

## CHROMOSOMES AND PHYLOGEOGRAPHY OF *SYLVILAGUS* (MAMMALIA, LEPORIDAE) FROM EASTERN BRAZIL

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### ABSTRACT

A large geographic differentiation has been reported in *Sylvilagus brasiliensis* based on external characters of a small number of specimens. A new karyotype with  $2n = 40$  and  $FNa = 74$  from Rio de Janeiro is herein reported. Phylogenetic analyses based on cytochrome *b* DNA of eight samples from the states of Paraíba, Tocantins, Goiás, Minas Gerais and Rio de Janeiro suggested a strongly structured population. Altogether, molecular and karyotypic data suggested that the diversity of *Sylvilagus* in eastern Brazil may be larger than the one reported in the literature on the basis of external characters.

**Keywords:** cytochrome *b*; genetic diversity; karyotype; Lagomorpha; phylogeny; tapiti.

### INTRODUCTION

*Sylvilagus* Gray, 1867 (Lagomorpha) is the most speciose genus of the Leporidae, comprising 17 species of New World cottontail rabbits (“tapitis”) distributed throughout North, Central and the northern part of South America (Hoffmann and Smith 2005). A revision of South American cottontail rabbits recognized only two species: *S. floridanus* and *S. brasiliensis* (Hershkovitz 1950) while another species, *S. gabii* Allen 1877 (Ruedas and Salazar-Bravo 2007), has recently been recognized in Central

America. Some cottontails are widely distributed, like *S. brasiliensis* (Linnaeus, 1758) and *S. floridanus* (J.A. Allen, 1890) while other species are extremely restricted, like *S. graysoni* (J.A. Allen, 1877) and *S. mansuetus* Nelson, 1904 (Angermann *et al.* 1990). The range of *S. brasiliensis*, the most widespread species, extends from Mexico to Central to Peru, Bolivia, Northern Argentina and Southern Brazil (Hoffmann and Smith 2005). Its large intraspecific diversity led to the description of 18 *S. brasiliensis* subspecies (Hoffmann and Smith 2005, Ruedas and Salazar-Bravo 2007).

The recognition of South American *Sylvilagus* taxa has been based, almost exclusively, on the external characteristics such as pelage coloration of a limited number of specimens, while few taxonomic studies have relied on karyotypic and phylogenetic data (Robinson *et al.* 1983, Halanych and Robinson 1997, Matthee *et al.* 2004, Robinson and Matthee 2005, Ruedas and Salazar-Bravo 2007). Phylogenetic relationships between different forms and the identification of cryptic species may be enhanced by karyotypic analysis (Schroder and Van der Loo 1979). Robertsonian translocations (like centric fusions) are the main rearrangements responsible for karyotypic evolution in *Sylvilagus*, accounting for the wide range of variation in diploid number between species, from  $2n = 36$  to  $2n = 52$  chromosomes (Guereña-Gándara *et al.* 1983). In this work, we analyze the chromosome complement of *S. brasiliensis* and the phylogenetic relationships of specimens from several localities of eastern Brazil, including areas of Atlantic Forest and Cerrado, based on cytochrome *b* DNA.

## MATERIAL AND METHODS

### *Samples*

Eight *Sylvilagus* were collected in seven Brazilians localities of Cerrado and Atlantic Forest. The geographic coordinates of the principal city of the municipality of collecting locality were taken from IBGE. Numbers in parentheses, corresponding to the localities plotted in Figure 1 and field numbers, are listed below.

Paraíba state: (1) Cruz de Espírito Santo,  $7^{\circ}08' S$   $35^{\circ}05' W$ , SBS1743; Tocantins state: (2) Axixá,  $5^{\circ}36'59'' S$   $47^{\circ}47'10'' W$ , LBCE13109; Goiás state: (3) Teresina de Goiás,  $16^{\circ}23' S$   $49^{\circ}04' W$ , MN43004; Minas Gerais state: (5) Conceição do Rio Verde,  $21^{\circ}52' S$   $45^{\circ}05' W$ , in the road between municipalities of Águas de Contendas and Conceição do Rio Verde, JFV367, (6) Juiz de Fora (CB227, CB 515); Rio de Janeiro state: (7) Areal,  $22^{\circ}13'50'' S$   $43^{\circ}06'20'' W$  444 m, MN79183 (CB97), (8) Itaboraí,  $22^{\circ}44' S$   $42^{\circ}51' W$ , RBP3555. (9) Magé,  $22^{\circ}39' S$   $43^{\circ}02' W$ , MN53377, MN50555, (10) Petrópolis (CB571).

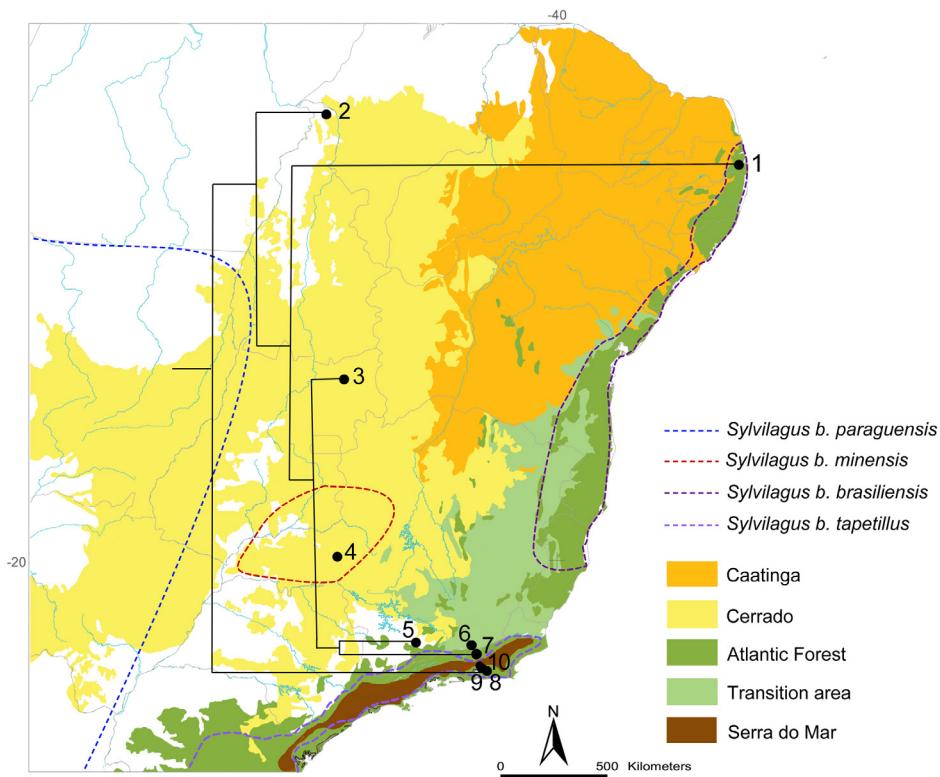
Skins and skulls of specimens are (or will be) housed in the mammal collections of Museu Nacional, Universidade Federal do Rio de Janeiro (MN), Rio de Janeiro, and Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios (LBCE), IOC, Rio de Janeiro. The following abbreviations refer to field numbers: CB = C Bueno, JFV = JF Vilela, RBP = RB Pineschi.

### *Karyotypic analysis*

Chromosome preparations were obtained from 2-hour bone marrow cultures at  $37^{\circ}C$  in RPMI 1640 supplemented with 20% fetal calf serum, ethidium bromide ( $5\mu g/mL$ ) and colchicine ( $10^{-6}M$ ). FNa refers to autosomal fundamental number.

### *Phylogenetic and phylogeographic analyses*

DNA was isolated from tissue samples preserved in 100% ethanol following with PCR using primers



**Figure 1.** Collecting localities of studied *Sylvilagus* samples. Paraíba state: (1) Cruz de Espírito Santo; Tocantins state: (2) Axixá; Goiás state: (3) Teresina de Goiás; Minas Gerais state: (4) Pedrinópolis (locality of karyotyped specimen from Langguth and Sousa 2003), (5) Conceição do Rio Verde, (6) Juiz de Fora; Rio de Janeiro state: (7) Areal, (8) Itaboraí, (9) Magé, (10) Petrópolis. Geographic distribution of *S. brasiliensis* subspecies was based on Herhskovitz (1950).

L14724 (5'-CGAACGCTTGATATGA AAAACCATCGTTG-3' (Irwin *et al.* 1991) and CIT-REV (5'-GAATATCA GCTTTGG-3' (Casado *et al.* 2010). Amplicons were purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Brazil) and labeled with primers L14724 and Citb AOT (5'-CATGAGGCCAAAT ATCATTCTGAGG-3 (Menezes *et al.* 2010), SOT-In1 (5'-TTTTTRGAT

CCTGTTTCRTG-3' (Cassens *et al.* 2000) and SOT-In2 (5'- TGAGGACA AATATCATTYTGAG-3' (Cassens *et al.* 2000). Sequencing was carried out in an ABI PrismTM 3730 automatic DNA platform. Electropherograms were manually checked with BIOEDIT (version 7.0.8.0, Hall 1999) and CHROMAS (version 1.45, MacCarthy 1998). Mitochondrial, cytochrome *b* DNA was referred as MT-CYB

following HGNC rules (Eyre *et al.* 2006, latest accession on January 2010).

The eight DNA sequences generated by us and six GenBank sequences of *S. obscurus* (AY292725), *Sylvilagus floridanus* (AY292724), *Sylvilagus palustris* (AY292727), *Sylvilagus aquaticus* (AY292726), *Sylvilagus nuttallii* (AY292723), *Sylvilagus audubonii* (AY292722) were manually aligned with BIOEDIT (Hall 2007). Three other sequences of *Lepus brachyurus* (AB058616), *Romerolagus diazi* (AY292734) and *Pronolagus rupestris* (AY292735) were used as outgroups.

Pairwise genetic distances were estimated with Kimura's two-parameters implemented in MEGA (version 4, Tamura *et al.* 2007). For phylogenetic reconstructions, the DNA substitution model HKY (Hasegawa *et al.* 1985) and gamma distributed substitution rates (HKY+G) were selected with MODELGENERATOR (version 0.85, Keane *et al.* 2006) with a Bayesian information criterion (BIC). Maximum likelihood (ML) reconstructions were estimated with PHYML (version 3.0, Guindon and Gascuel 2003). The tree topology space was searched with the best of Nearest Neighbor Interchange and Subtree Pruning and Regrafting algorithms starting from five random starting trees generated by BioNJ (Guindon *et al.* 2010, Guindon and Gascuel 2003). Branch support was calculated using the approximate likelihood ratio test (aLRT) with SH-like interpretation (Anisimova and Gascuel 2006, Guindon *et al.* 2010), and bootstrap applying 1,000 replicates.

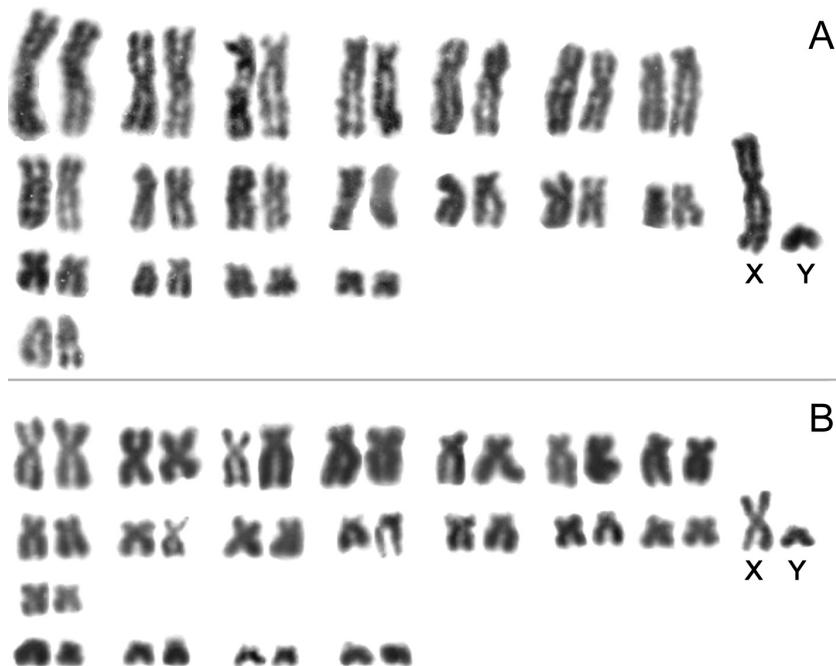
Furthermore, Bayesian reconstructions (BY) were obtained with MrBayes 3.2.3 (Ronquist *et al.* 2012). Posterior probabilities distribution estimates of tree topologies and branch lengths were obtained by Markov chain Monte Carlo (MCMC) sampling every 100 MCMC steps over a total of 1,000,000 steps. The first 1,000 trees were discarded as burning.

A dataset comprising all *S. brasiliensis* was used for constructing a median joining (MJ) network with NETWORK (Bandelt *et al.* 1999, Posada and Crandall 2001, <http://www.fluxus-engineering.com>) to evaluate population structure. This analysis was carried out only with variable sites and without sites with missing data in at least one haplotype.

## RESULTS

Karyotypic analyses of two specimens (male MN50555 and female MN53377) from Magé municipality, Rio de Janeiro state, showed  $2n = 40$  and  $FNa = 74$  (Table 1; Figure 2A). The autosome complement comprised 18 banded chromosome pairs, varying in size from large to small, and one median to small acrocentric pair. The X chromosome was a large sized submetacentric and the Y chromosome a small sized acrocentric chromosome.

Cytochrome *b* (1,143 bp) of *Sylvilagus brasiliensis* specimens showed seven haplotypes, three of which present in more than one specimen (Table 2). ML and BY topologies were coincident in showing the monophyly of the genus *Sylvilagus* and *S. brasiliensis*,



**Figure 2.** Karyotype with Giemsa coloration of (A) *Sylvilagus b. tapetillus* male MN50555 from Magé, Rio de Janeiro state, and (B) *S. b. minensis*, UFPB 1610, from Pedrinópolis, Minas Gerais state (Langguth and Sousa 2003). X and Y are sexual chromosomes.

both with high support (Figures 3 and 4) but differed with respect to the relationship between *S. brasiliensis* and other co generic species. ML showed a basal offshoot leading to (*S. floridanus*, *S. obscurus*) and two well-supported clades, one grouping *S. brasiliensis* and another with ((*S. audubonii*, *S. nuttallii*) (*S. aquaticus*, *S. palustris*)). On the other hand, the consensus BY topology showed *S. brasiliensis* as the most basal offshoot with respect to all other species (Figure 4).

Within *S. brasiliensis*, specimens from the Atlantic Forest of eastern Rio de Janeiro state (Itaboraí, Magé and Petrópolis municipalities), “EastRJ”

in the following, diverged as a basal lineage respective to specimens from the northern Cerrado in Tocantins, “TO” in the following, and the Northern Atlantic Forest in Paraíba, “PB” in the following, while specimens from the Central Cerrado transition to the Atlantic Forest of Minas Gerais, Goiás and western Rio de Janeiro, “GO/MG/westRJ” in the following, were more derived, grouping in a well-supported clade (aLRT = 0.99 / pp = 1).

Median-joining of *S. brasiliensis* haplotypes showed several median vectors and nucleotide substitutions between haplotypes belonging to three different groups, (a) the northern

**Table 1.** Karyologic data for *Sylvilagus*. Source: ts= this study, 1= Guereña-Gándara *et al.* (1983), 2= Robinson *et al.* (1984), 3= Ruedas and Salazar-Bravo (2007), 4= Robinson *et al.* (1983), 5= Ruedas and Elder (1994), 6= Langguth and Sousa (2003), 7= Lorenzo and Cervantes (1995), 8= Palmer and Armstrong (1967), 9= Worthington and Sutton (1966), 10= Schroder and Van der Loo (1979), 11= Lorenzo *et al.* (1993), 12= Holden and Eabry (1970), 13= Holden and Eabry (1970; Lorenzo *et al.* (1999-2000), 14= Diersing and Wilson (1980), 15= Ruedas *et al.* (1989), 16= Worthington (1970), 17= Cervantes *et al.* (1996), 18= Holden and Eabry (1970), 19= Wilson (1981).

Taxon	2n	FNa	Locality	Source
<i>S. brasiliensis truei</i>	36	68	Mexico: Puebla, Cuetzalan	1
<i>S. palustris</i>	38	68	USA: NC, Halifax County	2
<i>S. gabbi</i>	38	72	Panama: Peninsula del Azuero, Estancia La Catalina	3
<i>S. aquaticus</i>	38	72	USA: LA, Cameron Parish	4,5
<i>S. brasiliensis minensis</i>	40	68	Brazil: Minas Gerais, Pedrinópolis (40 Km SE of Rio Jordão)	6
<i>S. brasiliensis tapetillus</i>	40	72	Brazil: Rio de Janeiro, Magé	TS
<i>S. brasiliensis</i>	40	76	Mexico: Chiapas	7
<i>S. audubonii</i>	42	72	USA: California	9
<i>S. cunicularius</i>	42	72-74	Mexico, D.F., Parres	11
<i>S. floridanus</i>	42		USA	8
<i>S. floridanus</i>	42	74, 80	USA: Connecticut; Mexico, D.F.	12, 13
<i>S. graysoni</i>	42	78	Mexico: Tres Marias Islands	14
<i>S. nuttallii</i>	42	72	USA: California, Mono County	9, 10
<i>S. idahoensis</i>	44	76	USA: California, Mono County	10
<i>S. obscurus</i>	46	84	USA: West Virginia, Maril and Virginia, Garret	4, 15
<i>S. bachmani</i>	48	80	USA: OR, Benton County	16
<i>S. mansuetus</i>	48	80	Mexico: San Jose Island	17
<i>S. transitionalis</i>	52	94	USA: New York, Connecticut, New England and New Hampshire	15, 18, 19

**Table 2.** List of *Sylvilagus brasiliensis* specimens included in this study, their haplotype number (H), field or museum identification number (ID) and Brazilian localities. For acronyms see material and methods.

H	ID	Locality
1	MN43003	GO: Teresina de Goiás
2	CB97, CB227	RJ: Areal; MG: Juiz de Fora
3	CB515, JFV367	MG: Conceição do Rio Verde and Juiz de Fora
4	CB571, MN50555, MN53377	RJ: Petrópolis and Magé
5	LBCE13109	TO: Axixá
6	SGS1743	PB: Cruz de Espírito Santo
7	RBP355	RJ: Itaboraí

Cerrado in “TO”, (b) “GO/MG/WestRJ” grouping with “PB”, and (c) “EastRJ” (Figure 5). The single haplotype from the Northern Cerrado (TO) was more closely related to haplotypes from Atlantic Forest in EastRJ, with two medium vectors between them. This analysis also showed that haplotypes from GO/MG/WestRJ were separated from the haplotype from the Northern Atlantic Forest (PB) by two median vector and few mutations.

The highest K2-p genetic distance between haplotype groups (0.039) occurred between TO and GO/MG/WestRJ, while the lowest distance (0.009) was observed between PB and GO/MG/WestRJ (Table 3). These distance estimates were lower than between all *Sylvilagus* species (9.1 to 15.3).

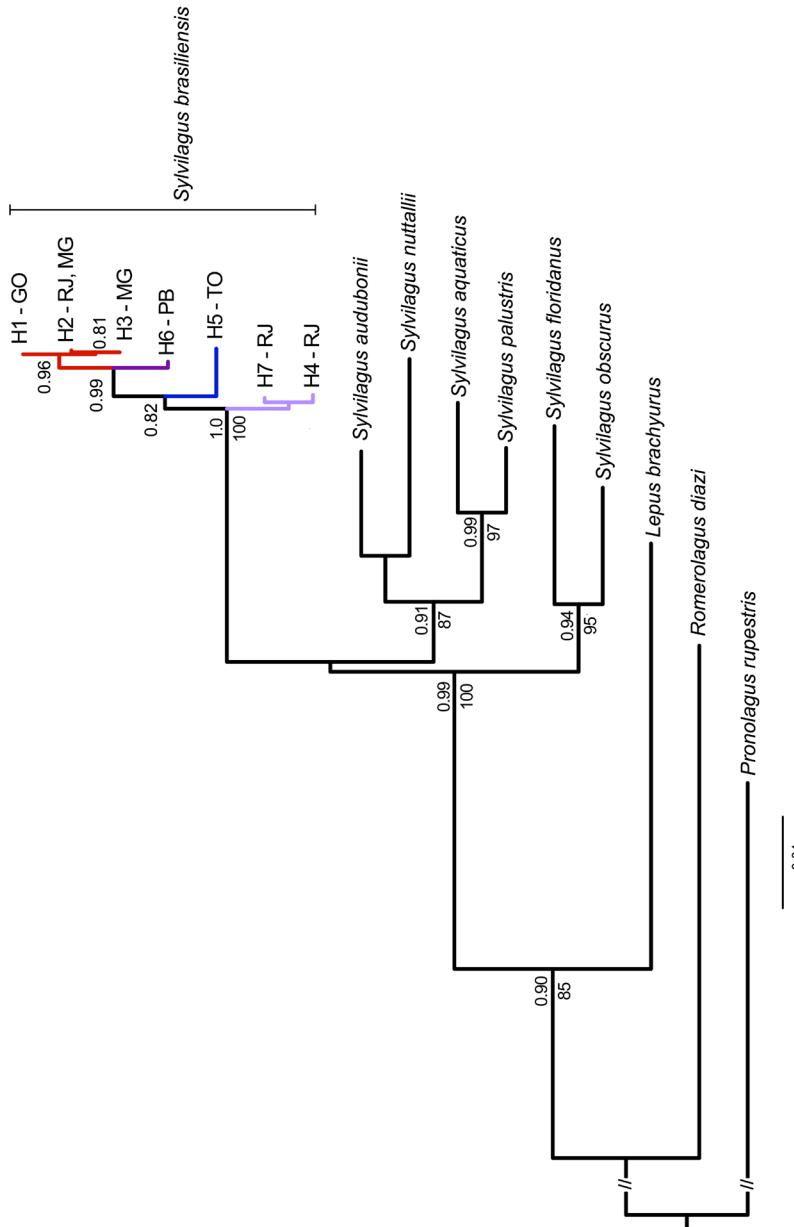
## DISCUSSION

### Karyotypic variation

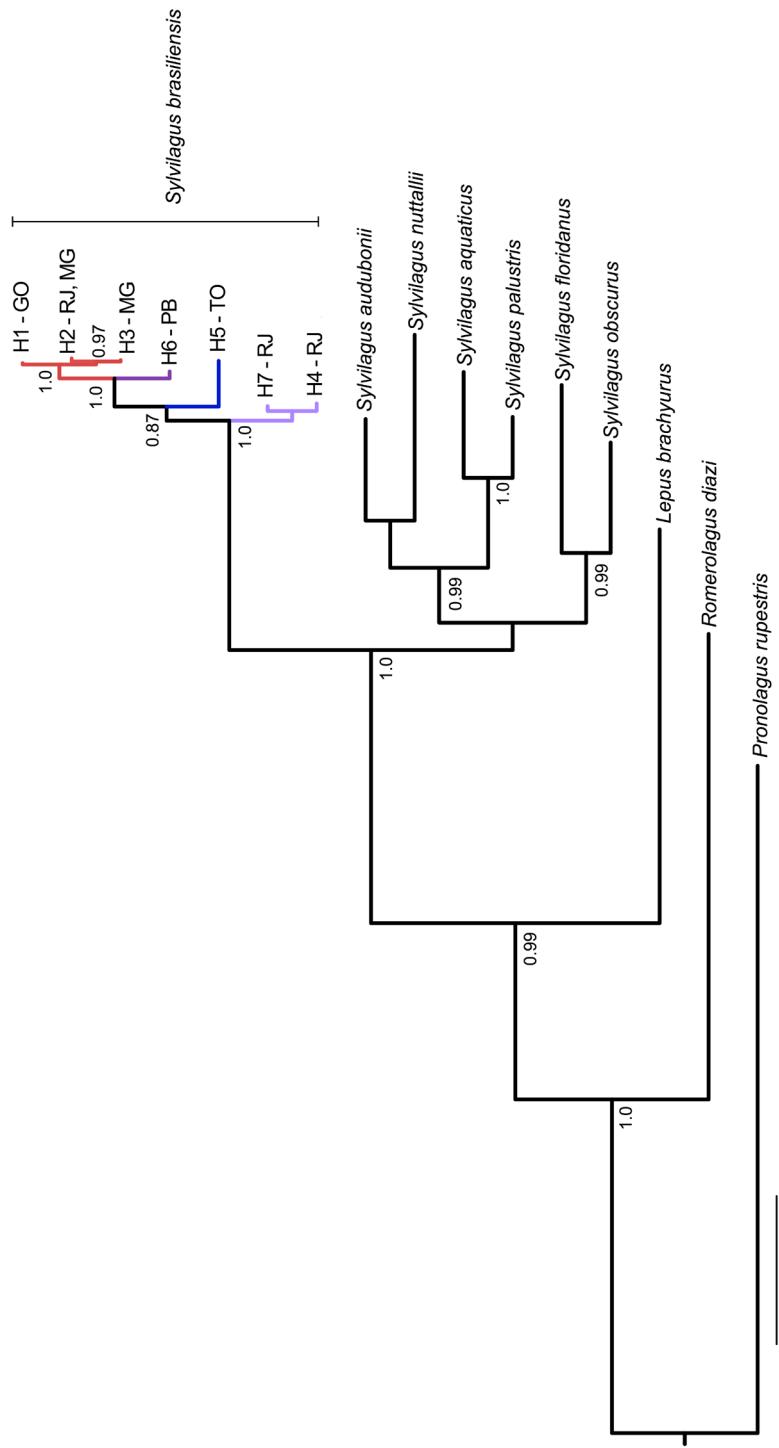
The genus *Sylvilagus* shows a high variation in diploid number ( $2n$ ), from

$2n = 36$  to  $2n = 52$  (Table 1). Only few *S. brasiliensis* have been analyzed, *S. b. minensis* from Pedrinópolis, Minas Gerais state, Brazil with  $2n = 40$  and FNa = 68 (Langguth and Sousa 2003), *S. b. tapetillus* from Rio de Janeiro state, herein reported, with  $2n = 40$  and FNa = 74, *S. brasiliensis* from Puebla, Cuetzalan, Mexico with  $2n = 36$  and FNa = 68 (Guereña-Gándara *et al.* 1983), and specimens from Chiapas, Mexico, with  $2n = 40$  and FNa = 76.

Differences between the fundamental autosome number (FNa) of Brazilian specimens, FNa = 68 in *S. b. minensis* and FNa = 74 in *S. b. tapetillus*, may be explained by three pericentric inversions affecting three autosome pairs which are biarmed in the  $2n = 40$  and FNa = 74 karyotype and acrocentric in the  $2n = 40$  and FNa = 68 karyotype. These inversions have been apparently fixed because heterozygotes (specimens with FN = 73, 72, 71, 70 or 69), were neither found in the present study nor reported by other authors. Although karyotypes did not appear to be geographically



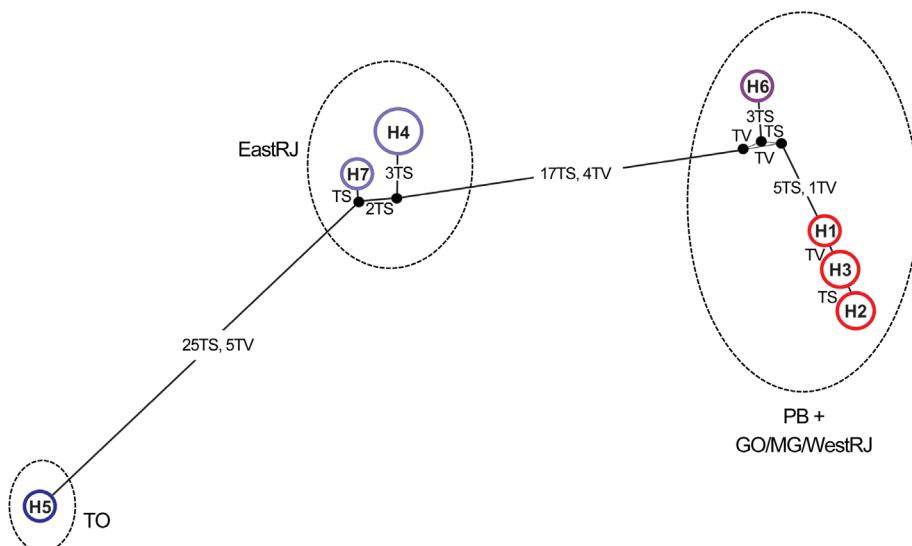
**Figure 3.** Maximum likelihood (ML) topology for *Sylvilagus*, numbers above nodes are aLRT values above 0.7, and below nodes bootstrap values above 70, ML bootstrap consensus tree did not recover the same taxon relationships for *S. brasiliensis* specimens so no support values are shown. Letters on terminals represent states of origin of samples, PB= Paraíba, GO= Goiás, TO= Tocantins, MG= Minas Gerais, RJ= Rio de Janeiro. Vertical lines identify lineages.



**Figure 4.** Bayesian analysis (BY) topology for *Sylvilagus*, only posterior probability  $>0.80$  are shown. Letters on terminals represent states of origin of samples, PB= Paraíba, GO= Goiás, TO= Tocantins, MG= Minas Gerais, RJ= Rio de Janeiro. Vertical lines identify lineages.

**Table 3.** Genetic distance estimates between *Sylvilagus* specimens. For haplotypes (H) identification see Table 1 Shaded areas indicate differences between *S. tapetillus* and *S. brasiliensis*.

	Taxon (H)	1	2	3	4	5	6	7	8	9	10	11	12
1	<i>S. brasiliensis</i> (H1)												
2	<i>S. brasiliensis</i> (H2)	.002											
3	<i>S. brasiliensis</i> (H3)	.001											
4	<i>S. brasiliensis</i> (H5)	.037	.039										
5	<i>S. brasiliensis</i> (H6)	.009	.011	.010									
6	<i>S. tapetillus</i> (H4)	.026	.028	.027	.032								
7	<i>S. tapetillus</i> (H7)	.028	.030	.029	.028	.025							
8	<i>Sylvilagus obscurus</i>	.131	.133	.132	.140	.129	.122						
9	<i>Sylvilagus palustris</i>	.137	.137	.138	.149	.136	.136	.136					
10	<i>Sylvilagus floridanus</i>	.145	.145	.146	.161	.145	.147	.146	.102				
11	<i>Sylvilagus aquaticus</i>	.147	.147	.149	.163	.149	.147	.149	.124	.065			
12	<i>Sylvilagus audubonii</i>	.149	.149	.151	.158	.151	.152	.155	.122	.091	.140		
13	<i>Sylvilagus nuttallii</i>	.150	.150	.152	.158	.153	.148	.151	.122	.124	.136	.128	.103



**Figure 5.** Median-joining network of *Sylvilagus brasiliensis*. Circle sizes correspond to number of individuals carrying a given haplotype. Dotted circles indicate clades shown in the cladogram of figures 3 and 4. Black points represent median vectors. TV= transversion, TS=transition.

structured, differences between *S. b. tapetillus* and *S. b. minensis* were strongly indicative of two evolutionary lineages. Even when chromosome rearrangements (*e.g.*, inversions) might show limited effects on hybrid fitness, they might reduce gene flow by suppressing recombination due to mechanical pairing problems and will facilitate speciation by extending the effects of linked, isolation genes rather than by reducing fitness (Reiseberg 2001, Kirkpatrick 2010).

#### Phylogenetic relationships

Conventional phylogenetic reconstructions based on single evolutionary markers have failed to recover robust phylogenies (*e.g.*, Irwin

*et al.* 1991, Matthee *et al.* 2004), while supermatrix approaches are more informative for constructing more reliable trees (*e.g.*, Cognato and Vogler 2001, Murphy *et al.* 2002). However, our phylogenetic reconstruction was similar to the one provided by a supermatrix comprising five nuclear introns and two mitochondrial genes (Robison and Matthee 2005), and to a previous report of non-Brazilian *Sylvilagus* based on 12S rDNA data (Halanych and Robison 1997).

ML and BY analyses (Figures 3 and 4) showed the monophyly of *Sylvilagus* and the Brazilian forms of this genus. These latter comprised three evolutionary lineages, (1) one grouping haplotypes from PB and GO/MG/

WestRJ (west of Serra do Mar mountain chain), (2) another one from TO, and (3) a third one grouping haplotypes from EastRJ, east of the Serra do Mar mountain chain. The low Kimura-2p distance estimates between these lineages were indicative of their recent separation. The discordance between geographic and genetic proximity was a strong indication that divergence between geographically close lineages, from the EastrJ and the GO/MG/WestRJ belong to different evolutionary lineages while haplotypes from distant regions, like PB and GO/MG/WestRJ were more closely related.

At the population level, networks are more appropriate than traditional phylogenetic reconstructions because they consider ancestral nodes, multifurcations and reticulations (Crandall and Templeton 1993, Posada and Crandall 2001). This is because lack of phylogenetic resolution with strictly dichotomous trees (traditional methods) when a low level of genetic diversity is expected between populations (Morrison 2005, Woolley *et al.* 2008).

Median-joining network (Figure 5) partially confirmed ML and BY topologies (Figures 3 and 4), showing three lineages. The TO lineage (TO) was more closely related to the EastRJ lineage that is closely related to the Northern Atlantic Forest (PB) and the GO/MG/WestRJ. Median vectors separated haplotypes between lineages, indicating that some extant haplotypes have not been identified or, alternatively, lack of intermediate haplotypes (Bandelt *et al.* 1999). Despite the close geographic

distance between the different localities of EastRJ (21.17 km), haplotypes were separated by two median vectors, indicating a high level of genetic diversity. On the other hand, some haplotypes from GO/MG/WestRJ were not separated by median vectors despite the large distance between localities (1,031.21 km).

The last revision of South American tapitis recognized 23 subspecies of *S. brasiliensis* (Hershkovitz 1950), based, exclusively, on pelage coloration. Subsequently, Ruedas and Salazar-Bravo (2007), based on morphologic characters karyotypic data, raised the taxonomic status of *Sylvilagus brasiliensis gabbi* to the full species level (*Sylvilagus gabbi*).

The following subspecies were identified by Hershkovitz (1950) in the study area.

- (1) *S. brasiliensis brasiliensis* (Linnaeus, 1758), described in 3 localities, two in Pernambuco (Recife and Poçâo) and one in Bahia state (Lamarão). The type locality was later restricted to the state of Pernambuco by Thomas (1911:146). Feijó and Langguth (2013) provided new records, from Paraíba to Sergipe states.
- (2) *S. b. minensis* Thomas, 1919, from Rio Jordão and Rio das Velhas, in Minas Gerais state. Type locality is Rio Jordão, southwest of Minas Gerais state.
- (3) *S. b. tapetillus* (Thomas, 1913), from Porto Real and Teresópolis, in Rio de Janeiro state, São Sebastião and São Paulo in São Paulo state, and Passo Fundo in Rio Grande

- do Sul state. Type locality is Porto Real, Rio Paraíba do Sul.
- (4) *S. b. paraguensis* (Thomas, 1901), from Santa Ana de Chapada, Caiçara in Mato Grosso, Corumbá, Salobra, Piraputanga in Mato Grosso do Sul state. Type locality is Sapucay, east of Asunción, Paraguay.

Data herein reported showed three lineages within Brazilian population of *Sylvilagus*: (1) EastRJ in Atlantic Forest, (2) TO in Northern Cerrado and (3) PB in Northern Atlantic Forest plus GO/MG/westRJ in the Central Cerrado and transition to Atlantic Forest. The locality of the Northern Atlantic Forest specimens was very close to the *S. b. brasiliensis* type locality in Pernambuco state, while specimens from the Atlantic Forest of Rio de Janeiro were captured near (131 and 151 km) Porto Real, the type locality of *S. b. tapetillus*. These localities in the Atlantic Forest of Rio de Janeiro are separated from all others by the Serra do Mar mountain chain. This scenario suggested a revision of the taxonomic status of this subspecies raising it to the species level (*Sylvilagus tapetillus*). This, however, must be reassessed by morphologic studies.

Although the genetic distance between *tapetillus* and *brasiliensis* was lower than between any pair of *Sylvilagus* species, cytochrome *b* data and karyotypic attributes supported their species status. Furthermore, the *S. tapetillus* karyotype differed from the karyotypes of *minensis* from the other side of the Serra do Mar mountain chain. The *Sylvilagus brasiliensis* populations are strongly structured, indicating

that further analyses are necessary for clarifying the taxonomic status of each lineage.

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