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Integrative lineage delimitation in rodents of the *Ctenomys* Corrientes group

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Abstract: The tuco-tucos rodents (genus *Ctenomys*) of the Corrientes group comprise several populations that inhabit the vast area under the influence of the Iberá wetland. Lineage delimitation within the recently diverged Corrientes group is a challenging task as morphological differentiation is not conspicuous between populations. However, delimitation is crucial for evolutionary studies and conservation issues. In this study, we performed a phylogenetic analysis including cytochrome b (cyt-b) sequences from taxa that had never been studied in a comprehensive context. We integrated previously published chromosomal studies, mitochondrial phylogenies and simple sequence repeat (SSR) variability analyses, and applied a delimitation criterion over the basis of chromosomal incompatibilities and genetic exclusivity. Under this integrative approach seven independently evolving lineages were delimited in the Corrientes group: *Ctenomys roigi*, which conserves its former definition, *Ctenomys dorbignyi* and *Ctenomys perrensi* complex which were redefined, Sarandicito which includes the population of Paraje Sarandicito and probably a group of nearby poorly studied populations, and Iberá i, ii and iii distributed at both sides of the Iberá wetland. We discuss future perspectives to evaluate the proposed lineages and conservation issues concerning these tuco-tucos.

Keywords: Corrientes group; *Ctenomys*; Iberá wetland; integrative taxonomy; lineage delimitation.

Introduction

Tuco-tucos, South American rodents of the genus *Ctenomys* (Blainville, 1826), are the most speciose group

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of extant subterranean rodents counting more than 60 species (Woods and Kilpatrick 2005, Bidau 2015). These rodents have undergone enormous levels of chromosomal evolution with diploid numbers varying from $2n=10$ to $2n=70$, mainly due to Robertsonian translocations, but also heterochromatin additions/losses, pericentric inversions and/or tandem fusions had been postulated (Reig and Kiblicky 1969, Cook et al. 1990, Reig et al. 1990, Masarini et al. 1991, Freitas 2006, Novello and Villar 2006, Caraballo et al. 2015). Their subterranean lifestyle has been associated to high population subdivision and low vagility which may have promoted the fixation of chromosomal novelties by genetic drift (Reig and Kiblicky 1969, Ortells 1995). Within the genus at least eight species groups were defined, although their relationships have not been completely solved, as several groups fall into polytomies (Parada et al. 2011) which may reflect the explosive nature of the radiation of tuco-tucos (Lessa and Cook 1998, Castillo et al. 2005). One of these clades, the torquatus group comprises species and populations that inhabit the eastern part of *Ctenomys*' distribution: *Ctenomys torquatus* Lichtenstein, 1830 in the grasslands of southern Rio Grande do Sul state, Brazil and northern Uruguay; *Ctenomys ibicuiensis* Freitas et al., 2012 in western Rio Grande do Sul, Brazil; *Ctenomys minutus* Nehring, 1887 and *Ctenomys lami* Freitas, 2001 distributed on the southern Brazilian coastal plains; *Ctenomys pearsoni* Lessa and Langguth 1983 complex distributed along the coastal plains of southern Uruguay; and the Corrientes group, a complex of populations distributed in the homonymous province of Argentina. Recent phylogenies based on mitochondrial markers distinguished four reciprocally monophyletic clades within the torquatus group (Freitas et al. 2012, Caraballo et al. 2016): *C. torquatus*, *C. ibicuiensis*, *C. lami* + *C. minutus* and *C. pearsoni* complex + Corrientes group. In turn, the Corrientes group and the *C. pearsoni* complex are reciprocally monophyletic clades with higher values of intergroup compared to intragroup genetic distances (Caraballo et al. 2016).

The tuco-tucos of the Corrientes group inhabit a vast area under the influence of the Iberá wetland, the second largest in South America, a geological depression covering 13,000 km² of lakes, swamps, marshes, grasslands and forests. Demes of tuco-tucos of the Corrientes group are patchy distributed in sandy soils, predominant in this

area, which are suitable for their digging activity. However, proximity to the wetlands makes these habitats unstable as the system's water-level fluctuates depending on rainfall and surface as well as underground rivers flow. To date, 26 populations have been thoroughly studied in the Corrientes group (Figures 1 and 2), some of which were alleged to belong to three species on the basis of morphological observations and karyotype: *Ctenomys roigi* (Contreras 1988, $2n=48$, FN=80), *Ctenomys dorbignyi* (Contreras and Contreras 1984, $2n=70$, FN=84) and *Ctenomys perrensi* (Thomas 1896, $2n=50$, FN=84), but most of them have an undefined taxonomic status (Contreras et al. 1985, Contreras and Scolaro 1986, Contreras 1988, Ortells et al. 1990). As occurs in most lineages of the torquatus group, the 26 populations of the Corrientes group showed high levels of chromosomal variability, with more than 17 different karyomorphs described (Caraballo et al. 2015 and references therein), which suggests that chromosomal evolution is an ongoing and recurrent process in this group.

Many contributions have been made to the study of the Corrientes group, depicting a remarkable complexity. Morphological analyses (Contreras and Scolaro 1986, Contreras 1988, Ortells 1990), cytogenetic studies (Ortells et al. 1990, García et al. 2000b, Argüelles et al. 2001, Caraballo et al. 2015), chromosome banding (Ortells 1995), satellite DNA dynamics (Caraballo et al. 2010), allozymic distances (Ortells and Barrantes 1994), population genetic studies (Mirol et al. 2010, Gómez Fernández et al. 2012), and mitochondrial phylogenies (Giménez et al. 2002, Caraballo et al. 2012) have been performed. From a morphological perspective, the populations comprising the Corrientes group, even the ones considered to belong to different nominal species showed little or no differentiation (Figure 1, Ortells 1990) and hence are likely to be cryptic ones. This fact may be reflecting selective pressures derived from the tuco-tucos underground lifestyle: the homogeneous subterranean niche may prevent the differentiation via divergent



Figure 1: Tuco-tucos captured at different Correntinean populations.

Sampling localities are listed according to the order of the photographs (beginning from the upper left, in left-right direction, ending in the lower right): Loreto, Loreto (another specimen from the same population as preceding one), Manantiales, Mburucuyá, Pago Alegre, Santa Rosa, Saladas Centro, Saladas Sur, Estancia San Luis and Sarandicito.

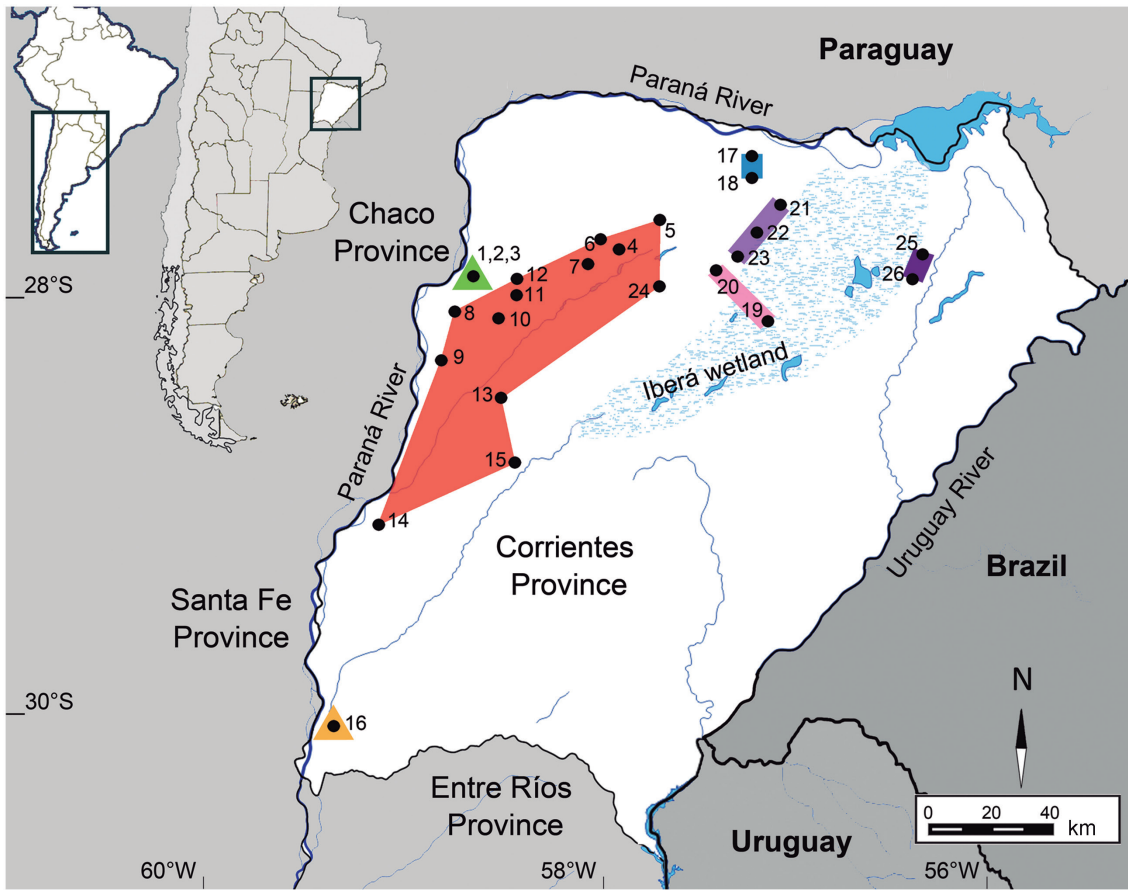


Figure 2: Map showing Corrientinean populations (black dots) studied by cytogenetics, mtDNA phylogeny and SSR clustering. Numbered black dots correspond to the following localities: 1-Arroyo Pehuajó, 2-Costa Mansión, 3-Estancia San Luis, 4-Mburucuyá, 5-Manantiales, 6-Loma Alta, 7-Pago Alegre, 8-Rincón de Ambrosio, 9-Colonia 3 de Abril, 10-Saladas Sur, 11-Saladas Centro, 12-Saladas Norte, 13-San Roque, 14-Goya, 15-Chavarría, 16-Paraje Sarandicito, 17-Mbarigüí, 18-Paraje Angostura, 19-San Alonso, 20-Paraje Caimán, 21-Loreto, 22-Curuzú Laurel, 23-San Miguel, 24-Santa Rosa, 25-Estancia la Tacuarita, 26-Contreras Cué. The extent of occurrence of the seven Corrientinean lineages delimited in this paper was drawn according to the IUCN Red List Categories and Criteria (Anonymous 2012). We used different colors for depicting lineages: *Ctenomys roigi* (green), *C. dorbignyi* (blue), *C. perrensi* complex (red), Sarandicito (orange), Iberá i (pink), Iberá ii (light purple), Iberá iii (violet).

adaptations, more likely to occur in less restricted habitats, e.g. surface rodents. In addition, among-population genetic distances based on allozymic data indicated that the Corrientes group has very low levels of genetic differentiation, comparable to intraspecific distances in other species of *Ctenomys* (Ortells and Barrantes 1994). Taken together, the low differentiation observed at both morphological and allozymic levels in the Corrientes group are congruent with a recent divergence scenario. Indeed, the age of the Corrientes group has been estimated at 0.77 Million years (0.51–1.02 Million years) in the context of a multi-calibrated phylogeny of the genus based on complete cytochrome b (cyt-b) sequences (Caraballo and Rossi, unpublished). This recent divergence explains not only the observed little differentiation but also the existing discordance among character

sets that may reflect ancestral polymorphisms, random lineage sorting as well as hybridization and introgression.

From an operative point of view, delimiting species is crucial for conserving biodiversity. Underestimation or inflation of the number of species could adversely affect biodiversity conservation and management efforts (Zachos et al. 2013 and references therein). The Mburucuyá National Park and the Iberá Provincial Reserve are two protected areas in Corrientes, which cover 176 km² and 13,000 km², respectively. Populations of tuco-tucos inhabit these territories; species delimitation could serve to decide which stocks should be conserved. From a theoretical point of view, species delimitation implies elaborating refutable hypotheses that can be tested by the addition of new character sources, populations, individuals, and methods, which will contribute to defining the species category itself.

The aim of this work is to delimit independently evolving lineages within the *Ctenomys* Corrientes group adopting an integrative approach under the light of all available sources of evidence. This criterion could be identified with the “integration by cumulation” framework defined by Padial et al. (2010), according to which evidence from all character sets is taken into account as a whole, with no need of total congruence among them to delimit a lineage. Hence, we took into consideration evidence indicating the existence of reproductive barriers (differences in FN), as well as exclusivity in SSR clusters and mitochondrial monophyly. However, chromosomal, SSR clusters and mtDNA data, offer different kinds of information about potential gene flow between populations, and hence can be taken into account with different hierarchy when delimiting lineages. Regarding chromosomes, with exception of heterochromatic arm additions, differences in FN between populations reflect rearrangements known to have strong negative heterotic effects or to promote isolation via recombination suppression (King 1995, Dobigny et al. 2005, Faría and Navarro 2010) and thus are good indicators of lineage independence. Additionally, highly structured populations which will form exclusive SSR clusters or reciprocally monophyletic groups would not be exchanging genes at the present or at the recent past and hence are also good candidates to form independent evolutionary lineages. However, in allopatric taxa, exclusivity could be driven only by genetic drift and could occur without detriment of the potential for gene exchange (Harrison and Larson 2014).

Under this criterion we delimited seven independent lineages within the *Ctenomys* Corrientes group which constitute not only a good working hypothesis but also establish a useful framework for conservation in this group of rodents.

Materials and methods

We ran a *cyt-b* based phylogeny including sequences that had never been subjected to broader sampling studies. We included sequences from the Entrerriean populations of Paso Vera and San Joaquín de Miraflores to test their membership to the Corrientes group. A sequence from the topotype, Costa Mansión (Parada et al. 2011) was also included to test the monophyly of *Ctenomys roigi*. Additionally we retrieved from GenBank nine octodontine sequences and 129 *Ctenomys* sequences, encompassing all sequences included in Parada et al. (2011) and Caraballo et al. (2016) (Supplemental Table 1). The sequences corresponding to Paso Vera and San Joaquín de Miraflores are 426 bp long,

and were obtained by Giménez et al. (2002). Sequences were aligned with CLUSTAL X2 (Larkin et al. 2007). We split the data into two character partitions: the 1st + 2nd codon positions, and the 3rd codon position. Substitution models were estimated for each partition using MrModeltest 2.3 (Nylander 2004). Phylogenetic inferences were performed in BEAST 2.3.0 (Bouckaert et al. 2014), running two separate analyses for 10^7 MCMC generations, sampling every 1.000 generations, with a burnin of 25% and also an extra run was performed sampling from the prior. We used Tracer 1.6.0 (Rambaut et al. 2014) to determine posterior distribution convergence. Log files and trees were combined using LogCombiner v2.3.0, and trees were summarized with the “maximum clade credibility” option using TreeAnnotator 2.3.0 (Bouckaert et al. 2014).

We established an operative criterion which states that two populations are considered members of different lineages if *they differ in their FN or belong to different SSR clusters and are reciprocally monophyletic*. It should be noted that as the exclusivity condition might not be sufficient to reflect evolutionary independence, we decided to consider it only when exclusivity was reached at both the mtDNA phylogeny and SSR clusters.

Results and discussion

Cytochrome b phylogenetic inference

The phylogenetic tree obtained is shown in Figure 3. All parameters reached convergence in both runs, and showed EES values over 500. Sequences from the Entrerriean populations of San Joaquín de Miraflores and Paso Vera do not belong to the Corrientes group, but instead fall into its sister clade, together with the also Entrerriean population of Médanos and the Uruguayan *Ctenomys pearsoni* complex. In turn, the sequence from Costa Mansión, *Ctenomys roigi* topotype, forms a monophyletic group with the ones from Estancia San Luis, in agreement with chromosomal (Caraballo et al. 2015) and SSR analyses (Gómez Fernández et al. 2012).

Lineage delimitation

In this section we will discuss previous classification, integrating congruences and discordances between chromosomes, SSR clustering and mtDNA phylogeny, and apply the lineage delimitation criterion. The main outcome of this analysis is the delimitation of seven independently

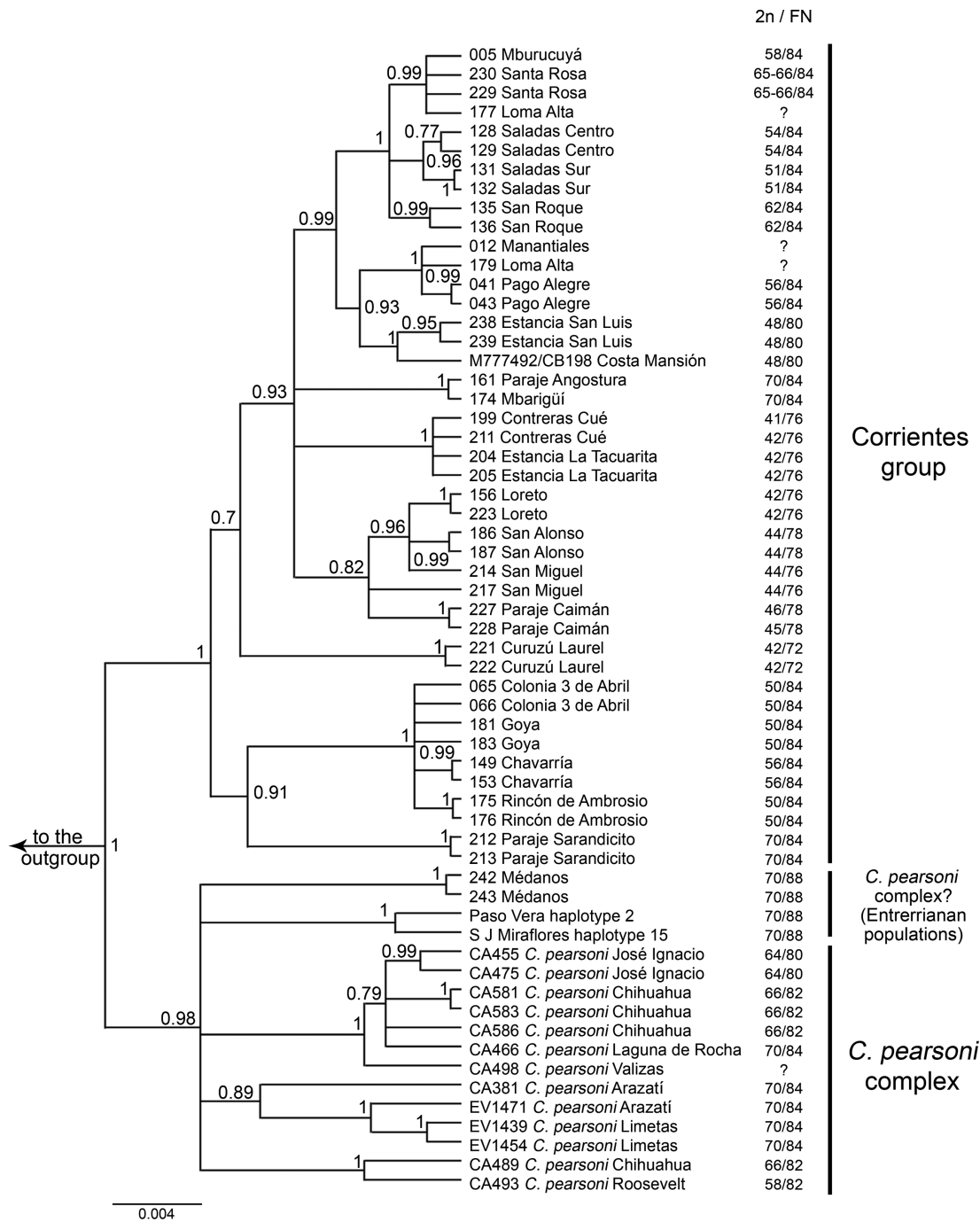


Figure 3: Phylogeny based on the complete cyt-b sequence of 138 Octodontid taxa. Nodes with Bayesian posterior probability value < 0.7 were collapsed into polytomies. Diploid (2n) and fundamental (FN) numbers are shown next to each terminal, based on data published by Argüelles et al. (2001), Tomasco and Lessa (2007), Caraballo et al. (2015) and references therein.

evolving lineages in the *Ctenomys* Corrientes group (Table 1, Figure 2). We decided to hold the species status of those previously described species which remain circumscribed to a particular lineage supported by all sources of evidence integrated in this study, i.e. *Ctenomys dorbignyi* and *Ctenomys roigi*.

Ctenomys roigi

This lineage corresponds to the species firstly described by Contreras (1988) who pointed out its morphological distinctiveness. This species is distributed in an extremely limited area, within less than 100 km², and thus was

Table 1: Detailed grouping of SSR clustering (Mirol et al. 2010, Gómez Fernández et al. 2012), mtDNA phylogenetic clades (Caraballo et al. 2012) as well as diploid and chromosomal fundamental numbers (2n/FN, Caraballo et al. 2015 and references therein) of the 26 studied populations of the *Ctenomys* Corrientes group.

	Locality	Mirol et al. 2010	Gómez Fernández et al. 2012	Hierarchical clustering	mtDNA phylogeny	2n/FN	Lineage
1	Arroyo Pehuajó	III	A		1 ^a	48/80	<i>C. roigi</i>
2	Costa Mansión	III	A		1 ^a	48/80	
3	Estancia San Luis	–	A		1 ^a	48/80	
4	Mburucuyá	VI	B	A + B	1	58/84	<i>C. perrensi</i> complex
5	Manantiales	VI	B		1	?	
6	Loma Alta	–	B		1	?	
7	Pago Alegre	VI	B		1	56/84	
8	Rincón de Ambrosio	V	I		2	50/84	<i>C. perrensi</i> complex
9	Colonia 3 de Abril	V	I		2	50/84	
10	Saladas Sur	V	I	I	1	51/84	
11	Saladas Centro	V	I		1	54/84	
12	Saladas Norte	V	I		1	54–56/84	
13	San Roque	IV	J		1	62/84	<i>C. dorbignyi</i>
14	Goya	IV	J		2	50/84	
15	Chavarría	IV	K	J + K + L	2	56/84	
16	Paraje Sarandicito	I	L		2 ^b	70/84	Sarandicito
17	Mbarigüí	II	C		3	70/84	<i>C. dorbignyi</i>
18	Paraje Angostura	II	C	C	3	70/84	
19	San Alonso	–	F		4	44/78	Iberá i
20	Paraje Caimán	VII	H		4	45–46/78	
21	Loreto	VII	D		4	42/76	Iberá ii
22	Curuzú Laurel	VII	H	D + F + G + H	5	42/76	
23	San Miguel	VII	G		4	44/76	
24	Santa Rosa	IV	G		1	65–66/84	<i>C. perrensi</i> complex
25	Estancia la Tacuarita	VIII	E		6	42/76	Iberá iii
26	Contreras Cué	VIII	E	E	6	41–42/76	

Their ascription to lineages delimited in this paper is shown in the last column. Samples employed by Mirol et al. (2010) were included in Gómez Fernández et al. (2012).

considered as Critically Endangered in the IUCN Red List of Threatened Species (Bidau et al. 2008a). To date, three nearby populations have been ascribed to *Ctenomys roigi*: Arroyo Pehuajó, Costa Mansión and Estancia San Luis, located in the northwest extreme of the Corrientes Province (Figure 2). All three populations share a distinctive karyotype, $2n=48$ FN=80, with a unique FN in the Corrientes group (Ortells et al. 1990, Table 1). *Ctenomys roigi* is also a well supported lineage in terms of genetic exclusivity, since it forms a unique SSR cluster and also results monophyletic in the mtDNA phylogeny (Table 1, Figure 3).

Ctenomys dorbignyi

This species was originally described by Contreras and Contreras (1984) establishing a narrow distribution in

the north of the Corrientes Province. Later, its range was extended, considering four disjunct groups of populations (nucleuses) over the assumption of a common chromosome number $2n=70$ (Contreras and Scolaro 1986). These nucleuses were designated North (Mbarigüí), South (Paraje Sarandicito, Los ángeles, Esquina and Laguna Itá), East (Contreras Cué) and West (Loma Alta and Mburucuyá), and were differentiated over the base of external and cranial morphometry (Contreras and Scolaro 1986). Based on discriminant analyses, these authors stated that each nucleus “behaves as a good taxonomic entity”. Karyotypical information was then published by Ortells et al. (1990) who found that Mbarigüí and Paraje Sarandicito had undistinguishable $2n=70$ FN=84 karyotypes (Table 1). Hence, these two disjunct nucleuses, although they are 350 km away, maintained their ascription to *Ctenomys dorbignyi* and were identified as relicts

of a widespread nearly continuous ancestral stock of the Corrientes group. More recently Argüelles et al. (2001) ascribed to this species a third karyotypically related group of populations from the localities of Paso Vera and San Joaquín de Miraflores, at the Entre Ríos Province. Taking these three nucleuses into consideration *C. dorbignyi* was considered Near Threatened in the IUCN Red List (Bidau et al. 2008b).

Notwithstanding, the Entrerriean populations have a different chromosomal arm number, FN=88 (Argüelles et al. 2001), which indicates that, under the applied delimitation criterion this third nucleus is a separate lineage. In addition, when included into the cyt-b phylogeny, the populations of San Joaquín de Miraflores and Paso Vera fall into the clade containing the *Ctenomys pearsoni* complex (Figure 3), as occurs with the also Entrerriean population of Médanos which also has a FN=88 (García et al. 2000a), confirming these populations are not members of the Corrientes group. The northern (Mbarigüí and Paraje Angostura) and the southern (Paraje Sarandicito) nucleuses split apart in the basal clades of the Corrientes group (Figure 3), and also form unique separate SSR clusters (Table 1, see below). Thus, we propose to restrict *Ctenomys dorbignyi* to the northern populations: Mbarigüí (topotype) and Paraje Angostura. It should be remarked that Contreras et al. (1985) included at least seven additional neighboring populations in the northern nucleus of *C. dorbignyi*. However, no genetic or cytogenetic studies have been carried out in these populations.

Ctenomys perrensi complex

Ctenomys perrensi was initially described by Thomas (1896) based on a sample from Goya, its topotype. Afterwards, this population was ascribed to *Ctenomys torquatus* (Cabrera 1961, Reig et al. 1965) and also to what would be later called *Ctenomys pearsoni* (Languth and Abella 1970). During the decade of 1980 Contreras made a reassessment of the taxonomy and geographic range of Correntinean tuco-tucos, and extended the range for *C. perrensi* covering the central-west region of the province, from the margins of the Paraná River to the west margins of the Iberá wetland (Figure 2). This geographical range redefinition included all Correntinean populations except for the four nucleuses formerly ascribed to *Ctenomys dorbignyi* (Contreras and Sclaro 1986, see above), the group of populations which were later alleged to *Ctenomys roigi* (Contreras 1988), and several unstudied populations between Goya and the former southern nucleus of *C. dorbignyi* (Contreras et al. 1985). However, the species range was further restricted by

Ortells et al. (1990) to the topotype (Goya) and two populations that share the same karyomorph $2n=50$ FN=84: Rincón de Ambrosio and Colonia 3 de Abril (Table 1). Besides, a group of populations formerly attributed to this species was indicated to be probably related to this species based on morphological similarity and the sharing of a common FN=84 (Ortells et al. 1990). These highly polymorphic or polytypic populations, with $2n$ varying from 54 to 62, were: Saladas (and nearby locations), Mburucuyá (and nearby locations), San Roque, Yatayí Calle and M.F. Mantilla. Additionally, Ortells et al. (1990) acknowledged the membership of the population of Curuzú Laurel ($2n=42$, FN=76) to a different lineage since it had a clearly divergent karyotype. They suggested it should be related to *C. torquatus*, a species with similar $2n$ and FN. According to the IUCN Red List, *C. perrensi* is listed as Least Concern (Bidau et al. 2008c), although its range of occupancy does not coincide with the one described by Contreras et al. (1985) or Ortells et al. (1990).

Under an integrative approach we delimit an extended lineage, the *Ctenomys perrensi* complex, encompassing all $2n < 70$ FN=84 forms of the Corrientes group (Table 1). These karyomorphs are the products of Robertsonian rearrangements (Ortells et al. 1990, Lanzone et al. 2007, Caraballo et al. 2015), which not necessarily constitute reproductive barriers. In fact, Lanzone et al. (2007) showed that, in Correntinean tuco-tucos, heterozygotes bearing one or two centric fusion/fission rearrangements depicted normal pairing of homologous chromosomal arms. These results suggest that at least two Robertsonian changes would not act as a barrier to gene flow and can persist as intraspecific polymorphisms or polytypisms. However, heterozygotes for three or more centric fusions/fissions have not been reported in the Corrientes group (Ortells et al. 1990, Lanzone et al. 2007, Caraballo et al. 2015), suggesting that highly Robertsonian-rearranged karyotypes are likely to produce hybrid sterility (Baker and Bickham 1986). Karyomorphs with FN=84 and $2n=50$ to $2n=66$ could form part of a chromosomal cline of neighboring interfertile or semi-interfertile forms, as occurs in the case of the Brazilian species *Ctenomys minutus* and *Ctenomys lami* (Gava and Freitas 2003, Lopes et al. 2013).

To evaluate the *Ctenomys perrensi* complex from an integrative point of view, we should revise coincidences and discordances between SSR clustering and mtDNA phylogeny. The mtDNA phylogeny splits this complex into two clades: clade 2 including the populations of *Ctenomys perrensi sensu* Ortells et al. (1990), Chavarría and Paraje Sarandicito (Table 1) and clade 1, including the remaining populations as well as *Ctenomys roigi* which is, as discussed above, a different monophyletic lineage. The members of

clades 1 and 2 of the *C. perrensi* complex resulted intermixed in SSR clusters (Table 1). With the exception of cluster G, that groups Santa Rosa and San Miguel which are highly divergent populations in terms of their karyotypes and mtDNA identity and hence this grouping is likely to be artificial, all members of the *C. perrensi* complex fall into exclusive SSR clusters (Table 1). Taken together, the mtDNA phylogeny and SSR clusters suggest these are recently diverged populations which share randomly assorted ancestral variants and/or are or have been genetically connected.

We consider Santa Rosa as a member of this complex, not only because it shares the same FN and is included in this group in the mtDNA phylogeny, but also because it falls into an exclusive cluster with all *Ctenomys perrensi* complex populations (San Roque, Chavarría and Goya) in previous SSR clusters (Mirol et al. 2010). In turn, Paraje Sarandicito is not considered part of this lineage and will be discussed in the following item.

Sarandicito

Paraje Sarandicito, located at the southern extreme of the Corrientes group distribution (Figure 2), has been previously ascribed to *Ctenomys dorbignyi* since they share a karyotype $2n=70$ FN=84, but, as discussed above, it resulted a clearly differentiated SSR and mtDNA lineage in relation to the northern nucleus (Table 1). This population shows a secondary affinity to the *Ctenomys perrensi* complex, being basal to the clade 2 in the mtDNA phylogeny and, although it forms an exclusive SSR cluster (L), it is included into a hierarchic cluster together with San Roque, Goya and Chavarría (Table 1). In fact, when members of San Joaquín de Miraflores (Entre Ríos) are included in the analysis, Paraje Sarandicito falls into a SSR cluster with this population (Mirol et al. 2010). However, as stated above, San Joaquín de Miraflores falls within a clade which also includes the *C. pearsoni* complex in the cyt-b based phylogeny (Figure 3) and hence belongs to a different lineage. These results confirm that Paraje Sarandicito is a unique lineage within the Corrientes group. This population is likely to be related to other seven nearby populations that Contreras et al. (1985) attributed to the former southern nucleus of *C. dorbignyi*, but further studies should be carried out in order to determine their membership to this lineage.

Iberá i, Iberá ii and Iberá iii

The populations located within the Iberá wetland have the lowest FN and $2n$ of the Corrientes group. As mentioned,

Curuzú Laurel, the first studied population of this group had been originally considered to be the eastern nucleus of *Ctenomys dorbignyi* (Contreras and Scolaro 1986), but after its karyotypic description it was treated as a separate lineage, related to *Ctenomys torquatus* (Ortells et al. 1990). A total of seven populations have been studied up to now in the proximities to the Iberá wetland: four located at the western margins of the wetland, two in the east and one isolated on a sandy elevation surrounded by waterlogged lowlands located in the center of the wetland (Figure 2). In mtDNA studies (Fernandes et al. 2009, Caraballo et al. 2012) their membership to the Corrientes group was confirmed rejecting its affinity to *C. torquatus*. With the exception of Curuzú Laurel, which falls apart probably due to incomplete lineage sorting, all the Iberanan populations form a monophyletic group (Caraballo et al. 2012, 2016). Within this clade, the eastern populations Estancia la Tacuarita and Contreras Cué form a well-supported clade (Table 1). Similarly, all these populations are grouped into intermixed SSR clusters (Table 1) that group together in a hierarchical clustering (Table 1). The only exceptions are, again, Contreras Cué and Estancia la Tacuarita, which form an exclusive SSR cluster (Table 1).

Two different FN have been described in the Iberanan populations. Paraje Caimán and San Alonso (Figure 2) possess the same FN=78, while they have $2n=45-46$ and $2n=44$, respectively. These karyomorphs are related by Robertsonian rearrangements (Caraballo et al. 2015). The remaining populations have diploid numbers in the range $2n=41-44$ and a constant FN=76, and are also related by centric fusions/fissions (Caraballo et al. 2015). The karyotype $2n=44$ FN=78 is undistinguishable from the $2n=42$ FN=76, with the exception of a small telocentric pair that was probably involved in a tandem fusion with a bivalent pair (Caraballo 2013, Caraballo et al. 2015). Tandem fusions are known to have powerful negative heterotic effects (King 1995). In mammals, heterozygotes' sterility for tandem fusions was found in controlled breeding experiments in rodents of the genera *Otomys* Couvier, 1824 (Pillay and Willan Meester 1992, Pillay et al. 1995, Taylor 2000) and *Taterillus* Thomas, 1910 (Dobigny et al. 2005) as well as in the antelope *Madoqua kirkii* Günther, 1880 (Ryder et al. 1989, Cernohorska et al. 2011). Since they are heterotically deleterious, once occurred, tandem fusions have an all or nothing fate: they reach homozygosis and promote instantaneous reproductive isolation from the non-rearranged stock, or they are lost (Dobigny et al. 2005). Thus, populations with FN=78 are likely to be reproductively isolated from the ones with FN=76, constituting independent evolving lineages. We designated the populations with FN=78, Paraje Caimán and San Alonso, as Iberá i.

Within the group of populations with FN=76, two lineages should be distinguished according to the established delimitation criterion. Estancia la Tacuarita and Contreras Cué accomplish exclusivity at both SSR clusters and mtDNA phylogeny, and hence are a separate lineage. San Miguel, Loreto and Curuzú Laurel comprise the lineage Iberá ii (Figure 2), while we designate Estancia la Tacuarita and Contreras Cué as Iberá iii.

The need for integrating different data

In past decades, specialists have advocated for distinct and partially incompatible species concepts (reviewed by Mayden 1997, de Queiroz 2005b), leading to different conclusions about species boundaries and numbers of species. These incompatibilities emerge from considering different biological properties upon which several alternative concepts are based (de Queiroz 2007 and references therein). The reason why different biological features lead to incompatible species concepts is that they evolve at different rates, and not even necessarily in the same order during the process of speciation. Before the acquisition of the first distinct biological property, there would be overall agreement in the existence of a single species. At the other extreme, where putative species become phenotypically distinguishable, reproductively incompatible, diagnosable, ecologically distinct, etc, there would be full agreement on the fact that there are two different species. The problem arises in what de Queiroz (2007) calls the gray zone where different species concepts would yield different species delimitation. Lineage delimitation becomes more confusing as divergence times are shorter, not only because there is little differentiation, but also because there is a greater discordance among different character sources (Harrison and Larson 2014). To overcome this problem de Queiroz (2005a,b, 2007) proposed a unified species concept that reflects an underlying property to all alternative conceptualizations. All modern species concepts equate species with separately evolving segments of metapopulation lineages (de Queiroz 2005a), where a metapopulation refers to an inclusive population made up of connected subpopulations (Hanski and Gaggiotti 2004), and a lineage is an ancestral-descendant series of populations/metapopulations. According to this concept, lineages do not have to be monophyletic, intrinsically reproductively isolated, phenetically distinguishable, etc., to be considered different species; they have to be evolving separately from other lineages. In this context, any property that provides evidence of lineage separation is relevant to the inference of species boundaries and the

number of species. Although the presence of a single property provides evidence for lineage separation, a highly corroborated hypothesis of lineage separation will require multiple lines of evidence. For instance, in the case of the Corrientes group, if only FN would have been taken into account only four lineages would be delimited hindering the clear SSR and mtDNA differentiation between *Ctenomys dorbignyi* and the *Ctenomys perrensi* complex. Likewise, if only SSR clusters were considered, Santa Rosa and San Miguel could be treated as members of the same lineage, in spite of their high chromosomal differences (that act undoubtedly as reproductive barriers, as stated above). On the contrary, *Ctenomys roigi* would represent a highly corroborated hypothesis, as it is supported by the three independent lines of evidence.

As mentioned (see Introduction) patterns of exclusivity for allopatric taxa could be a product of geographic isolation over time, without detriment of the potential gene exchange between them. For this reason we explicitly established a restrictive condition to achieve exclusivity (i.e. at both SSR clusters and mtDNA phylogeny). Under the applied lineage delimitation criterion, the populations of Estancia la Tacuarita and Contreras Cué were separated from other populations that share FN = 76 (Iberá ii) as they depict exclusivity at both SSR clusters and mtDNA phylogeny. The same occurs in the case of *Ctenomys dorbignyi*, that is separated from other populations of FN = 84. However, there is a fundamental distinction between both cases. The exclusiveness of Estancia la Tacuarita and Contreras Cué within the Iberanan group has been interpreted as the result of a strong bottleneck: almost all members of these populations were monomorphic for SSR alleles commonly found in other populations of the Corrientes group (Gómez Fernández et al. 2012). In contrast, *C. dorbignyi* has allele richness comparable to other populations/lineages such as *C. roigi*, Sarandicito, or San Alonso. Thus, genetic exclusivity could be the result of processes that have either occurred at the expense of variability (Iberá iii) or by differentiation without losing variability (*C. dorbignyi*).

Habitat stability and conservation

There are various reasons for the conservation of several Correntinean tuco-tucos populations. Although all tuco-tucos inhabit the subterranean niche and thus are ecologically exchangeable, the seven delimited lineages are genetically and/or karyotypically differentiated. Some lineages comprise one or few described populations and, although more intensive surveys should be carried out to

measure the area of occupancy of these tuco-tucos, they need a closer surveillance. *Ctenomys*, as a subterranean rodent, plays important ecological roles. As was demonstrated for the desert inhabiting tuco-tuco *C. cf. knighti*, Thomas, 1919 (Fracchia et al. 2011) the tuco-tucos are dispersal agents of root-associated fungi. This ecological role is particularly important for the populations of *Butia paraguayensis* and *Butia yatay* palm groves, broadly distributed in the Corrientes Province. Phosphorus intake and early growth of these palms depend on the association of their roots with arbuscular mycorrhizae (Ramos-Zapata et al. 2009, Phosri et al. 2010). Factors as the placement within a protected area, such as the Mburucuyá National Park or the Iberá Reserve, as well as the economic damage these animals can produce (see below) should also be taken into account to determine which stocks inside and outside these areas should be conserved.

There are six studied tuco-tuco populations that inhabit the Iberá Reserve (Figure 2): Loreto, Cruzú Laurel, San Miguel, Estancia la Tacuarita, Contreras Cué and San Alonso. The small population of San Alonso is prone to suffer inbreeding depression as it is located in an island surrounded by waterlogged wetlands. This population could be recovered only with individuals from the other population of the Iberá i lineage (Paraje Caimán) but not with individuals from Iberá ii/iii populations since the chromosomal differences (FN 76 vs 78) are reflecting a tandem fusion and hence, constitute a reproductive barrier. For the same reason, the population of San Miguel inhabiting the west margin of the Iberá wetland (or the inbred Estancia la Tacuarita or Contreras Cué, as argued above) could be genetically recovered with individuals from the allele-rich Loreto or Cruzú Laurel but not with individuals from Paraje Caimán, which is the nearest population (Figure 2).

Tuco-tucos populations located within rural settlements produce a negative economic impact on family farming, which is usually based on the cultivation of sweet potatoes, cassavas and other vegetables, since these rodents feed mainly on roots. Hence, these populations should not be included in conservation plans, unless they were unique representatives of a differentiated lineage. For instance, the Saladas Centro population lies in a farmhouses' area and farmers report the need for eradicating these tuco-tucos, which are considered a pest. As these tuco-tucos are members of the widespread *Ctenomys perrensi* complex, there is no need of conserving this population. In contrast, *Ctenomys roigi*, the only Critically Endangered species according the IUCN Red List of Threatened Species (Bidau et al. 2008a), should continue under strict protection since the three populations described are

distributed within a very restricted area (Figure 2) with anthropic influence. As argued above, the rest of the lineages should also be taken into account as regards conservation efforts. The lineage Sarandicito might include eight neighboring populations (Contreras et al. 1985) and then could be considered Vulnerable or Endangered according to the IUCN Red List Categories and Criteria (Anonymous 2012). The same categories should be applied to the lineages Iberá i, Iberá ii, Iberá iii and *C. dorbignyi*, since they have restricted areas of occurrence and probably they exist in no more than 10 locations. However, an exhaustive survey of tuco-tuco populations should be carried out in order to assess feasibly the conservation status of these lineages.

In relation to habitat stability, the Iberá system has been recently affected by the construction of the large Yaciretá dam, a few kilometers northwards on the Paraná River. This reservoir was filled in 1994, reaching 76 m above sea level. Since then, a considerable groundwater inflow has taken place from the Paraná River into the Iberá system, which has increased its water level with the subsequent loss of several hundreds of thousands of hectares of land (Canziani et al. 2006). Nevertheless, Úbeda et al. (2013) in a survey that spanned over the years 2000–2010, integrating climatological and hydrological models projected that water-level in the Iberá system would go through a moderate net decline over the next 90 years, with more extreme inter-annual fluctuations. Both climate change and anthropogenic stressors, and their interactions, turn the suitable areas for tuco-tucos occupancy highly unstable. However, the effects of these disturbances could be buffered by the system, given the slow slope of the Iberá Basin, the hydro-biological regulation mechanisms favored by the hydraulic characteristics of the *embalsados* (floating vegetation islands) and the morphology of the submersed soils, which contribute to water retention and ecological resilience.

Future perspectives

Nuclear loci could be incorporated as an additional line of evidence to infer a species tree by modeling coalescence between unlinked sequences (Heled and Drummond 2010). However, since in these tuco-tucos we did not recover enough variability in nuclear genes as the von Willebrand factor (*VWF*) and the recombination activating gene, *RAG1* (unpublished data), Next-Generation Sequencing techniques would be needed to expose greater nuclear variability. As a paradigmatic case, Wagner et al. (2013) demonstrated that high-throughput DNA sequence

data from restriction site-associated DNA (RAD) markers provide “unprecedented resolution of species boundaries” in Lake Victoria cichlid fish, a group which has yielded conflicting phylogenies due to recent divergence times and shared polymorphisms. Although large morphological differences are not expected in this group of species living in homogeneous underground environments (Ortells 1990), geometric morphology methodologies (Barčiová 2009, Sánchez et al. 2013, Ruane 2015) would offer a more detailed analysis of variable characters that could serve as another source of data to re-discuss lineages boundaries in this group.

Lineage delimitation in the Corrientes group was not a straightforward task. Three independent lines of evidence, SSR clustering, mtDNA phylogeny and karyotypic comparisons, yielded partially conflicting groupings when taken separately, but could be integrated to establish a criterion contemplating reproductive isolation indicators and genetic exclusivity evidences. Seven lineages are distinguished as well corroborated hypotheses in the Corrientes group. In the future, any undescribed population should be studied under this frame, to assess its membership to these lineages and to simultaneously test the robustness of the hypotheses proposed herein.

Conclusion

Under this integrative approach seven independently evolving lineages were delimited in the Corrientes group: *Ctenomys roigi*, which conserves its former definition, *Ctenomys dorbignyi* and *Ctenomys perrensi* complex which were redefined, Sarandicito which includes the population of Paraje Sarandicito and probably a group of nearby poorly studied populations, and Iberá i, ii and iii distributed at both sides of the Iberá wetland.

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