | Brazil, AM – Cuieiras River | – | – | – | – | – | – |
| | INPA 6746 | M | | – | – | – | – | – | – |
| | INPA 6744 | F | | – | – | – | – | – | – |
| | INPA 6750 | M | Brazil, AM – Jatapú River | – | – | – | – | – | – |
| | INPA 6755 | M | | – | – | – | – | – | – |
| | INPA 6748 | F | | – | – | – | – | – | – |

polymerase chain reaction (PCR) using the universal primers described by Ivanova et al. (2007). The PCR products were purified with the ExoSap® kit (GE Healthcare) and sequenced using the method described by Sanger et al. (1977) on an ABI 3130XL automatic sequencer. The resulting sequences were submitted to the NCBI database under the following accession numbers: KT258600–KT258632.

Sequences were manually aligned using BioEdit v7.2.2 software (Hall 2001) and compared with sequences deposited in GenBank using BLASTn (Basic Local Alignment Search Tool). A Bayesian phylogenetic analysis was conducted with MrBayes 3.2 (Ronquist and Huelsenbeck 2003). For this analysis, Markov Chain Monte-Carlo sampling was conducted every 20,000$^{th}$ generation until the standard deviation of split frequencies was <0.01. A burn-in period equal to 25% of the total generations was required to summarize the parameter values and trees. Parameter values were assessed based on 95% credibility levels to ensure that the analysis had run for a sufficient number of generations. A genetic distance matrix was constructed using the MEGA 6 program (Tamura et al. 2013) and was obtained according to the Kimura 2 parameter (K2p) model. For Bayesian analysis, 53 *Oecomys* COI sequences available in GenBank were included (Appendix 1). One specimen of *Euryoryzomys macconnelli* was used as an outgroup.

## Results

### Chromosome analysis

### *Oecomys auyantepui* – Jatapú River

Two different diploid numbers were observed along the same bank of the Jatapú River: Karyomorph "a" exhibited 2n=64 chromosomes, a fundamental number = 110, and a karyotypic formula of 16m+6sm+26st+14a+XY (Fig. 2), in which pairs 1, 4, 15, 22, 26, 28, 30 and 31 were metacentric; 2, 3 and 19 were submetacentric; 5-13, 23, 24, 25 and 27 were subtelocentric; and 14, 16, 17, 18, 20, 21 and 29 were acrocentric (Fig. 2a). Karyomorph "b" exhibited 2n=66 chromosomes, a fundamental number = 112 and a karyotypic formula of 12m+10sm+26st+16a+XY (Fig. 3), in which pairs 1, 4, 17, 21, 24 and 29 were metacentric; 2, 3, 15, 16 and 27 were submetacentric; 5, 6, 7, 8, 9, 12, 18, 19, 20, 23, 25, 26 and 28 were subtelocentric; and 10, 11, 13, 14, 22, 30, 31 and 32 were acrocentric (Fig. 3a). Chromosomes X and Y were submetacentric for 2n=64 (Fig. 2a), whereas for 2n=66, chromosome X was metacentric, and chromosome Y was submetacentric and half the size of chromosome X (Fig. 3a). The heterochromatin was predominantly centromeric for both 2n=64 and 2n=66 chromosomes, ranging between subtle and conspicuous (Figs 2b, 3b). The Y chromosome exhibited a heterochromatic long arm in both karyotypes, while the X chromosomes presented a centromeric block and bitelomeric labeling. G-banding patterns enabled the identification of homologous pairs for each karyomorph (Figures 2c, 3c) and homology detection among the largest pairs of the complement. Pairs

1, 2, 3, 4, 5, 6, 12 and 13 from the 2n=66 chromosome karyomorph were homologous to pairs 1, 3, 2, 4, 5, 6, 12 and 13 from the 2n=64 chromosome karyomorph, respectively. Silver nitrate staining of the 2n=64 karyomorph resulted in labeling of three terminal sites, on one of the chromosomes of pair 10 and on both of the pair 14 homologs (Fig. 2d). The 2n=66 karyomorph also exhibited labeling of three terminal sites, two on pair 10 and one on one of the pair 14 chromosomes (Fig. 3d).

18S rDNA loci were visualized on chromosome pairs 10 and 14 of both karyomorphs, while the single 5S rDNA loci was located on pair 5 of karyomorph "a" and pair 7 of karyomorph "b" (Figs 2e, 3e). Both karyomorphs presented interstitial telomeric sequences (ITSs) in the centromeric region of the X chromosome (Figs 2f and 3f).

### *Oecomys bicolor* – Jatapú, Negro and Purus rivers

*Oecomys bicolor* was found to exhibit a diploid number 2n=80 chromosomes, a fundamental number = 142, and a karyotypic formula of 18m+10sm+36st+14a+XX or XY, wherein pairs 12, 32, 33, 34, 35, 36, 37, 38 and 39 were metacentric; pairs 7, 20, 25, 26 and 27 were submetacentric; 1, 2, 5, 6, 10, 11, 13, 14, 15, 16, 17, 19, 21, 22, 23, 24, 29 and 30 were subtelocentric; and 3, 4, 8, 9, 18, 28 and 31 were acrocentric (Figs 4a, 4b), with no differences being observed among individuals from the three collection sites. Sex chromosome X is the largest submetacentric chromosome of the complement, while sex chromosome Y is an average subtelocentric chromosome (Fig. 4b). Heterochromatin can be found in conspicuous blocks in the centromere region of all chromosomes, and in the case of the majority of metacentric, submetacentric (Fig. 4c), and the sex X chromosome, it also extends into the short arm (Fig. 4d). G-banding patterns enabled the correct identification of homologous pairs (Fig. 4e). Silver nitrate staining showed multiple terminal type-NORs on both homologous chromosomes of pairs 18 and 26 and on one of the homologous chromosome of pairs 15, 21 and 22 (Fig. 4f). 18S rDNA loci were identified on both homologous chromosomes of pairs 2, 3, 13, 15, 16, 18, 19, 21, 22, 25, 26 and 30, whereas 5S rDNA locus was located only on pair 7 (Fig. 4g). No ITSs could be observed (Fig. 4h).

### *Oecomys rutilus* – Cuieiras, Jatapú and Negro rivers

*Oecomys rutilus* was characterized as showing a diploid number 2n=54 chromosomes and a fundamental number = 90, with a karyotypic formula 24m+6sm+8st+14a+XX or XY, in which pairs 3, 5, 11, 12, 13, 14, 15, 19, 21, 22, 25 and 26 were metacentric; 7, 8 and 20 were submetacentric; 1, 2, 4 and 6 were subtelocentric; and 9, 10, 16, 17, 18, 23 and 24 were acrocentric (Figs 5a, 5b), with no differences being detected between the specimens collected at three different sites. The X chromosome was large and submetacentric, while the Y chromosome was subtelocentric and approximately 3/4 of the size of chromosome X (Fig. 5b). Heterochromatic regions were characterized

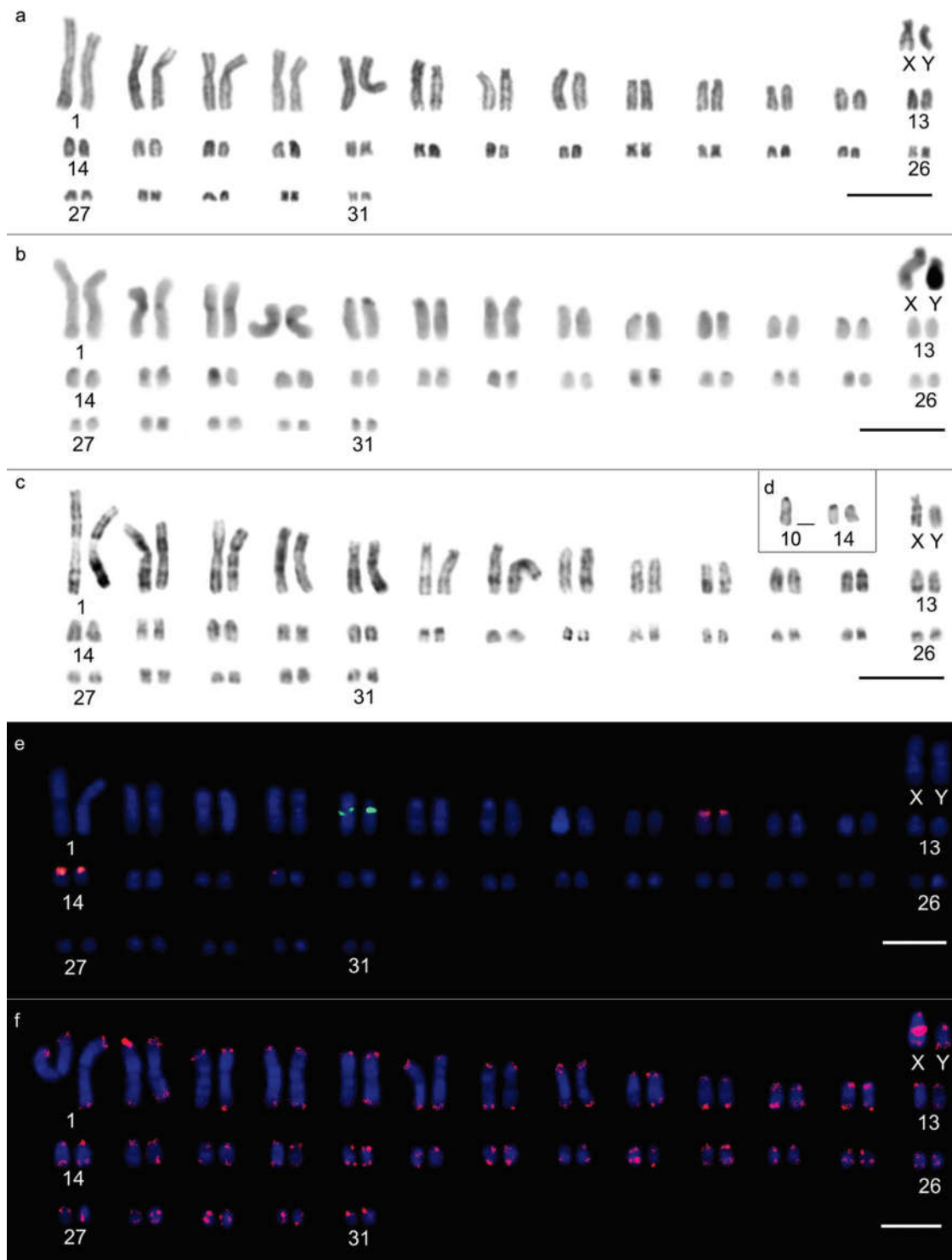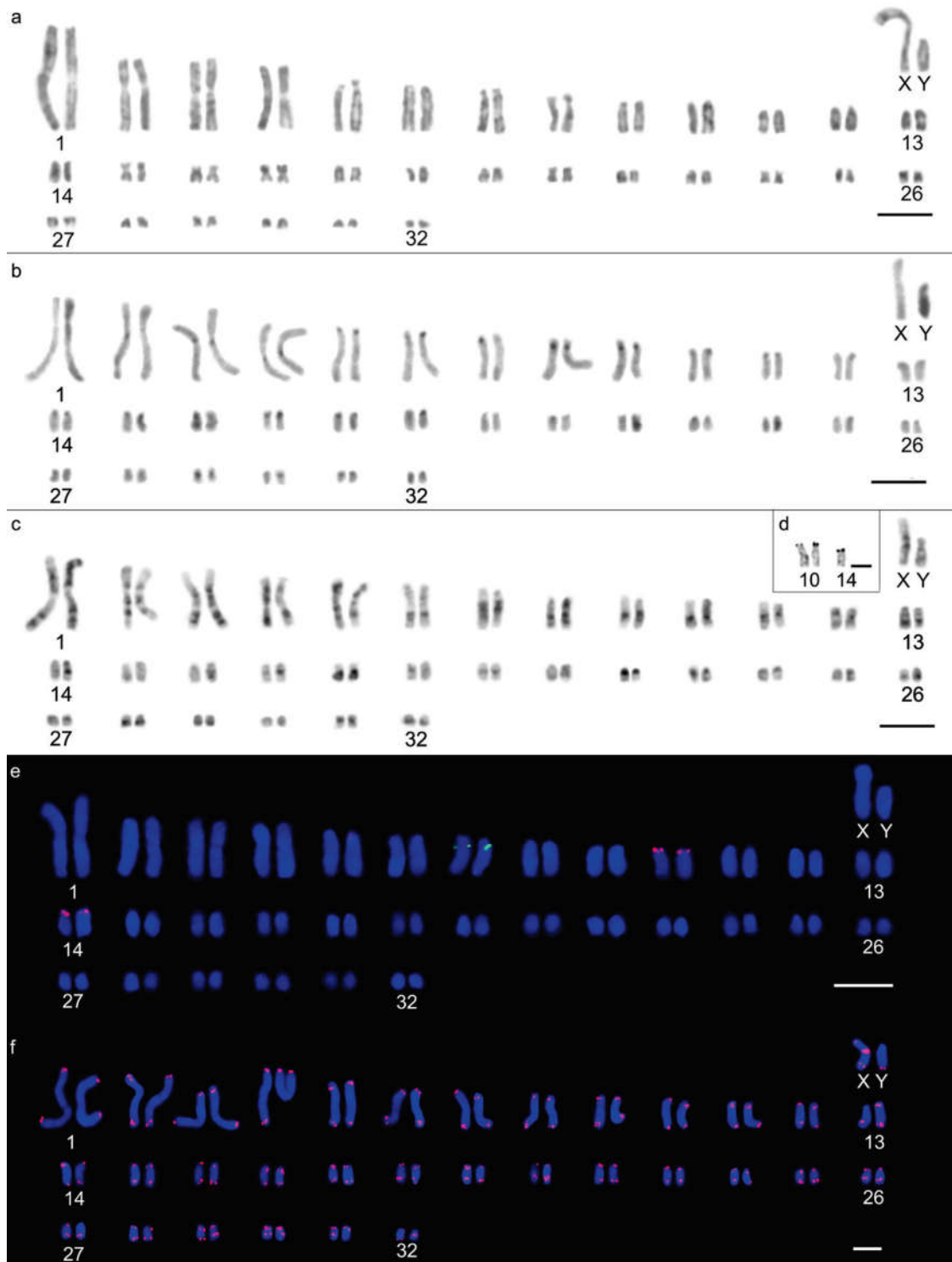**Figure 2.** Karyotypic characteristics of male *Oecomys auyantepui*, karyomorph "a" (INPA 6754) with 2n=64: **a** conventional Giemsa staining **b** heterochromatic regions highlighted by C-banding **c** G-banding **d** nucleolus organizing region-carrying pairs evidenced by silver nitrate staining **e** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes **f** karyotype indicating the presence of telomeric sites as well as interstitial telomeric sequence in the sex X chromosome. Bars: 10 μm.

**Figure 3.** Karyotypic characteristics of male *Oecomys auyantepui* karyomorph "b", with 2n=66: **a** conventional Giemsa staining (INPA 6751) **b** heterochromatic regions highlighted by C-banding (INPA 6751) **c** G-banding (INPA 6751) **d** nucleolus organizing region-carrying pairs revealed by silver nitrate staining (INPA 6751) **e** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes (INPA 6751) **f** karyotype indicating the presence of telomeric sites as well as an interstitial telomeric sequence on the X sex chromosome (INPA 6754). Bars: 10 μm.
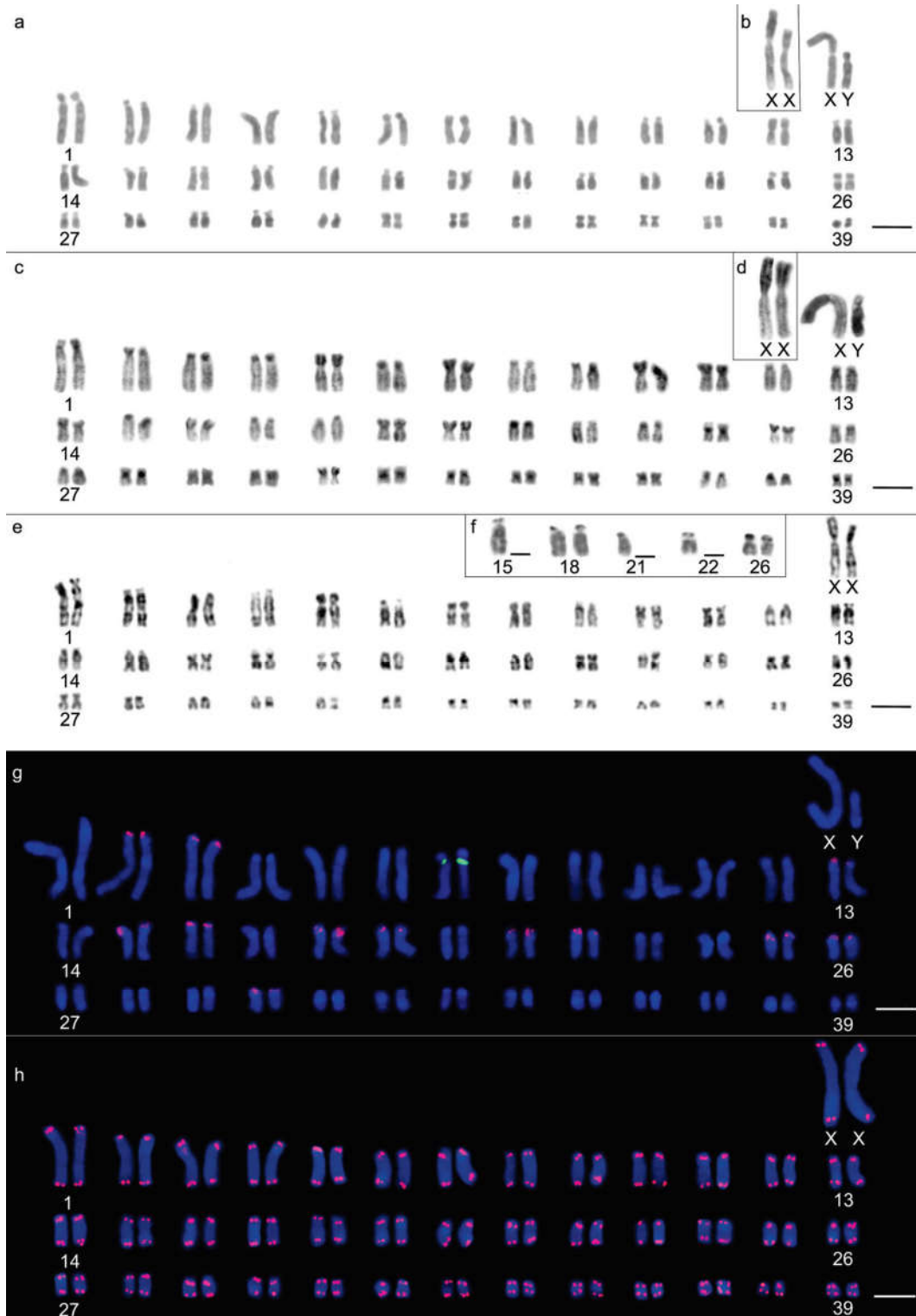
**Figure 4.** Karyotypic characteristics of *Oecomys bicolor*: **a** conventional Giemsa staining of a male (INPA 6749) **b** highlighted sex chromosomes of a female (INPA 6749) **c** heterochromatic regions revealed by C-banding in a female (INPA 6772) **d** highlighted C-banding on a male's sex chromosomes (INPA 6772) **e** G-banding of a female (INPA 6772) **f** nucleolus organizing region-carrying pairs revealed by silver nitrate staining (INPA 6749) **g** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes (INPA 6758) **h** karyotype indicating the presence of telomeric sites (INPA 6772). Bars: 10 μm.
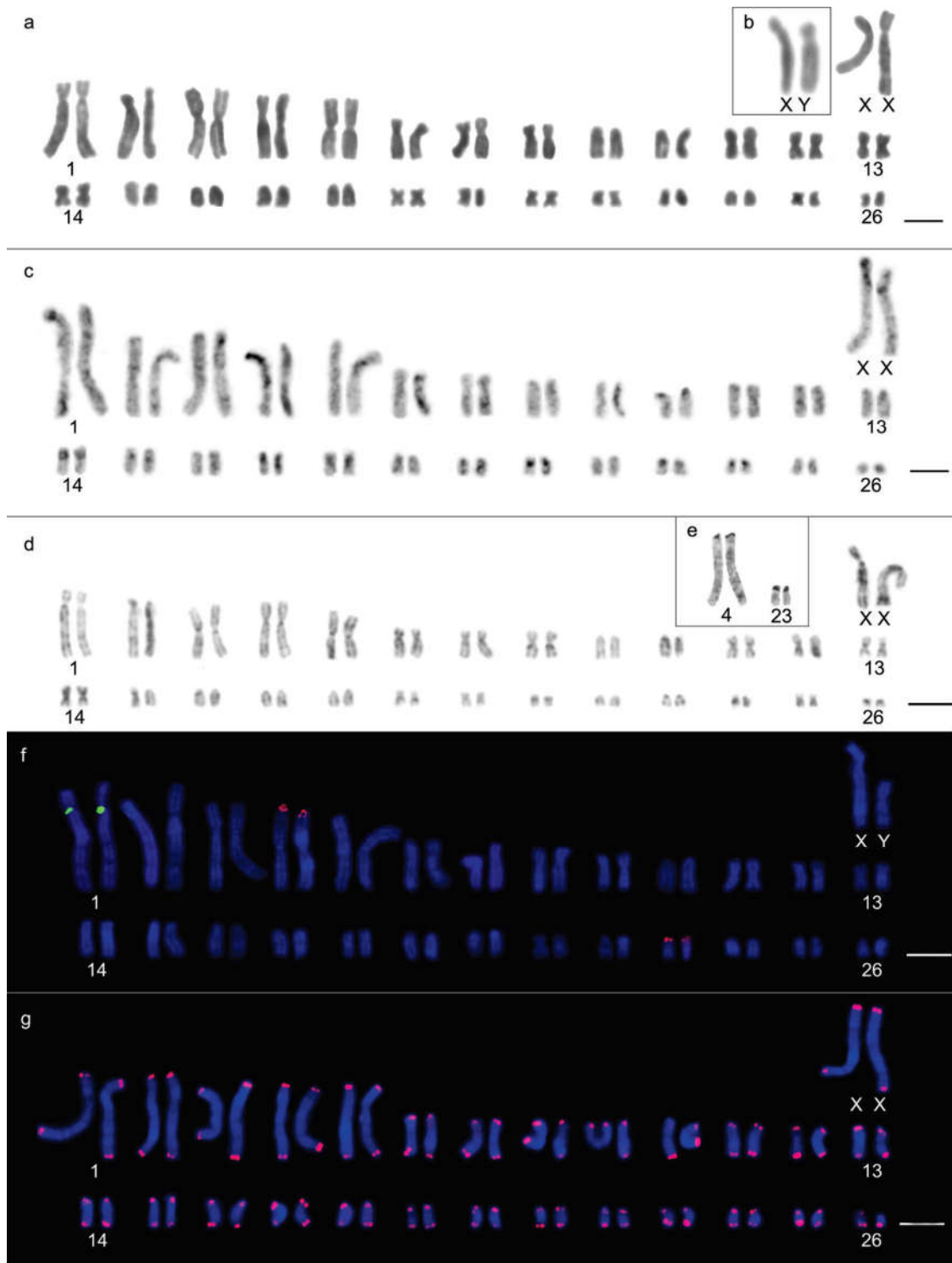
**Figure 5.** Karyotypic characteristics of *Oecomys bicolor*: **a** conventional Giemsa staining of a male (INPA 6761) **b** highlighted sex chromosomes of a male (INPA 6768) **c** heterochromatic regions revealed by C-banding of a male individual (INPA 6754) **d** G-banding of a female (INPA 6761) **e** nucleolus organizing region-carrying pairs revealed by silver nitrate staining (INPA 6762) **f** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes (INPA 6761) **g** karyotype indicating the presence of telomeric sites (INPA 6761). Bars: 10 μm.

by subtle or conspicuous centromeric labeling on some chromosome pairs (Fig. 5c). G-banding patterns enabled correct homologous pairing (Fig. 5d). Multiple NORs were revealed by silver nitrate staining in the terminal regions of both homologous chromosomes of pairs 4 and 23 (Fig. 5e), coinciding with 18S rDNA loci (Fig. 5f), which were also observed on both homologous chromosomes of pair 1, in a proximal position on the long arms (Fig. 5f). No ITSs were observed (Fig. 5g).

## Mitochondrial DNA identification

A total of 86 *Oecomys* mitochondrial COI gene sequences were compared: 33 originating from the present work and 53 deposited in GenBank (Appendix 1). NJ, Bayesian and ML tree retrieved the same topology and showed differences mainly in relation to branch support values. The similarity index was greater than 98%, which allowed molecular identification of the species. The phylogenetic trees (Figure 6) grouped *O. rutilus* into two clades, one comprising individuals from Brazil, Suriname and Guyana (I), while the other consisted of one individual from Ecuador (J). The genetic distance between clades I and J was 7.33%, whereas the genetic distance within clade I was 1.62%. The individuals of *O. auyantepui* were grouped into a single clade (H), comprising individuals from Brazil, Guyana and Suriname, with an intraspecific genetic distance of 1.41%. One individual from Ecuador, whose species was not defined in GenBank, belonged to a distinct lineage (clade E). Two other specimens without species level-definition were grouped with *Oecomys concolor* (branch F), with a genetic distance of 0.79%. One other individual (clade G), also identified as *O. concolor* in GenBank, exhibited a distinct lineage, showing a large genetic distance (12.88%) from branch F. All *O. roberti* specimens were grouped together (clade D), with a genetic distance of 0.39%. *Oecomys bicolor* formed three clades (A, B and C) with large genetic distances: individuals from the Guyanas and Suriname were grouped together with high support, forming a moderately supported clade (C) with an individual from the Central Amazon (INPA 6775). *Oecomys bicolor* and *Oecomys* sp. from Ecuador and the Negro river (INPA 6770) formed a group with moderate-to-high support (clade B). One individual from the Purus River (clade C) showed a highly supported association with clade B, with a genetic distance of 7.12%. The genetic distance between clades A and B was 8.4%, and that between A and C was 9.89%. *Oecomys rex* also formed two clades (K and L), with a large genetic distance between them (11.92%).

## Discussion

The identification of Rodentia species is often difficult using morphological criteria alone (Granjon et al. 2002, Lecompte et al. 2005, Ben Faleh et al. 2010). Such difficulties are evident in this order mainly because of the existence of cryptic species (Granjon et al.

2002, Musser and Carleton 2005, Lecompte et al. 2005) and new species are continually described (Helgen 2005, Musser et al. 2005). Species identification via molecular methods, such as molecular barcoding using a short genetic marker (Hebert et al. 2003), has been proposed to overcome some of the weaknesses of the traditional approach, which will aid non-taxonomists by fulfilling the urgent requirement for rapid and accurate species identification tools (Teletchea 2010). This approach is potentially useful in the study of rodents (Borisenko et al. 2008, Tamrin and Abdullah 2011, Barbosa 2013). In the present work, employing COI sequences as a tool for species identification was shown to be satisfactory, as the obtained distance patterns provided sufficient information for the identification of specimens whose taxonomic identification at the species level is not straightforward. Most of the species were recovered as monophyletic groups.

The available chromosomal data for *Oecomys* species consist mostly of descriptions of diploid and fundamental numbers, which restricts comparisons with the data obtained in the present work (Table 1). However, high karyotypic diversity can be observed, with countless chromosomal rearrangements between *Oecomys* species being responsible for this diversity. Neither of the two *O. auyantepui* karyomorphs reported in this work had been previously described in the literature. Both individuals (INPA 6751, INPA 6754) showing these two karyomorphs were captured on the same bank (right) of the Jatapú river, approximately 1 km from each other. The karyomorphs only exhibited one ITS, located on X chromosome. ITSs have been observed in other rodents as well (Castiglia et al. 2007, Rovatsos et al. 2011, Suárez-Villota et al. 2013). Short telomeric sequences $(TTAGGG)_n$ have been primarily classified as components of satellite DNA (Adegoke et al. 1993). These sequences may be located in subtelomeric and interstitial chromosome positions (Garrido-Ramos et al. 1998) and are subjected to amplification (Arnason et al. 1998, Castiglia et al. 2006). They may also appear during the double-stranded DNA nick repair process (Nergadze et al. 2004, 2007). However, the most commonly accepted scenario is that ITSs signal recent chromosomal rearrangements, such as the transposition of functional telomeric sequences to an interstitial position (Dobigny et al. 2003, Zhdanova et al. 2005), or chromosome fusion events, with the latter being the main source of ITSs in many organisms (Lee et al. 1993, Slijepcevic 1998). Nevertheless, it was not possible to determine the occurrence of either an increase in the diploid number from 2n=64 as a result of a fission event or a decrease from 2n=66 due to a fusion event.

Establishing the evolutionary direction of chromosomal rearrangements is not always possible because most of the available painting data for the Sigmodontinae group are incomplete, and it is not possible to draw definitive conclusions regarding the composition of a putative Sigmodontinae ancestral karyotype (Romanenko et al. 2012). The same is true for *Oecomys*, where it cannot be determined whether the diploid number has increased or decreased because the *in situ* hybridization method used in this study likely does not detect very short (< 1 kb) stretches of (TTAGGG)n sequences. Thus, even if chromosome fusions that would result in a decrease in diploid number have occurred, the fused chromosomes will not always possess an ITS, which

may have been lost prior to the fusion or been subjected to molecular erosion (Mandrioli et al. 1999).

Both *Oecomys auyantepui* karyomorphs exhibit similar, predominantly centromeric, subtle heterochromatic blocks. Their NORs are also similar, with three different labeled sites being observed on the same chromosome pairs. The largest chromosomes of both karyomorphs are homologous - those carrying 5S rDNA loci in particular - sharing the same chromosomal region (subtelocentric chromosomes), position (long arm, proximal) and number of labeled sites, as inferred based on the increased resolution provided by G-banding. Thus, much like the NOR-carrying pairs, these chromosomes were not involved in chromosomal alteration processes leading to the occurrence of two different diploid numbers in *O. auyantepui*. Mitochondrial DNA analysis grouped all *O. auyantepui* specimens onto a single branch (Fig. 6) with a high support value and low intraspecific genetic distance (1.41%), indicating that the occurrence of these two karyomorphs may be due to chromosomal polymorphism and not to the existence of two differentiated evolutionary units, as the intraspecific genetic distance is consistent with available data for other Sigmodontinae and the family Cricetidae in general (Smith and Patton 1993, Patton 1999,Ventura 2009).

Current phylogenetic and karyotypic data suggest the existence of a complex of *O. bicolor* species (Smith and Patton 1999, Flores 2010, Andrade and Bonvincino 2003). Four different diploid numbers have previously been characterized in the Brazilian Amazon, varying from 54 to 86 chromosomes, with 2n=80 being the most common (Gardner and Patton 1976, Patton et al. 2000, Andrades-Miranda et al. 2000, Andrades-Miranda et al. 2001, Lira 2012). Comparison of the karyotypic patterns of *O. bicolor* captured along the Jatapú and Purus rivers revealed a similar chromosomal organizational pattern for individuals with 2n=80 chromosomes. However, the karyotypic pattern of individuals collected on the banks of the Jari river diverges, with a diploid number 2n=82 and FN=116 (Lira 2012). The NORs described in the present work (7 labeled sites) occurred in larger numbers than what had been previously described for the species (1 to 4 labeled sited) (Andrades-Miranda 2001, Lira 2012). These NORs do not refer to the labeling of acidic heterochromatic regions, as fluorescent *in situ* hybridization using 18S rDNA probes revealed the existence of twelve chromosome pairs carrying these sequences. A larger number of sites compared with the number identified through silver nitrate staining, which is a common occurrence and is observed in other groups (Lira 2012). This disparity stems from the fact that the latter technique labels proteins associated with the nucleolar structure and not ribosomal DNA regions, thus identifying only NORs that had been active in the preceding interphase (Miller et al. 1976). Thus, the difference in silver-stained sites between different populations may stem from the activity of ribosomal RNA genes. Because rDNA sequence hybridization had not been performed in individuals from the analyzed populations in previous studies, this hypothesis cannot be verified. In contrast, the heterochromatin distribution pattern is similar, with centromeric blocks extending to the short arms of the majority of metacentric and submetacentric chromosomes and both sex chromosomes.
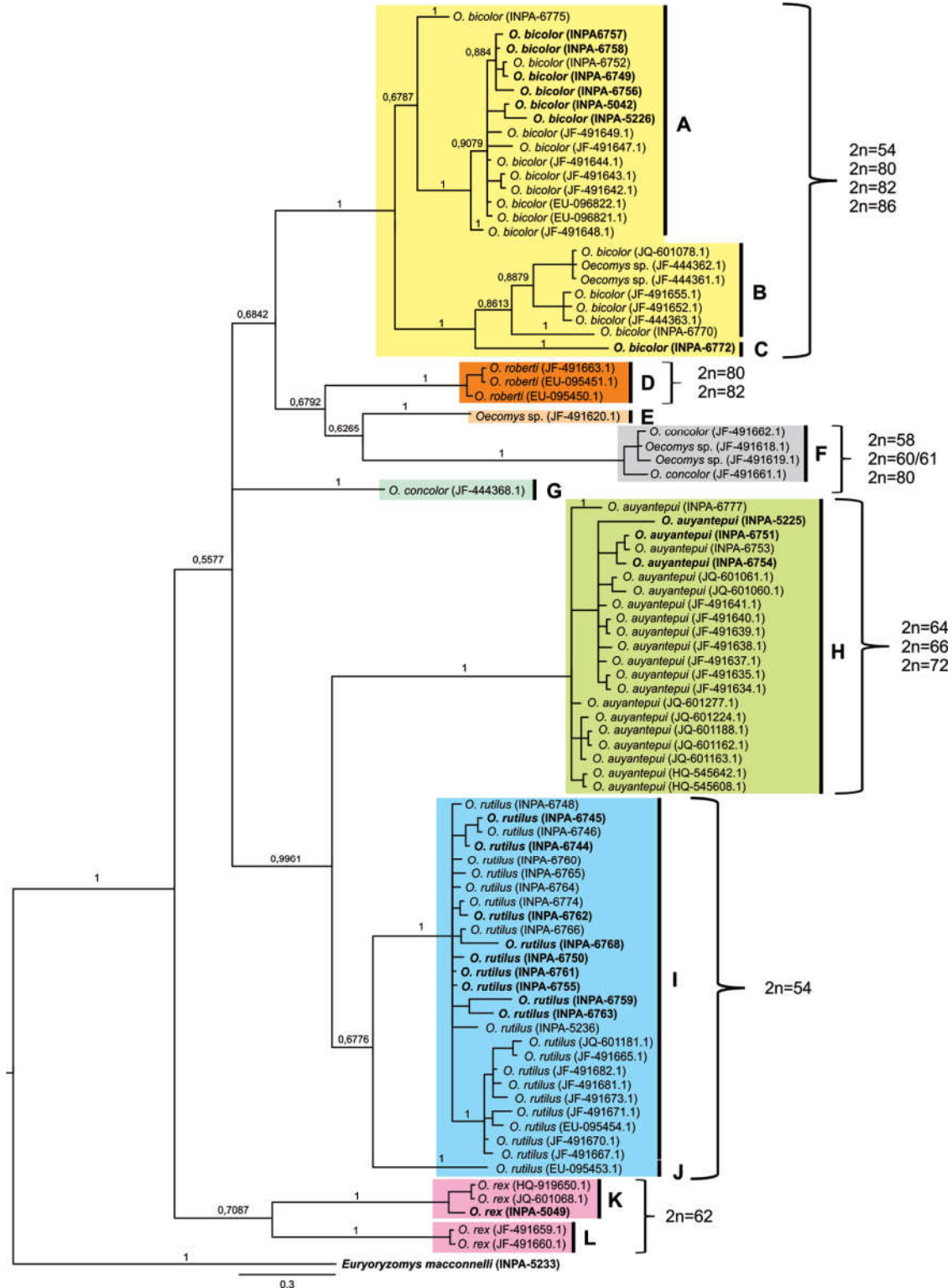
**Figure 6.** Bayesian tree of the cytochrome oxidase I gene. The probabilistic support is presented above the branches. Letters (**A–L**) represent the groups formed based on the analysis of the genetic distances between them. Sequences in bold were analyzed in the present work.

The diploid number determined for *O. rutilus* (2n=54, first described in Lira (2012) did not vary, regardless of the collection site, and no variations in karyotypic structure were observed in the present work. However, the three specimens described previously (Lira, 2012) exhibited differences in their autosomal fundamental number (82, 84 and 86). Such variation may be related to karyotype interpretation, given that it depends on the quality of chromosome preparations, DNA compaction patterns, size and number of chromosomes and errors in the measurement of chromosomal arms. The C-banding pattern observed in *O. rutilus* consisted of very subtle labeling on the majority of chromosomes but was consistent with the expected locations previously described for other *Oecomys* species and the tribe Oryzomyini (Yonenaga-Yassuda et al. 1987, Svartman and Almeida 1992, Silva and Yonenaga-Yassuda 1998, Aniskin and Volobouev 1999, Volobouev and Aniskin 2000, Andrades-Miranda et al. 2002, Bonvicino et al. 2005, Lira 2012).

Based on the amplitude of the genus distribution, Langguth et al. (2005) suggested *O. catherinae* (2n=60) as the ancestral taxon; the same finding was reported by Weksler (2006), based on phylogenetic analysis of the IRBP gene and morphological data for *O. bicolor*, *O. catherinae*, *O. concolor*, *O. mamorae* and *O. trinitatis*. Although the current phylogenetic analysis based on COI sequences was limited to a single marker and did not consider several of the taxa analyzed by Flores (2010), it showed similar results with high support values, such as monophyly of the genus *Oecomys*, which was also observed in molecular studies using other markers (Smith and Patton 1999, Weksler 2006). However, considering *O. rex* as a sister group of *O. catherinae*, which would classify both species as ancestral taxa (Flores 2010), the basal diploid number would be approximately 60/62 chromosomes. Therefore, it must be noted that molecular analyses did not detect an increasing or decreasing tendency in the diploid number between the branches, suggesting a complex karyotypic structure, as shown by the different diploid numbers obtained for the same morphological species. Moreover, the phylogenetic analysis placed all individuals in a single group.

In the present work, NORs were found to be preferentially located in the terminal regions of chromosomes, and their number increased with the diploid number; this pattern is also present in other members of the family Cricetidae (Lira 2012, Romanova et al. 2006, Ventura 2009, Fagundes et al. 1997). These data agree with FISH results obtained using the 18S ribosomal DNA probe, confirming the presence of two labeled pairs for *O. auyantepui* and *O. rutilus*. Labeling of four NORs was observed in *Oecomys rutilus*, whereas three were detected in *O. auyantepui*. Lira (2012) described four labeled sites in *O. rex*, again suggesting that it may constitute a basal taxon. In *Oecomys bicolor*, five chromosome pairs exhibited labeling, though not all displayed labeling on both homologous chromosomes. The multiple 18S rDNA sites observed in *O. bicolor* likely derive from duplication and dispersion. Di Meo et al. (1993) reported that the difference in the NOR distribution in correlated species is ascribed to rearrangements that have accumulated since the divergence of the common ancestor, mainly via inversions and Robertsonian translocations. Grozdanov et al. (2003) and Britton-Davidian et al. (2012) stated that NOR diversity among rodents is an indica-

tor of high intrachromosomal transposition rates in the absence of visible rearrangements, suggesting, once again, that this character represents a derived state for this taxon. Despite this fact, the interstitial position of 5S rDNA is related to sequence protection, thereby avoiding possible crossing-over or transposition events, which are more frequent in terminal regions (Martins and Galetti Jr., 1999). This scenario is made evident by comparing the degree of conservation in the position and location of this sequence compared with 45S rRNA. Ventura et al. (2012) described a similar situation in Akodontini, which shows conservation of 5S rDNA chromosomal sites, despite large chromosomal variability within the group.

*Oecomys* species have undergone intense chromosomal alteration processes, as confirmed by the observed karyotypic patterns, indicating high local diversity and an ample distribution for the taxa under study. However, the limited taxonomic sample available, in terms of both *Oecomys* individuals and molecular data renders the determination of which evolutionary processes have led to the variability in karyotype morphology more difficult. Furthermore, the current data reinforce the necessity for integrative taxonomy, where genetic tools should be used in conjunction with morphological analysis to delimit *Oecomys* taxa.

## Conclusions

The intra- and interspecific variations observed in the diploid number of *Oecomys* species indicate that chromosomal rearrangements such as fusions/fissions, translocations and duplications have led to the appearance of different diploid numbers and karyotypic formulas. However, telomere sequence hybridization was not found to be a good indicator of autosomal chromosome rearrangements in the *Oecomys* species under study, as no autosomal ITSs could be observed. *O. bicolor*, which is considered to be a derived taxon of the genus (Flores 2010), exhibits the highest diploid number, possibly arising from chromosomal fission events that occurred during its evolutionary history.

## Acknowledgments

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## Appendix

COI sequences of *Oecomys* deposited in GenBank. The voucher, species and collection sites are listed.

| Species | Genbank n° | Collection sites |
|---------|-----------|------------------|
| *O. auyantepui* | JQ601277.1 | Suriname – Sipaliwini river |
| | JQ601224.1 | Suriname – Sipaliwini river |
| | JQ601188.1 | Suriname – Kutari River |
| | JQ601163.1 | Suriname – Kutari River |
| | JQ601162.1 | Suriname – Kutari River |
| | JQ601061.1 | Suriname: Brownsberg Nature Park |
| | JQ601060.1 | Suriname: Brownsberg Nature Park |
| | JQ601049.1 | Suriname: Brownsberg Nature Park |
| | HQ545642.1 | Suriname: Sipaliwini River |
| | HQ545608.1 | Suriname |
| | HQ545608.1 | Suriname |
| | JF491641.1 | Guiana: Upper Demerara-Berbice, West Pibiri, Mabura |
| | JF491640.1 | Guiana: Upper Takutu-Upper Essequibo |
| | JF491639.1 | Guiana: Upper Takutu-Upper Essequibo |
| | JF491638.1 | Guiana: Potaro-Siparuni |
| | JF491637.1 | Guiana: Potaro-Siparuni |
| | JF491635.1 | Guiana: Cuyuni-Mazaruni |
| | JF491634.1 | Guiana: Cuyuni-Mazaruni |
| *O. bicolor* | JQ601078.1 | Equador: Parque Nacional Yasuni |
| | JF491655.1 | Equador: Napo, Parque Nacional Yasuni |
| | JF491652.1 | Equador: Orellana, Onkone Gare |
| | JF491649.1 | Guiana: Demerara-Mahaic |
| | JF491648.1 | Guiana: Barima-Waini |
| | JF491647.1 | Guiana: Barima-Waini |
| | JF491644.1 | Guiana: Upper Takutu-Upper Essequibo |
| | JF491643.1 | Guiana: Potaro-Siparuni |
| | JF491642.1 | Guiana: Potaro-Siparuni |
| | JF444363.1 | Equador: Orellana |
| | EU096822.1 | Suriname: Sipaliwini |
| | EU096821.1 | Suriname: Sipaliwini |
| *O. concolor* | JF491662.1 | Equador: Napo, Parque Nacional Yasuni |
| | JF491661.1 | Equador: Orellana, Onkone Gare |
| | JF444368.1 | Equador: Orellana |
| *O. rex* | JF491660.1 | Guiana: Potaro-Siparuni |
| | JF491659.1 | Guiana: Potaro-Siparuni |
| | JQ601068.1 | Guiana: 40 Km NE of Surama |
| | HQ919650.1 | Suriname |
| *O. roberti* | JF491663.1 | Guiana: Potaro-Siparuni |

| Species | Genbank n° | Collection sites |
|---|---|---|
| *O. rutilus* | JQ601181.1 | Suriname: Kutari River Camp |
| | JF491682.1 | Guiana: Potaro-Siparuni |
| | JF491681.1 | Guiana: Potaro-Siparuni |
| | JF491673.1 | Guiana: Potaro-Siparuni Kabukalli Landing, Iwokrama Forest |
| | JF491671.1 | Guiana: Siparuni river |
| | JF491670.1 | Guiana: Barima-Waini, Baramita, Old World |
| | JF491667.1 | Guiana: Barima-Waini, Baramita, Old World |
| | JF491665.1 | Guiana: Upper Takutu-Upper Essequibo |
| | EU095454.1 | Guiana: Upper Demerara-Berbice |
| | EU095453.1 | Equador: Napo |
| *Oecomys sp.* | JF491619.1 | Equador: Napo, Parque Nacional Yasuni |