Short Note

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The karyotype of *Trinomys paratus* (Rodentia: Echimyidae) with comments about its phylogenetic relationship

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Abstract: The spiny rat *Trinomys*, with ten endemic species in eastern Brazil, has a complex taxonomy. We carried out a revision of the karyotypes of *Trinomys*, described for the first time the karyotype of *Trinomys paratus* and performed the first phylogenetic analysis including all *Trinomys* species based on the mitochondrial cytochrome *b* gene. The *T. paratus* karyotype showed diploid number of 58 and fundamental autosomal number of 112. Diploid and fundamental autosomal numbers (FNa), and chromosomes' morphology, are similar to those described for *Trinomys eliasi*. *T. paratus* appears as sister taxa of *T. eliasi*; in turn, this clade was recovered as the sister group of *Trinomys setosus*, as previously reported, confirming that sister species of *Trinomys* has conserved karyotypes, and suggesting that karyological evolution in this genus could be slower than species differentiation.

Keywords: C-band; morphology; phylogeny; spiny rat; *Trinomys paratus*.

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Trinomys Thomas, 1921 is one of the most taxonomically complex genera within the suborder Hystricomorpha, with ten currently recognized species distributed in eastern Brazil (Moojen 1948, Pessôa et al. 2015). Most species are morphologically similar (e.g. Dalapicolla and Leite 2015), and several have been synonymized or placed as subspecies in different taxonomic arrangements due to the complexity of morphological characters (e.g. Moojen 1948, Pessôa et al. 1992, 2015, Pessôa and Reis 1993, Lara et al. 1996, Lara and Patton 2000). Nevertheless, phylogenetic analyses clearly demonstrate that the genus exhibits independent evolutionary lineages (Lara and Patton 2000, Tavares et al. 2015, 2016), and the current taxonomic arrangement recognizes 13 taxa in ten species (Pessôa et al. 2015).

The karyotypes of all *Trinomys* species have been described, with the exception of *Trinomys paratus* (Moojen 1948) and *Trinomys mirapitanga* (Lara, Patton and Hingst-Zaher 2002). The basic diploid number (2n) in species of the genus ranges from 54 in *Trinomys yonenagae* (Rocha 1996) to 60 in *Trinomys iheringi* (Thomas 1911), *Trinomys dimidiatus* (Günter 1876), and *Trinomys albispinus* (Geoffroy 1838), while fundamental autosomal number (FNa) varies from 104 to 116 (Yonenaga-Yassuda et al. 1985, Leal- Mesquita et al. 1992, Corrêa et al. 2005, Pessôa et al. 2005, Souza et al. 2006). Sister species, in general, have very similar karyotypes (Souza et al. 2006) and some related species, such as *T. dimidiatus* and *T. iheringi*, share the same basic karyotype, although the former species also displays supernumerary chromosomes (Yonenaga-Yassuda et al. 1985).

Trinomys paratus has a very restricted distribution in Southeastern Atlantic Forest, in the states of Espírito Santo and Minas Gerais. Its type locality is Capela de São Braz, Santa Teresa municipality in Espírito Santo state, where it occurs in sympatry with *Trinomys gratiosus* (Moojen 1948) (Tavares and Pessôa 2010, Dalapicolla and Leite 2015, Pessôa et al. 2015). The close relationship of *T. paratus* with *Trinomys eliasi* (Pessôa and Reis 1993) is well established (Lara and Patton 2000, Tavares et al. 2015, 2016), but no single paper has presented a hypothesis of

phylogenetic relationships including all species of this genus.

The present study describes the karyotype of *Trinomys paratus* from Espírito Santo state, evaluate the genetic variation within this species, and analyze its phylogenetic position within the genus *Trinomys*, based on mitochondrial gene cytochrome *b*. This study also presents the first phylogenetic analysis including all recognized species of *Trinomys*.

The five *Trinomys paratus* specimens studied in this paper were collected in June 2012 in Guarapari municipality, Espírito Santo state, in two localities: (1) Buenos Aires (20°34′44.5″ S/40°32′16.8″ W) in peridomicile habitat (female LBCE17652, male LBCE17662), and (2) Baia Nova (20°31′15.2″ S/40°3418.2″ W) in secondary vegetation (females LBCE17654 and LBCE17658, male LBCE17653). Specimens were captured with Tomahawk (four specimens) and Sherman (one specimen) live traps, and voucher material was deposited in the mammals' collection of Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil (LBCE). One female specimen was pregnant with three embryos, each of them with 14 mm of total length suggesting that this species is reproductive in winter (June).

Morphological identification of *Trinomys paratus* was based on characters described by Dalapicolla and Leite (2015) and Pessôa et al. (2015): mid-dorsal aristiforms hairs dark and wide; tail much longer than head and body length (approx. 120%) with a developed hairy brush in the tail tip; incisive foramen oval with complete septum; molariforms with four lophs (Figure 1).

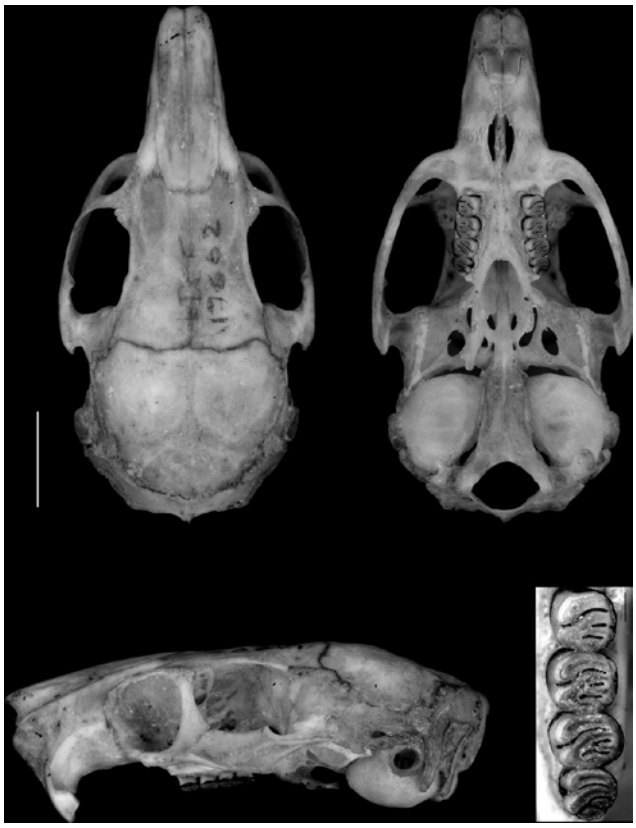

Figure 1: Dorsal, ventral and lateral view of the skull and superior molar row of *Trinomys paratus* (LBCE17662) from Guarapari, Espírito Santo state (scale bar = 1 cm).

Chromosome preparations were obtained from two specimens (LBCE17653 and LBCE17654) following short-term bone marrow cultures (Andrade and Bonvicino 2003). Conventional staining with Giemsa 5% was used to observe chromosome morphology, diploid (2n) and fundamental autosomal numbers (FNa). Chromosomes were ordered according to morphology and decreasing size. The constitutive heterochromatin distribution pattern (C-bands) was revealed using the barium hydroxide method (Sumner 1972).

DNA was isolated from tissue samples preserved in 100% ethanol using phenol chloroform extraction protocol (Sambrook et al. 1989). Nucleic acid concentrations were quantified using a NanoDrop spectrophotometer. Polymerase chain reaction (PCR) was carried out on DNA extractions to amplify the complete cytochrome *b* (*mt-Cytb*) sequence (1140 bp) in four samples with primers L14724 (Irwin et al. 1991) and CIT-REV (Anderson and Yates 2000). Each PCR had a reaction volume of 25 µl and contained 1.0 µl of DNA template, 2.5 µl 10× reaction buffer, 2.0 µl of 50 mM $MgCl_2$, 0.5 µl of 25 µM premixed deoxynucleotide triphosphates (dNTPs), 0.2 µl of 5 U/µl PlatinumTaq DNA Polymerase (Invitrogen, Life Technologies, São Paulo, Brazil), 0.5 µl of each 10 µM primer and 18.3 µl of double-distilled H_2O (dH_2O). Touchdown PCRs conditions followed: a pre-denaturation step at 94°C (2 min), five cycles of denaturation at 94°C (45 s), annealing at 42°C with a 0.4°C decrease per cycle (45 s), and extension at 72°C (45 s), followed by five cycles of denaturation at 94°C (45 s), annealing at 40°C with a 0.4°C decrease per cycle (45 s), and extension at 72°C (45 s), and 25 cycles of denaturation at 94°C (45 s), annealing at 38°C (45 s) and extension at 72°C (45 s) and final extension at 72°C (3 min).

Amplicons were purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, São Paulo, Brazil) and labeled with primers L14724, CIT-REV, CB-In1 and CB-In2 (Cassens et al. 2000). Sequencing was carried out in an ABI PrismTM 3730 automatic DNA platform. Electropherograms were manually checked using CHROMAS PRO 1.41 (Technelysium Pty Ltd., South Brisbane, Australia). Sequences were aligned manually with MEGA 6 (Tamura

et al. 2013). Fifteen additional *Trinomys* sequences previously published in GenBank were included (Lara and Patton 2000, Tavares et al. 2015): *Trinomys albispinus* (accession number KM014008), *Trinomys dimidiatus* (U35168 and U35170), *Trinomys eliasi* (U35166 and KJ707247), *Trinomys iheringi* (U35171 and EU44664), *Trinomys gratiosus* (KJ707248), *Trinomys mirapitanga* (U35173), *Trinomys moojeni* (Pessôa et al. 1992) (KF562097), *Trinomys paratus* (U35165 and KF562094), *Trinomys setosus* (Desmarest 1817) (KF562095 and KF562096) and *Trinomys yonenagae* (U35172). Sequences of *Proechimys cuvieri* (Petter, 1978) (AJ251403), *Clyomys laticeps* (Thomas, 1909) (AF422918), and *Euryzygomatomys spinosus* (G. Fischer, 1814) (EU54667) were used as outgroups.

Maximum likelihood (ML) reconstructions were carried out with RAXML 7.2.8 (Stamatakis 2006) using the GTR + G + I model selected using MODELGENERATOR 0.85 (Keane et al. 2006) using the AKAIKE criterion corrected for complexity (AIC2; Posada and Crandall 2001). Branch support was calculated using bootstrap applying 1000 replicates using the GTR + CAT model.

Karyotypic analyses of two *Trinomys paratus* specimens from Guarapari revealed 2n = 58 chromosomes and FNa = 112, with 27 pairs of biarmed autosomes varying in size from large to small, and another small pair with a dubious morphology, and a secondary constriction in the long arm of a median size autosome pair (Figure 2A). The X chromosome is a large submetacentric, intermediate in size between pairs 2 and 3 and the Y chromosome is a small biarmed chromosome. C-banded karyotype showed conspicuous pericentromeric heterochromatic blocks in 24 pairs of autosomes, a band in the terminal region of the long arm of chromosome 7, and an interstitial band in the long arm and a telomeric band in the short arm of the X chromosome. The Y chromosome is partially heterochromatic (Figure 2B).

Trinomys paratus share the same diploid and autosome fundamental number with those described to *Trinomys eliasi* with 2n = 58 and FNa = 112 (Pessôa et al. 2005). The X chromosome of *T. paratus* is clearly a submetacentric, the third greatest chromosome of the complement, whereas the X chromosome of *T. eliasi*, as reported by Pessôa et al. (2005), is the second largest chromosome of the complement. However, the *T. eliasi* karyotype described by Pessôa et al. (2005) apparently has a mismatch, as one of the chromosomes of pair n.2 looks like to be in fact the X chromosome, whereas the chromosome identified as X chromosome is one of the chromosomes from pair n.2. If this hypothesis is confirmed based on G-banded karyotypes, both karyotypes would be very similar in basic morphology, including the X chromosome.

Four haplotypes were identified among the seven *mt-Cytb* (1140 bp) sequences of *Trinomys paratus* (Table 1): the two specimens captured in the peridomicile in Guarapari shared one haplotype, while the three specimens collected in the secondary vegetation in the same locality shared a second haplotype; the two GenBank sequences correspond to the other two haplotypes. Genetic distance among haplotypes, estimated with Kimura's 2-parameter method, varied from 0.004 to 0.011, with mean equal 0.0075.

ML analysis recovered a fully resolved tree (Figure 2C) showing *Trinomys* as monophyletic and with most clades displaying high nodal support (bootstrap > 95%); one notable exception is the clade including all *Trinomys* species, which received medium support (bootstrap = 75%). Three basal lineages are identified: (I) a clade containing (*Trinomys moojeni, Trinomys gratiosus*) as sister group to [*Trinomys mirapitanga* (*Trinomys dimidiatus, Trinomys iheringi*)]; (II) a clade containing (*Trinomys paratus, Trinomys eliasi*) as sister group to (*Trinomys yonenagae, Trinomys setosus*); and (III) a single species lineage containing *Trinomys albispinus*. The two latter lineages are recovered as sister groups, but with very low support (bootstrap < 50%).

This is the first phylogenetic analysis encompassing all the currently recognized species of *Trinomys*. The close relationship between *Trinomys paratus* and *Trinomys eliasi*, and the recovery of this clade as sister group to *Trinomys setosus*, are congruent with previous studies (Figure 2; Lara and Patton 2000, Upham and Patterson 2012, Tavares et al. 2015). The similarity between the karyotypes of *T. paratus* and *T. eliasi*, together with the fact that they appear as sister taxa, corroborate previous postulation that these two species diverged recently (Upham and Patterson 2012, Tavares et al. 2015). Further studies encompassing additional markers, however, are necessary to better understand the species relationships and diversity within the genus.

Although *Trinomys paratus* and *Trinomys eliasi* share the same karyotype and are closely related sister species, the two taxa have allopatric distributions; the first is an inhabitant of ombrophilous lower montane Atlantic Forest formations in Espírito Santo and Minas Gerais states, and *T. eliasi* is an inhabitant of Restingas, an open vegetation formations of Atlantic Forest, and of lowland Atlantic forests in Rio de Janeiro state. In turn, *Trinomys gratiosus*, which is sympatric with *T. paratus*, has a different chromosome complement (2n = 56 and FNa = 108; Zanchin 1988, Paresque et al. 2004) and is phylogenetically distant related to *T. paratus*. Other closely related *Trinomys* species, such as *Trinomys dimidiatus* and

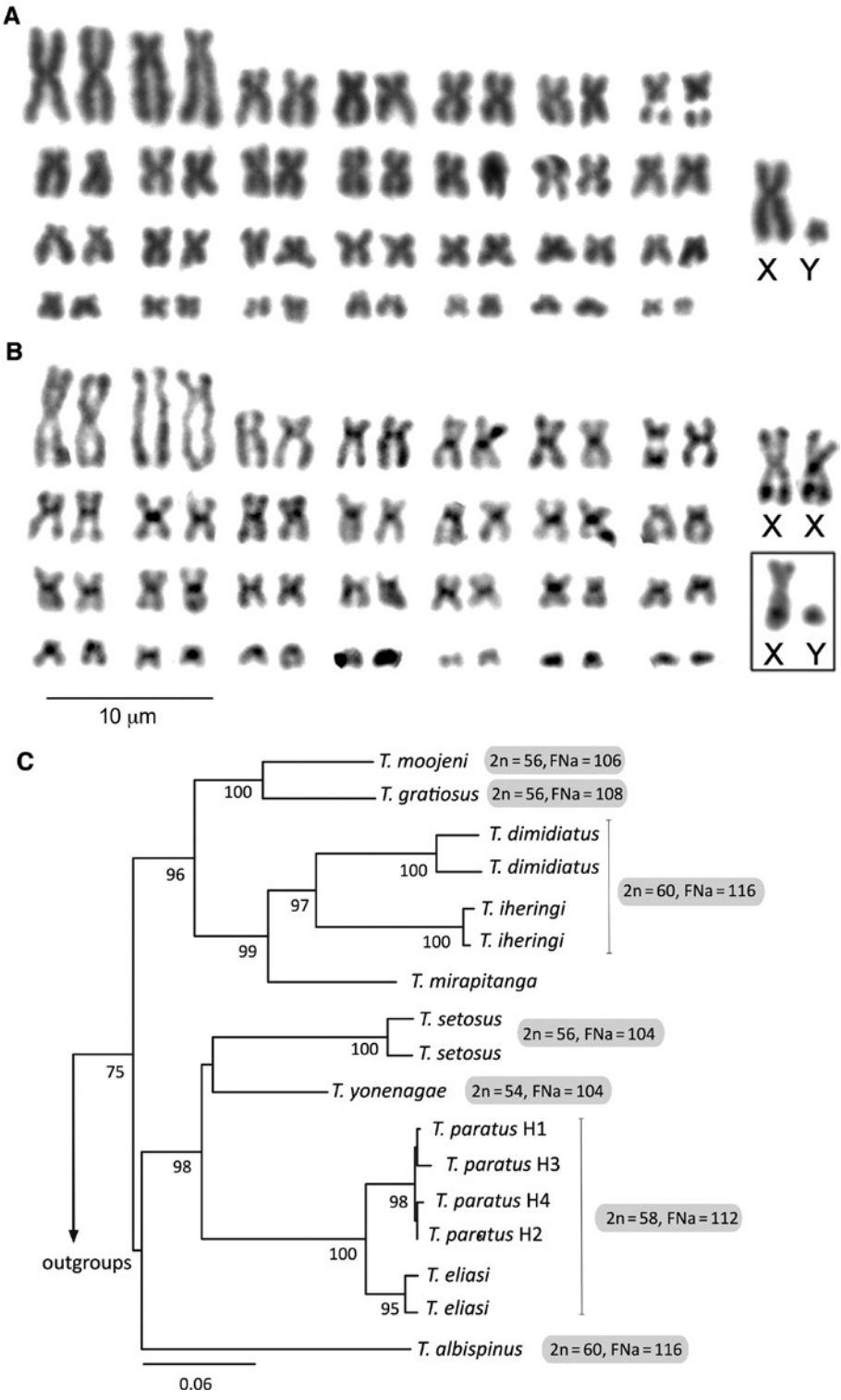

Figure 2: Karyotype and phylogeny of *Trinomys paratus* with 2n = 58 and FNa = 112.
(A) Giemsa-stained karyotype of male LBCE17653; (B) C-banded karyotype of female LBCE17654, with male LBCE17653 sex chromosomes in evidence; (C) maximum likelihood phylogeny based on *mt-Cytb* of *Trinomys*; numbers close to branches are bootstrap values.

Trinomys iheringi, also share the same karyotype (2n = 60 and FNa = 116; Yonenaga-Yassuda et al. 1985, Pessôa et al. 2005, see also Figure 2). The closely related species *T. gratiosus* (2n = 56 and FNa = 108) and *Trinomys moojeni* (2n = 56 and FNa = 106) also share similar karyotypes, with the same 2n and different FNa due to a probable pericentric

Table 1: List of *Trinomys* specimens from Brazilian Espírito Santo state (ES) included in this study with field voucher number, haplotype number (H), GenBank number, diploid and fundamental numbers (2n/FNa), locality and reference.

Taxon	Voucher	H	GenBank number	2n/FNa	Locality	Reference
Trinomys paratus	LBCE17652	1	KY553151	N/A	ES: Guarapari	Present study
Trinomys paratus	LBCE17653	2	KY553152	58/112	ES: Guarapari	Present study
Trinomys paratus	LBCE17654	2	KY553153	58/112	ES: Guarapari	Present study
Trinomys paratus	LBCE17658	2	KY553154	N/A	ES: Guarapari	Present study
Trinomys paratus	LBCE17662	1	KY553155	N/A	ES: Guarapari	Present study
Trinomys paratus	YL34	3	U35165	N/A	ES: Aracruz	Lara et al. 1996
Trinomys paratus	MBML3033	4	KF562094	N/A	ES: Ibiraçu	Tavares et al. 2015

inversion in a small sized pair. The other two closely related species, *Trinomys setosus* (2n = 56 and FNa = 104) and *Trinomys yonenagae* (2n = 54 and FNa = 104), share the same FNa and differ in 2n, probably due to a centric fusion.

These findings corroborate previous hypothesis that sister species of *Trinomys* share similar karyotypes (Souza et al. 2006), and suggests a karyologic evolution slower than species differentiation. This pattern was already detected in other echimyid rodents such as *Thrichomys*, where different and allopatric evolutionary lineages share the same chromosome complement (Nascimento et al. 2013).

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