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Short Communication

The use of PCR-RFLP as an identification tool for three closely related species of rodents of the genus *Akodon* (Sigmodontinae, Akodontini)

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Abstract

Three cryptic species of the rodent *Akodon (A. cursor*, *A. montensis* and *Akodon* sp.) were analyzed. The two former species are sympatric in the Brazilian states of São Paulo, Rio de Janeiro and Minas Gerais, where hybrids have already been found. The third species, *Akodon* sp., occurs in an isolated area in Central Brazil. The identification of these species is difficult by the need of living animals. At present, karyotyping is the only method used in the identification of specimens. We used PCR-RFLP of the mitochondrial cytochrome gene to test the distinctiveness of the three species, which was confirmed by the absence of shared species-specific haplotypes. We also detected a geographical pattern of haplotypes distribution with highly polymorphic populations of *A. cursor* from Espírito Santo and of *A. montensis* from Rio Grande do Sul.

Key words: Akodon cursor, *Akodon montensis*, PCR-RFLP, Sigmodontinae.

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Akodon Meyen, 1833 (Muridae, Sigmodontinae) is widely distributed throughout South America (Smith and Patton 1993) and is one of the richest genus of sigmodontines, with around 41 species (Musser and Carleton 2005). The taxonomy of the genus is complex because the limits of some species are not clear and morphology is not always useful in distinguishing related species. One specific case regards a group of three species of *Akodon* from Brazil, the so-called *Akodon cursor* species group, composed of *A. cursor* Winge, 1887; *A. montensis* Thomas, 1913; and an undescribed species *Akodon* sp.

The distribution of *Akodon cursor* is restricted to lowlands and mid-elevation areas in the "floresta ombrófila densa" (dense rainforest) from northern (Paraíba, Pernambuco, Bahia) throughout southern Brazil (Espírito Santo, Rio de Janeiro, São Paulo, Minas Gerais and Paraná) (Yonenaga-Yassuda 1979, Maia and Langguth 1981, Liascovich and Reig 1989, Rieger *et al.* 1995, Sbalqueiro and Nascimento 1996, Fagundes *et al.* 1998, Geise *et al.* 2001).

For a long time, *A. montensis* was considered a junior synonym of *A. cursor* (Musser and Carleton, 2005) because of their morphological similarity in integumental, cranial and dental features (Ximenez and Langguth 1970). The recurrent misidentification of *A. montensis* and *A. cursor* was an issue in the taxonomy of Atlantic species of *Akodon*, which were basically defined by their karyotypes: 2n = 14-16 in *A. cursor* and 2n = 24-26 in *A. montensis*. Currently, *A. montensis* is recognized as a species with 2n = 24-26, distributed from the Atlantic coast of Rio de Janeiro, Minas Gerais throughout southern Brazil, Uruguay, Misiones in Argentina and eastern Paraguay (Ximenez and Langguth 1970, Yonenaga-Yassuda 1979, Liaschovich and Reig 1989, Rieger *et al.* 1995, Geise *et al.* 2001, Pardiñas *et al.* 2003).

Both species are simpatric in the states of Rio de Janeiro, Minas Gerais, São Paulo and in northern Paraná. Natural hybrids have been found in São Paulo (Fagundes *et al.* 1997a, b) and sterile hybrids have been produced in the lab (Yonenaga *et al.* 1975). Christoff (2002) used external anatomy and 20 cranium-dental features to determine the morphological characteristics of *A. cursor* and *A. montensis*. This author concluded that no external or cranio-dental feature could be used to discriminate between both species, which could only be achieved through karyotyping. Geise *et al.* (2004) suggested that the absence of a gall bladder in *A. montensis* could be a good discriminating feature between this species and *A. cursor* but they noted the usefulness of karyotyping for species identification. There is no information about the presence of a gall bladder in *Akodon* sp.

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A. cursor presents 2n = 14 to 16 with polymorphisms due to autosomes fusions and inversion (Fagundes *et al.* 1997a, 1998). *A. montensis* has 2n = 24, 25 and 26 and polymorphisms resulting from variable numbers of supernumerary chromosomes (Yonenaga-Yassuda 1979, Rieger *et al.* 1995, Fagundes *et al.* 1997b). *Akodon* sp., the third species of the *A. cursor* species group, presents 2n = 10 (Silva and Yonenaga-Yassuda 1998) and was collected in Mato Grosso State, Central Brazil, in a transitional area between the Amazonian forest and the Cerrado. This species is morphologically undistinguishable from the other two (A.U. Christoff, unpublished data).

The validity of the species status for these three taxons is questioned because they share a similar morphology, and two of them are sympatric in part of their distribution area. Karyotyping is currently the only way to discriminate each of these three species.

The major aim of this study was to develop molecular markers that would allow to distinguish these three *Akodon* species and thus avoid misidentifications. The PCR-RFLP is a rapid, easy and cheap technique used in species identification. Although it is a low cost technique, karyotyping requires the sacrifice of live animals or the establishment of cell cultures, which may be expensive and sometimes unfeasible due to ecological issues. DNA is easily obtained from blood, muscle, skin, ear or tail biopsies from living animals. The presence of distinct haplotypes in *A. cursor*, *A. montensis* and *Akodon* sp would allow their use for taxonomy and to genetically discriminate the three species, which would then be considered as full species under the biological species concept.

In this work, 78 samples were used: 50 samples of *Akodon cursor* from 12 localities, 23 of *A. montensis* from six localities, and five of *Akodon* sp from two localities. Specimens of *A. cursor* and *A. montensis* were from the Atlantic rainforest and specimens of *Akodon* sp. were from a transitional area between the Amazonian forest and the Cerrado (Table S1). The samples used are deposited at the Tissue Collection of "Laboratório de Genética Animal" at Universidade Federal do Espírito Santo (LGA-UFES) and "Laboratório de Citogenética de Vertebrados" at Universidade de São Paulo (CIT-USP).

Total genomic DNA was extracted from liver or muscle preserved in 70% ethanol following Bruford *et al.* (1992). Polymerase Chain Reaction (PCR) was used to amplify the complete sequence of the cytochrome b mitochondrial gene (1140 bp), with primers MVZ 05 (5'-CGAA GCTTGATATGAAAAACCATCGTTG-3') and MVZ 14 (5'-GGTCTTCATCTYHGGYTTACAAGAC-3') (Smith and Patton, 1991, 1993). PCR amplifications were carried out in a 50 µL reaction mix containing 1xTaq buffer, 3.0 mM $MgCl_2$, 0.4 mM of each dNTP, 0.4 mM of each primer, 0.6 U Platinum Taq DNA polymerase (Invitrogen) and 50 ng of DNA. PCR cycles were one cycle at 92 °C for 5 min, 37 cycles of 92 °C for 1 min, 47 °C for 1 min and 72 °C for 1 min. A final extension at 72 °C for 5 min was performed to completely extend the amplified product. After amplification, the PCR products were checked by electrophoresis in a 1% agarose gel and fragment sizes were estimated using a 100 bp or a 1 kb marker ladder (Invitrogen).

For PCR-RFLP analysis, the 1140 bp PCR products were digested with seven restriction endonucleases which recognize four to seven base pairs (*Alu*I, *Bsa*I, *Hae*III, *Mbo*I *Hinf*I, *Rsa*I and *Taq*I, Invitrogen). Restriction fragments were separated by electrophoresis in a 5% acrylamide gel and their sizes were estimated using the molecular markers. The results were taken into account when the sum of all the restriction fragments for each enzyme were in the range of 1140bp ± 100. Bands generated for the same enzyme that migrated similarly in the same gel were considered homologous.

Each set of bands obtained after the digestion of the PCR product by each enzyme (the cleavage pattern) was at-

Table 1 - Cleavage patterns (identified by upper cases in the left) of four restriction endonucleases for the three species of *Akodon*. Band sizes are given in base pairs.

Species	*Alu*I	*Hae*III	*Hinf*I	*Mbo*I
A. cursor	A 620, 400, 220 B 680, 480 C 680, 280, 220 D 1140	A 480, 420, 200, 180 B 540, 230, 200, 110, 100 C 520, 230, 200, 150, 100 D 460, 310, 230, 180, 100 E 530, 330, 200, 100 F 290, 230, 200, 180, 150, 110 G 540, 400, 330	A 520, 350, 220, 150 B 530, 520, 150 C 530, 380, 180, 150 D 700, 520	A 390, 370, 270, 170 B 560, 360, 160, 150 C 420, 370, 270, 160 D 620, 350, 170 E 480, 360, 170, 160, 140 F 380, 360, 170, 160, 140
A. montensis	B 680, 480 E 620, 540 F 640, 480, 100 G 660, 320, 250	H 680, 200, 180, 110 I 430, 230, 200, 180, 110, 100	A 520, 350, 220, 150 B 530, 520, 150 C 530, 380, 180, 150 E 530, 380, 220, 170, 150	A 390, 370, 270, 170 G 480, 360, 170, 140, 90 H 400, 370, 170, 90 I 480, 370, 180, 170 J 390, 360, 170, 140, 90 K 540, 360, 170, 90
Akodon sp.	H 660, 320, 220	J 540, 330, 200, 150	F 520, 510, 210	L 680, 380, 170

Table 2 - Collection sites and haplotypes of *Akodon cursor*, *A. montensis* and *Akodon* sp.

Species	State[1]	Locality	Ni	Haplotype[2]
Akodon cursor	PE	Rio Formoso	1	1 AGAF
	BA	Una	13	2 AAAB
	ES	Cariacica	1	3 BCBC
		Castelo	2	4 BCBA
			1	5 BBBA
			2	6 BECA
		Domingos Martins	2	7 BCBD
			2	5 BBBA
			1	4 BCBA
			1	8 BBCA
			1	9 DCBA
			2	10 BDBC
		Santa Teresa	2	3 BCBC
			1	11 CBBC
			1	12 DBBA
	SP	Ariri	1	4 BCBA
			2	5 BBBA
		Iguape	8	5 BBBA
		Capão Bonito	1	5 BBBA
		Picinguaba	2	5 BBBA
		Boracéia	1	5 BBBA
		Ilha do Cardoso	2	13 DFDE
Akodon montensis	SP	Iguape	9	14 EHAG
			1	15 EHAJ
		Juquiá	1	14 EHAG
		Luiz Antônio	1	16 EHCA
		Pilar do Sul	1	14 EHAG
	RS	Canela	1	17 EHAK
		Maquiné	1	18 BHAH
			2	19 BHCH
			2	20 GHEI
			1	21 BHAJ
			1	22 FHAJ
			1	23 FHAH
			1	24 BIBA
Akodon sp	MT	Gaúcha do Norte	4	25 HJFL
		Vila Rica	1	25 HJFL
Total			78	

[1]PE = Pernambuco, BA = Bahia, ES = Espírito Santo, SP = São Paulo, RS = Rio Grande do Sul and MT = Mato Grosso states.
[2]Letters correspond to cleavage patterns obtained with the endonucleases *Alu*I, *Hae*III, *Hinf*I and *Mbo*I, respectively.
Ni: Number of individuals.

tributed a letter. Some enzymes showed more than one cleavage pattern (polymorphisms). The mtDNA haplotype of each individual was defined as the cleavage patterns obtained with all of the enzymes used.

Four of the seven tested restriction endonucleases were considered informative (*Alu*I, *Hae*III *Hinf*I and *Mbo*I), *i.e.*, they generated species-specific band patterns (Table 1). The informative cleavage patterns were: *Alu*I, four cleavage patterns for *A. cursor* (A, B, C and D); four patterns for *A. montensis* (B, E, F and G) and one pattern for *Akodon* sp. (H); *Hae*III, seven cleavage patterns for *A. cursor* (A, B, C, D, E, F and G); two for *A. montensis* (H and I) and one for *Akodon* sp. (J); *Hinf*I, four cleavage patterns for *A. cursor* (A, B, C and D); four patterns for *A. montensis* (A, B, C and E) and one for *Akodon* sp (F).; and *Mbo*I, six cleavage patterns for *A. cursor* (A, B, C, D, E and F); seven for *A. montensis* (A, G, H, I, J and K) and one for *Akodon* sp (L).

Some of the cleavage patterns were shared by *A. cursor* and *A. montensis*, but the haplotypes (the cleavage patterns of all four enzymes) were distinct for each of the three species (Table 2). Haplotypes 1 to 13 belonged to *A. cursor*, 14 to 24 occurred in *A. montensis* and haplotype 25 was found in *Akodon* sp.

Some intraspecific variability was detected in *A. cursor and A. montensis*. A phylogeographic pattern was observed with particular *A. cursor* haplotypes related to specific geographic areas: haplotypes 1 and 2 in the northern localities of Pernambuco and Bahia, haplotypes 3 to 13 in the southern localities of Espírito Santo and São Paulo. The haplotypes of *A. montensis* from the southern population of Rio Grande do Sul (haplotypes 17, 18, 19, 20, 21, 22, 23 and 24) were not shared by the northern populations of São Paulo (haplotypes 14, 15 and 16). This pattern of geographic variation may reflect hitherto unknown molecular differences between populations. This contrasts with results from karyologic and morphometric analyses that revealed heterogeneous population patterns across the distribution of *A. cursor* in Espírito Santo with ten haplotypes versus three in São Paulo and of *A. montensis* in Rio Grande do Sul with eight haplotypes versus three in São Paulo (Table 2).

The fact that all 25 haplotypes were species-specific (Table 2) and that only few cleavage patterns were shared by *A. cursor* and *A. montensis* (B for *Alu*I, A, B and C for *Hinf*I and A for *Mbo*I) indicates that the three species are genetically divergent, although the time since speciation was not sufficient to allow morphological differences to accumulate.

This methodology can be widely used to identify non-karyotyped animals (which represent over 80% of the non-identified specimens) from museums or private collections, if tissue samples are preserved in ethanol or liquid nitrogen. Identification of ancient specimens without tissue samples remains a difficult task, since only highly degraded DNA is obtainable from the skins kept in museums.

Our main conclusions are that molecular data support the hypothesis that *A. cursor, A. montensis* and *Akodon* sp.

are distinct and isolated species and allow to easily distinguishing each of the three *Akodon* species.

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Supplementary Material

The following online material is available for this article: - Table S1

This material is part of the electronic version at: http://www.scielo.br/gmb.

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Table S1 Specimens used in the PCR-RFLP analysis, with collection localities and identification numbers. The total number of individuals for each species is in parentheses.

Akodon cursor (50) - **(1) Rio Formoso, PE** (8º40'S, 35º09'W): CIT 953 (2n=16 NA=26); **(2) Una, BA** (15º18'S, 39º04'W): CIT 878 (2n=16 NA=24), CIT 879 (2n=15 NA=22), CIT 880 (2n=15 NA=24), CIT 881 (2n=15 NA=24), CIT 883 (2n=16 NA=25), CIT 929 (2n=15 NA=21), CIT 930 (2n=16 NA=26), CIT 931 (2n=15 NA=23), CIT 933 (2n=14 NA=20), CIT 934 (2n=15 NA=24), CIT 1021 (2n=16 NA=23), CIT 1022 (2n=16 NA=25), CIT 1023 (2n=16 NA=26); **(3) Ariri, SP** (25º12'S, 48º02'W): CIT 306 (2n=15 NA=21), CIT 310 (2n=16 NA=22), CIT 314 (2n=15 NA=20); **(4) Capão Bonito, SP** (24º23'S, 47º55'W): CIT 20 (2n=14 NA=19); **(5) Iguape, SP** (24º42' S, 47º33' W): CIT 167 (2n=15 NA=23), CIT 222 (2n=15 NA=21), CIT 223 (2n=15 NA=22), CIT 248 (2n=14 NA=19), CIT 264 (2n=15 NA=22), CIT 267 (2n=16 NA=23), CIT 287 (2n=14 NA=20), CIT 288 (2n=14 NA=20); **(6) Boracéia, SP** (22°10'S, 48°45'W): CIT 309 (2n=14 NA=20); **(7) Picinguaba, SP** (23°22'S, 44°50'W): CIT 135 (2n=14 NA=21), CIT 146 (2n=14 NA=20); **(8) Ilha do Cardoso, SP** (25º09'S, 47º59'W): CIT 785 (2n=14 NA=20), CIT 786 (2n=14 NA=20); **(9) Cariacica, ES** (20°16'S, 40°25'W): CIT 327 (2n=14 NA=18); **(10) Castelo, ES** (20º36'S, 41º11'W): LGA 934 (2n=14 NA=20), LGA 936 (2n=14 NA=20), LGA 993 (2n=14 NA=20), LGA 995 (2n=14 NA=19), LGA 996 (2n=14 NA=21); **(11) Domingos Martins, ES** (20º22'S, 40º40'W, ES): LGA 956

(2n=14 NA=20), LGA 957 (2n=14 NA=20), LGA 960 (2n=14 NA=19), LGA 961 (2n=14 NA=20), LGA 962 (2n=14 NA=19), LGA 963 (2n=14 NA=18), LGA 964 (2n=14 NA=19), LGA 976 (2n=14 NA=20), LGA 983 (2n=14 NA=19); **(12) Santa Teresa, ES** (19º55'S, 40º36'W): LGA 37 (2n=14 NA=18), LGA 42 (2n=14 NA=20), LGA 50 (2n=14 NA=20), LGA 159 (2n=14 NA=18).

Akodon montensis (23) - **(5) Iguape, SP** (24º42'S, 47º33'W): CIT 181 (2n=23 NA=42), CIT 166 (2n=24 NA=42), CIT 201 (2n=24 NA=42), CIT 241 (2n=24 NA=42), CIT 243 (2n=24 NA=42), CIT 286 (2n=24 NA=42), CIT 157 (2n=25 NA=44), CIT 158 (2n=25 NA=44), CIT 165 (2n=25 NA=44), CIT 230 (2n=25 NA=44); **(13) Juquiá, SP** (24º19'S, 47º38'W): CIT 1291 (2n=24 NA=42); **(14) Luis Antônio, SP** (21º33'S, 47º43'W): CIT 938 (2n=24 NA=42); **(15) Pilar do Sul, SP** (23º49'S, 47º42'W): CIT 1230 (2n=24 NA=42); **(16) Canela, RS** (29°22'S, 50°50'W): LGA 405 (2n=26 NA=46); **(17) Maquiné, RS** (29º40'S, 50º12'W): LGA 316 (2n=24 NA=42), LGA 317 (2n=24 NA=42), LGA 318 (2n=24 NA=42), LGA 319 (2n=24 NA=42), LGA 320 (2n=24 NA=42), LGA 335 (2n=24 NA=42), LGA 409 (2n=24 NA=42), LGA 932 (2n=24 NA=42), LGA 965 (2n=24 NA=42).

Akodon sp1 (5) - **(18) Gaúcha do Norte, MT** (13º14'S, 53º05'W): CIT 541 (2n=10), CIT 579 (2n=10), CIT 580 (2n=10) and CIT 610 (2n=10); **(19) Vila Rica, MT** (10º01'S, 51º07'W): CIT 732 (2n=10).