

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

DIVERSIDADE GENÉTICA EM ESPÉCIES DO
GÊNERO *CAVIA* (RODENTIA, MAMMALIA)
E A HISTÓRIA EVOLUTIVA DO RARO
PREÁ DE MOLEQUES DO SUL

RICARDO KANITZ
SANDRO LUIS BONATTO (ORIENTADOR)

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FACULDADE DE BIOCÊNCIAS

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MAMMALIA) E A HISTÓRIA EVOLUTIVA DO RARO PREÁ DE MOLEQUES DO SUL

Ricardo Kanitz
Orientador: Dr. Sandro Luis Bonatto

DISSERTAÇÃO DE MESTRADO

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DEDICATÓRIA

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APRESENTAÇÃO

Apresento esta dissertação ao Programa de Pós-Graduação em Zoologia da Pontifícia Universidade Católica do Rio Grande do Sul como um dos requisitos para a obtenção do grau de mestre em Zoologia. Realizei este trabalho – juntamente com meu orientador Prof. Dr. Sandro Luis Bonatto – nas dependências do Laboratório de Biologia Genômica e Molecular na Faculdade de Biociências da mesma universidade.

A motivação inicial deste trabalho se deveu à descoberta de uma população de preás – gênero *Cavia* Pallas, 1766 – em uma ilha de apenas 10ha no litoral do estado de Santa Catarina no Brasil. Aparentemente isolados das populações continentais e com marcantes diferenças morfológicas, os preás de Moleques do Sul foram considerados uma nova espécie: *Cavia intermedia* (Cherem *et al.* 1999). Estudos previamente realizados (Salvador & Fernandez 2008a) demonstraram que o tamanho de censo da população destes roedores exibe uma média de 42 indivíduos com oscilações entre aproximadamente 30 e 60. Além disso, estima-se que o arquipélago de Moleques do Sul esteja separado do continente há pelo menos 8.000 anos. Cerca de 14 km perfazem a distância entre o arquipélago e o continente onde habita a população mais próxima de *C. magna* – a espécie-irmã continental de *C. intermedia*. Tal grau de isolamento em tempo e distância de qualquer outro grupo de preás, assim como o tamanho populacional extremamente pequeno desta espécie, fazem de *C. intermedia* um objeto de estudo interessantíssimo sob o âmbito da genética evolutiva: o mamífero naturalmente mais raro do planeta.

Pequenas populações são uma importante fonte de dados para estudos evolutivos. Entre as pequenas populações, as mais comuns são aquelas endêmicas de ilhas e uma das espécies de mamíferos com essas características é *Cavia intermedia*. Conhecer parâmetros tais quais a sua variabilidade genética intra-específica e sua diferenciação em relação à espécie mais próxima podem revelar aspectos evolutivos interessantes.

Entre as questões que existem sobre evolução em populações insulares, há um amplo

debate sobre o papel da seleção natural na manutenção de populações reduzidas e, logo, endocruzadas (Reed 2007). Alguns problemas de “saúde genética” podem surgir em populações com alto endocruzamento. A exposição de alelos deletérios que, em heterozigosidade, não seriam expressos resulta na diminuição do *fitness*. Essa redução do *fitness* pode se dar por malformações congênitas, susceptibilidade maior a doenças, menor tamanho de ninhada, entre outros efeitos prejudiciais aos indivíduos (Frankham *et al.* 2002).

Alguns trabalhos propõem que, nessas populações, ocorre uma forte seleção em favor dos heterozigotos aumentando sua frequência o que resulta, finalmente, em uma manutenção de diversidade na população como um todo (Gilligan *et al.* 1997; Kaeuffer *et al.* 2006). Outros fatores podem atuar em pequenas populações, tais como expurgo genético (*genetic purge*), que pode atenuar a depressão por endocruzamento. O expurgo ocorre por seleção purificadora que retira da população os alelos deletérios que são expostos pelos cruzamentos entre indivíduos aparentados. Apesar de não haver evidências de que esse efeito seja realmente eficiente na diminuição dos efeitos deletérios do endocruzamento a curto e médio prazos (Frankham *et al.* 2001), acredita-se que sua ação seja eficaz em longo prazo.

Adaptações morfo-fisiológicas também são descritas em táxons ilhéus. Boa parte dos trabalhos tratando de adaptações de espécies a condições insulares resalta a síndrome de ilhas como uma condição recorrente em mamíferos (Adler & Levins 1994). Essa síndrome, resumidamente, pode ser descrita pelas seguintes características. Organismos pequenos tendem ao gigantismo; enquanto que, espécies maiores tendem ao nanismo (Bromham & Cardillo, 2007). Exemplos dessas adaptações podem ser os musaranhos comuns (*Sorex araneus*) que ocorrem nas ilhas escocesas que têm seu tamanho corporal aumentado em relação às populações continentais (White & Searle 2007). No outro lado do espectro, há os extintos elefantes anões (*Stegadon* spp.) das ilhas de Flores que chegavam a ter somente 10% do tamanho dos elefantes asiáticos atuais (*Elephas maximus*) (Van den Bergh *et al.* 2001). Adaptações na história de vida também são comuns. Existe uma tendência à maior longevidade e menor fertilidade (Gliwicz

1980; Adler & Levins 1994). Essa síndrome parece se expressar, mantendo a regra, em *Cavia intermedia* (Salvador & Fernandez 2008b).

Além dos aspectos seletivos, outras forças evolutivas atuam sobre populações insulares. A deriva genética é a mais evidente e talvez a mais efetiva em alterar a frequência dos alelos na população. Ilhas pequenas, por sua área restrita *per se*, não suportam populações tão grandes quanto as continentais. Portanto, populações insulares são mais sujeitas às ações da deriva. Migrações também têm efeito mais acentuado em ilhas, pois a introgressão de alelos novos é potencialmente maior. O impacto dos novos alelos nas populações como um todo é maior, simplesmente, porque a população é menor. Além disso, a fixação do novo alelo pode se dar mais facilmente por efeito de deriva. Existem alguns casos relatados de espécies em condições semelhantes a *Cavia intermedia*. Um exemplo filogeneticamente próximo está no trabalho de Seddon & Baverstock (1999). Neste trabalho, os autores mostram um complexo de populações de ratos nativos (*Rattus fuscipes greyii*) dispostas em ilhas de diferentes tamanhos próximas à costa leste australiana. Viu-se que há uma correlação direta entre tamanho da ilha e diversidade genética presente nas populações tanto em marcadores neutros como em marcadores sujeitos à seleção natural – no caso – um gene de MHC de classe I: RT1.Ba. Geograficamente próximo, tem-se o exemplo de duas espécies de serpentes endêmicas de ilhas da costa: *Bothrops insularis* e *B. alcatraz*. Um trabalho envolvendo marcadores mitocondriais (Grazziotin *et al.* 2006) demonstrou que as duas espécies insulares, como esperado, apresentam diversidade genética bastante reduzida se comparadas com a espécie continental (*B. jararaca*).

Porém, todas as espécies de mamífero com pequeno tamanho populacional (com menos de 1.000 indivíduos conhecidos) estudadas geneticamente até o momento, são espécies cuja população foi reduzida, por ação antrópica, relativamente recentemente (poucas dezenas ou raramente alguns poucos séculos) (www.animalinfo.org/rarest.htm; www.iucnredlist.org). Portanto, talvez o aspecto mais importante de *C. intermedia* seja a hipótese de que seu tamanho populacional extremamente pequeno seja uma característica de longo prazo, talvez muitos

milhares de anos. Testar esta hipótese, e em caso de confirmação, estudar as consequências desta história idiossincrática para a diversidade genética da espécie são os principais objetivos deste estudo.

Então, para que obtivéssemos uma representação adequada do nível de diversidade genômica em *C. intermedia*, procuramos, em primeira instância, regiões de repetições curtas em seqüência ou microssatélites (*short tandem repeats: STR*). Usando uma abordagem menos ortodoxa, buscamos microssatélites diretamente nas seqüências genômicas de *C. porcellus* (o porquinho-da-Índia) procurando *STRs* especialmente longos. Sabe-se que quanto maior uma região de repetições, maior deve ser a sua taxa de mutação. Portanto, buscamos por microssatélites altamente variáveis para que – se houvesse alguma variabilidade em *C. intermedia* – ela pudesse ser captada através destes marcadores. A implementação desta técnica nas espécies continentais (*C. magna* e *C. aperea*), assim como a descrição dos *primers*, deverá ser publicada no periódico *Molecular Ecology Resources* na forma de uma *Primer Note*. O manuscrito inicial para esta publicação está detalhado no capítulo I.

No capítulo II, apresentamos o artigo principal desta dissertação. Sob a forma de um *Original Article*, devemos submetê-lo ao periódico *Molecular Ecology*. Neste trabalho, analisamos dois tipos de marcadores moleculares incluindo (i) os microssatélites descritos no primeiro capítulo e (ii) seqüências mitocondriais das regiões hipervariável I (*HVSI*) citocromo b (*cytochrome b*). Através da avaliação desses marcadores, nós pudemos estimar a diversidade neutra presente na população de *C. intermedia* e compará-la com sua espécie-irmã continental (*C. magna*) de maneira intra e interpopulacional.

Adicionalmente, estimamos os tamanhos efetivos populacionais (N_e) histórico e atual para *C. intermedia* baseados nos perfis genotípicos daquela espécie. O N_e histórico é também conhecido como N_e de endocruzamento (N_{el}). Este índice se baseia na diversidade genética encontrada dentro da população avaliada e remonta à diversidade acumulada e perdida ao longo da evolução desta população. Por outro lado, o assim chamado N_e atual pode ser medido através

da variância da composição alélica da população entre diferentes gerações: daí seu nome N_e da variância (N_{ev}). O princípio desta estimativa é mensurar a ação da deriva genética de uma geração para outra. Quanto maior for essa ação, menor o tamanho efetivo daquela população.

Acreditamos que este estudo pode ter uma importante contribuição para a área da biologia da conservação de populações insulares, assim como para a compreensão dos processos microevolutivos que atuam sobre a composição genética dessas populações. Além disso, é claro, este trabalho fornece inéditas estimativas, tanto da diversidade genética do mais raro mamífero conhecido, como dos seus tamanhos efetivos populacionais histórico e atual que, por si só, são resultados de grande validade à ciência. Finalmente, caso a hipótese seja confirmada, esta será a única espécie de mamífero conhecida até o momento com esta história evolutiva extrema, o que pode transformar *C. intermedia* em uma inestimável ferramenta de estudos evolutivos em geral e de genética e biologia da conservação em especial.

RESUMOS

CAPÍTULO I: “Caracterização de 16 *loci* de microssatélites para os roedores sul-americanos *Cavia magna* e *C. aperea*”

Baseando-nos no recentemente publicado genoma de *Cavia porcellus* e em outros cinco loci já descritos para *C. aperea*, nós aqui apresentamos 16 *loci* de microssatélites empregáveis em *C. magna* e *C. aperea*. Os *primers* foram projetados para serem usáveis em um arranjo de fluorescência múltipla (*multiplex*). O número médio de alelos para cada *locus* foi de 7,4 e a média da heterozigosidade esperada foi de 0,67. A combinação de alguns ou todos estes marcadores pode possibilitar trabalhos em genética populacional, ecologia molecular e outros estudos evolutivos nas espécies aqui avaliadas.

CAPÍTULO II: “Baixíssima diversidade genética do preá de Moleques do Sul (*Cavia intermedia*), o mamífero naturalmente mais raro do Mundo”

Ilhas têm chamado a atenção de biólogos evolucionistas há séculos pelos seus altos níveis de endemismos promovidos, tanto pelo isolamento, quanto pelo tamanho limitado a que estas populações estão sujeitas. *Cavia intermedia* é uma espécie recentemente descrita como endêmica de uma pequena ilha na costa meridional do Brasil que parece ser o mamífero naturalmente mais raro conhecido, apresentando uma população estável de aproximadamente 40 indivíduos. Apesar de algumas simulações demográficas terem estimado sua chance de extinção em 100 anos como 100%, dado a história da ilha, torna-se improvável que a população possa ter sido muito maior no passado recente. Utilizando marcadores mitocondriais e microssatélites, nós descobrimos que este preá insular apresenta uma diversidade genética extremamente reduzida com estimativas de tamanhos efetivos populacionais histórico e atual compatíveis com seu tamanho de censo atual, sem sinal de redução de tamanho populacional. Considerando a antiga divergência de *C. intermedia* em relação ao seu grupo-irmão continental, concluímos que a espécie mantém esse tamanho extremamente reduzido desde, pelo menos, a separação da ilha em relação ao

continente há cerca de 8.000 anos. Portanto, *C. intermedia* pode ser o melhor e mais extremo exemplo até agora conhecido de uma espécie de mamífero que sobreviveu por centenas de anos com um tamanho populacional tão extremamente reduzido. Essa espécie, então, estabelece novos desafios ao entendimento do papel da redução populacional e da diversidade genética na extinção de espécies. Finalmente, é importante enfatizar a necessidade de um cuidado especial na manutenção das boas condições do hábitat para a sobrevivência de *C. intermedia* em seu ambiente natural.

**CAPÍTULO I: CHARACTERIZATION OF 16 MICROSATELLITE LOCI FOR THE
SOUTH-AMERICAN RODENTS *CAVIA MAGNA* AND *C. APEREA***

(Artigo a ser submetido ao periódico *Molecular Ecology Resources*)

1 **Permanent Genetic Resources Note**

2

3 **Characterization of 16 microsatellite loci for the South-American**
4 **rodents *Cavia magna* and *C. aperea***

5

6 Ricardo Kanitz¹, Fritz Trillmich², Sandro L. Bonatto¹

7

8 ¹ Faculdade de Biociências, PUCRS. Av. Ipiranga 6681, prédio 12C. Porto Alegre, RS 90619-
9 900, Brazil.

10 ² Department of Animal Behavior, University of Bielefeld. P.O. Box 100131, Bielefeld 33501,
11 Germany.

12

13 *Keywords:* *Cavia*, *Cavia magna*, *Cavia aperea*, microsatellite, STR, molecular markers.

14

15 Corresponding author: Sandro L. Bonatto, Faculdade de Biociências, PUCRS. Av. Ipiranga
16 6681, prédio 12C, sala 172. Porto Alegre, RS 90619-900, Brazil. Fax: +55 (51) 3320-3612. E-
17 mail: slbonatto@pucrs.br

18 *Running title:* Microsatellites for the genus *Cavia*.

19 **Abstract**

20 Based on the recently published genome draft of *Cavia porcellus* and five other loci
21 described for *C. aperea*, we present 16 microsatellite loci applicable for *C. magna* and *C.*
22 *aperea*. We designed the primers to be used in a multiplex fluorescence array. The average
23 number of alleles for each locus was 7.4 and the mean expected heterozygosity was 0.67. The
24 combination of some or all of these markers may give a good framework for population
25 genetics, molecular ecology and other evolutionary studies in these species.

26 The genus *Cavia* Pallas, 1766 is a widespread rodent taxon in the Neotropics. Among
27 the six recognized species (Wilson & Reeder, 2005), stand the best known *Cavia porcellus*
28 (Domestic Guinea Pig) and *C. aperea* (Wild Guinea Pig). The latter seems to be the most
29 common in the wild, while the first is the likely domestic derivative of the Andean cavy, *C.*
30 *tschudii* (Spotorno *et al.* 2006) and also an important model species. *Cavia magna* (Greater
31 Guinea Pig) is endemic to a narrow coastal region in southern Brazil and Uruguay. As *C.*
32 *magna* and *Cavia aperea* also present unique ecological characteristics (Kraus *et al.* 2003;
33 Kraus *et al.* 2005; Asher *et al.* 2008), highly variable molecular markers is very important for
34 further studies on their biology. A previous work on the ecology and behaviour of *C. aperea*
35 described six microsatellite (STR) loci for that species (Asher *et al.* 2008), but these primers
36 were not tested for any other species. Moreover, additional loci are necessary for more
37 accurate inferences of population genetics parameters in the species. Here we aim to (i) test
38 the cross-amplification of these six loci using redesigned primers, and (ii) present 10 new
39 STR loci and their respective primers sequences applicable to *C. aperea* and *C. magna*.

40 First, we redesigned primers for the six microsatellite loci described by Asher *et al.*
41 (2008) trying to improve multiplexing possibilities. The new primers were designed using
42 Primer3 web-based software (Rozen & Skalesky, 2000). We renamed these loci to Cavy1
43 (formerly Asher *et al.*'s CAP49651), Cavy2 (CAP49653), Cavy3 (CAP49654) and Cavy4
44 (CAP49655). The additional two loci in Asher *et al.* (2008) did not provide satisfactory
45 results and were not further used in this study.

46 We additionally searched for other 17 loci in the *Cavia porcellus* genome draft (July,
47 2007). Of the 219 contigs we searched in *C. porcellus* genome (~37.8 million bp), 65
48 presented 78 microsatellite sequences. For the search for usable STR loci, given the massive
49 amount of data, we used the program MSatCommander (Faircloth, 2008) which has proved to

50 handle well with large datasets. We designed the primers for these loci in Primer3 (Rozen &
51 Skalesky, 2000) for the same annealing temperatures we applied for the previous primer pairs
52 (60°C).

53 To minimize the cost of fluorescent primers, we applied the M13 tail method as
54 proposed by Lorenz et al. (2001). The amplification tests were made on 20 subjects from each
55 *C. aperea* and *C. magna* species collected in North Uruguay and South Brazil. The
56 Polymerase Chain Reactions (PCR) were performed in a 10 µL reaction using 5-15 ng of
57 genomic DNA, with 0.2 mM of all dNTPs, 0.2 µM of each the reverse and the M13-
58 fluorescent primers, 0.013 µM of the forward primer, 2 mM MgCl₂ and 0.25 U of *Taq* DNA
59 polymerase *Platinum* (Invitrogen). The PCR thermal profile were as follows: 94 °C for 3 min
60 for initial denaturing, followed by 35 cycles of 94 °C for 1 min 30 s, 60 °C for 1 min 30 s and
61 72 °C for 45 s; after these 35 cycles, we added a final extension (72 °C) for 30 min followed
62 by cooling at 4 °C.

63 Genotyping was performed in an automated sequencer MegaBACE1000 (GE
64 Healthcare) using the internal size standard ET Rox-550 and inspecting the electropherograms
65 in Genetic Profiler 1.5 (GE Healthcare). Diversity indexes estimations, Hardy-Weinberg
66 equilibrium (HWE), and linkage disequilibrium tests with Bonferroni correction for multiple
67 comparisons were performed in Arlequin 3.1 (Excoffier *et al.* 2005).

68 We characterized 16 (*C. magna*) and 12 (*C. aperea*) working loci which are able to be
69 used in intra-specific studies. Twelve of the 16 loci were successfully amplified for both *C.*
70 *magna* and *C. aperea* and may be used for across species studies. Details on primer
71 sequences, repeat motifs, expected PCR product sizes and suggested dyes for multiplex
72 genotyping are given in Table 1. The summary statistics showed high levels of diversity for all
73 loci in both species. Three loci presented significant departure from the HWE in *C. magna*,

74 and none for *C. aperea* (Table 2). The excess of homozygous individuals in *C. magna* may be
75 due to inbreeding in our sample, which focused in individuals from one small and probably
76 isolated population. Besides, no linkage-disequilibrium was found between any loci for both
77 species.

78 The primers described here revealed are readily usable for evolutionary studies in both
79 species. Given their high variability, these markers may be applied for both inter-population
80 (e.g. genetic isolation, number of migrants, etc.) and intra-population studies (e.g. kinship
81 exploration). Furthermore, we believe that most of the primers presented here may also be
82 used in studies in the other three wild cavy species, *C. fulgida*, *C. tschudii*, and *C. intermedia*,
83 considering that *C. aperea* and *C. magna* are in two divergent clades within *Cavia* (results in
84 preparation by our group). Additionally, these loci may be also useful for studies on *C.*
85 *porcellus* biology, which is an important species in experimental biology.

86

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Table 1: Primers details for all 16 loci.

Locus	Repeat Motif	Forward Primer Sequence*	Reverse Primer Sequence	Dye**	Size****	Accession #
Cavy 1	[AG] _n	CGGTTCTTGATTGGTTCAT	GCCGTCTCCTGTTCTCTCTC	FAM	255	AJ496558
Cavy 2	[AC] _n	GGCCATTATGCCCCCAAC	AGCTGCTCCTTGCTGTAG	HEX	164	AJ496560
Cavy 3	[CT] _n	ACAGCCATCACAAATCTGCAC	GCAGTGGTAAACCCAGAAATGG	NED	225	AJ496561
Cavy 4	[GT] _n	GTTGTATCTTCAAAAGCCCTGAC	AATACAGTGTGCCCCAGCA	FAM	119	AJ496562
Cavy 5	[AAGG] _n	CTCCATTACAGAGTGGTCT	AAAAGTCTGTTAAATGGGA	FAM	423	AC190431
Cavy 6	[TCT] _n	GTACCAGGGATCAAACTCAG	GAGCTTTCGAGAGTACGAGA	FAM	388	AC190431
Cavy 7	[AGG] _n	TGGACCTCCAGGTACTACAC	GTGACCCCTGCAACATTTCT	NED	404	AC185540
Cavy 8	[TTC] _n	CCCTTCCCACCTCTTCTATT	CTGCCAGCTTAGCAATTTAT	FAM	290	AC189989
Cavy 9	[TCT] _n	CAGCGATCTTCTATGGAGAC	TCTTTAAATGGTGGGTTTCAG	HEX	192	AC192512
Cavy 10	[AAAG] _n	ATGAACTTCAACATGGATGG	CCCTCTGAGATCTTTCCCTCT	FAM	398	AC174822
Cavy 11	[CT] _n	TCAGAAAAGCTGGAAAATTCAAT	AATGTGATGTGCTGAACAGA	NED	379	AC192015
Cavy 12	[AG] _n	TCCCTGTTCTTTGCTACAAT	CTGCTTCATAGATCTTTGCCCT	HEX	236	AC182323
Cavy 13	[AGG] _n	AGGGAGGCCAGAGTGAGAG	TCCTACACTGCATTGCTTGC	NED	385	AC189135
Cavy 14	[CT] _n	AGTGTGGCAGCTTGATCCT	AGCTCACCCAGGGAAAAATGTG	FAM	367	AC190428
Cavy 15	[AG] _n	TTCATGCTACCTGGCACTTG	TTCAGGCAATATGGCATTAT	NED	238	AC191184
Cavy 16	[CT] _n	CCAGTGGATTGGGAGACATT	CTCACCAAGAATGCAAAAGCA	HEX	381	AC194996

* Forward primers include the M13 sequence (CACGACGTTGTAAAACGGAC) at the 5' edge.

** Suggested dyes for four multiplex panels.

*** Annealing temperatures standardized when possible for multiplex amplification.

**** Expected base pair PCR product size according to the original sequence in GenBank including the M13 tail.

Table 2: Diversity indexes for all 16 loci in both species. N_A is the number of alleles, H_e and H_o are the expected and observed heterozygosities respectively.

Locus	N_A		H_e		H_o	
	<i>C. magna</i>	<i>C. aperea</i>	<i>C. magna</i>	<i>C. aperea</i>	<i>C. magna</i>	<i>C. aperea</i>
Cavy 1	10	7	0.721	0.617	0.444*	0.550
Cavy 2	4	11	0.592	0.842	0.600	0.800
Cavy 3	6	10	0.677	0.806	0.700	0.750
Cavy 4	2	6	0.224	0.628	0.250	0.750
Cavy 5	7	-	0.554	-	0.500*	-
Cavy 6	8	6	0.822	0.697	0.789	0.750
Cavy 7	3	12	0.286	0.790	0.316	0.600
Cavy 8	5	6	0.747	0.656	0.533	0.778
Cavy 9	7	7	0.671	0.740	0.700	0.650
Cavy 10	7	9	0.832	0.681	0.450*	0.700
Cavy 11	7	16	0.821	0.882	0.900	0.765
Cavy 12	3	3	0.537	0.099	0.700	0.100
Cavy 13	5	-	0.659	-	0.550	-
Cavy 14	8	-	0.784	-	0.647	-
Cavy 15	5	-	0.721	-	0.500	-
Cavy 16	14	9	0.856	0.738	0.588	0.588
Mean	6.313	8.500	0.65647	0.68132	0.57302	0.64839
	7.406		0.669		0.611	

*Denotes significant HWE departure ($P < 0.01$).

**CAPÍTULO II: DEPAUPERATED GENETIC DIVERSITY OF THE MOLEQUES DO SUL
CAVY (*CAVIA INTERMEDIA*), THE NATURALLY RAREST MAMMAL IN THE WORLD**

(Artigo a ser submetido ao periódico *Molecular Ecology*)

1 **Original Article – Population and Conservation Genetics**

2

3 **Depauperated genetic diversity of the Moleques do Sul cavy**
4 **(*Cavia intermedia*), the naturally rarest mammal in the World**

5

6 Ricardo Kanitz¹, Carlos Henrique Salvador², Sandro L. Bonatto¹

7

8 ¹ Faculdade de Biociências, PUCRS. Av. Ipiranga 6681, prédio 12C. Porto Alegre, RS 90619-
9 900, Brazil.

10 ² Instituto de Biologia, UFRJ. Ilha do Fundão. Rio de Janeiro, RJ 21941-590, Brazil.

11

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13 demography, ABC.

14

15 Corresponding author: Sandro L. Bonatto, Faculdade de Biociências, PUCRS. Av. Ipiranga
16 6681, prédio 12C, sala 172. Porto Alegre, RS 90619-900, Brazil. Fax: +55 (51) 3320-3612. E-
17 mail: slbonatto@puers.br

18

19 *Running title:* Low genetic diversity of *Cavia intermedia*.

20 **Abstract**

21 Islands have been calling the attention of evolutionary biologists for centuries for their
22 high level of endemic species fostered by isolation and limited population size. *Cavia*
23 *intermedia* is a recently described cavy species endemic to a small island in the southern
24 Brazilian coast, that seems to be the naturally rarest known mammal, having a stable
25 population around 40 individuals. Although previous demographic simulations estimated its
26 probability of extinction in 100 years as 100%, given the island history, it is unlikely that its
27 population size could have been much larger in the recent past. Using mitochondrial and
28 microsatellite markers, we have found that this insular cavy presents an extremely reduced
29 genetic diversity with an estimated historical and present effective population size matching
30 its present census size, with no evidence of recent population reduction. Considering *C.*
31 *intermedia* long divergence from its sister continental species, we concluded that it keeps this
32 extremely reduced population size since at least the most recent separation of the island from
33 the continent around 8,000 years ago. *C. intermedia* may therefore be the best and most
34 extreme case so far of a mammal species that survived for thousands of years with an
35 extremely reduced population size. This species then poses new challenges to understanding
36 the role of population reduction and genetic diversity to species extinction. Finally, we
37 emphasize the need for special care in the maintenance of the pristine conditions of *C.*
38 *intermedia*'s habitat.

39 **Introduction**

40 In conservation biology, island endemic populations are of special interest and
41 concern. While in one hand the insular ecosystems maintain the major part of the biological
42 richness in the World (Myers *et al.* 2000); in the other, their biota may be more susceptible to
43 extinction than that in mainland given their limited population size (Frankham 1998, Alcover
44 *et al.* 1998). There are many examples in the literature of insular species that underwent
45 extinction in the historical time. Since the classical examples of the dodo extinction (Fuller
46 2002) by hunting in the 17th century to the more recent introduction of the brown treesnake
47 that initiated a series of extinction of several populations in the island of Guam during World
48 War II (Fritts & Rodda 1998). Other taxa did not become extinct, but faced severe problems
49 to survive (e.g. the Española's Giant Tortoises population in the Galápagos was reduced to
50 only 14 individuals in 1965 (MacFarland *et al.* 1974)). These populations were depleted by
51 human interference; therefore their reduced population sizes were caused by recent events
52 such as hunting, habitat destruction and the introduction of exotic predators.

53 One frequent fact that underpins examples of extreme population reduction is that
54 these populations also faced severe reductions in their effective population sizes (N_e ; Wright
55 1931). Populations whose N_e is depressed are considered more prone to extinction by both
56 environmental and genetic causes. Among the environmental reasons, there are three main
57 categories: (i) demographic and (ii) environmental stochasticity, and (iii) natural catastrophes
58 (Shaffer 1981). The first might be caused by the intrinsic properties of the population (e.g.
59 lowered number of breeders of one gender); the second, by the habitat characteristics (e.g.
60 variable climate, natural or introduced predators and competitors, etc.); and the third, mainly
61 by unpredictable events (e.g. earthquakes, floods, hurricanes, etc.).

62 The small size of a population may lead to reduced adaptability (loss of evolutionary
63 potential) and inbreeding depression (Frankham *et al.* 2002). These genetic problems might
64 not directly result in population extinction, but may intensify this process magnifying
65 environmental extinction causes (Brook *et al.* 2002). Considering all these environmental and
66 genetic factors, it has been proposed and it is generally accepted that, for short-term survival
67 in the wild, an isolated population should maintain a N_e of no less than 50; and for long-term
68 survival, an effective size of at least 500 would be necessary (Franklin 1980).

69 In 1999, a new mammal species was described endemic of a very small (10.5 ha)
70 coastal island in Southern Brazil (Figure 1): *Cavia intermedia* (Cherem *et al.* 1999). Moleques
71 do Sul is arguably the smallest known Island with an endemic mammal species (several times
72 smaller than that previously known in the California Channels Islands, Alcover *et al.* 1998,
73 Knowlton *et al.* 2007). The Moleques do Sul cavy is a hystricognath rodent related to the
74 guinea pig (*C. porcellus*). Although initially considered an isolated population of its putative
75 sister continental species *C. magna*, *C. intermedia* presents several morphological differences
76 from that taxon, such as feet and teeth shape, cranial structure, and coat color, the latter being
77 of an intermediary hue between the two continental species *C. magna* and *C. aperea* (Cherem
78 *et al.* 1999).

79 More recently, two thorough studies on *C. intermedia* ecology and demography
80 (Salvador & Fernandez 2008a; 2008b) showed that the species as a whole has an average of
81 42 individuals as its census population size (N_c) varied from around 30 to 60 during the
82 observed period of 15 months. Given its extremely restricted habitat and much reduced
83 population size, *C. intermedia* may likely be the naturally rarest known mammal.
84 Furthermore, Salvador (2006) using demographic simulations preliminarily estimated *C.*
85 *intermedia* extinction probability in 100 years as 100%. Such condition, with a much reduced

86 population size, placed the Moleques do Sul cavy in the IUCN Red List of Threatened
87 Species (Chapman 2008) as Critically Endangered (IUCN 1994, 2001).

88 Geological evidences show that Moleques do Sul largest island, where the species
89 lives, has been isolated and has maintained its current size for at least 8,000 years, since the
90 rise of the sea level in the present interglacial period (Cherem *et al.* 1999). This suggests that
91 *C. intermedia* may have been isolated in this extremely small island for a long time. This
92 scenario is reinforced since *C. intermedia* exhibits a chromosome number different from the
93 other species of *Cavia* – as studied by Gava *et al.* (1998) before the species' description – and
94 an almost complete island syndrome [which is a combinations of adaptive traits commonly
95 observed in insular taxa (Adler & Levins 1994)] with lower reproduction and greater
96 longevity (Salvador & Fernandez 2008b). If this scenario is true, *C. intermedia* poses an
97 interesting challenge to conservation genetics and an extraordinary model for evolutionary
98 biology, since it would be the first known example of a mammal species with a very long
99 history of a naturally extremely reduced population size. However, no genetic study has been
100 done so far in this species to test this hypothesis and, if true, to evaluate its consequences for
101 the genetic architecture of the species.

102 Here, we present a thorough assessment of the genetic diversity of the species using
103 both mitochondrial DNA and nuclear microsatellite markers to test the above scenario. For
104 this, we estimated both the historical and the current effective population sizes for *C.*
105 *intermedia* using several approaches including the newly developed approximate Bayesian
106 computation (ABC). Moreover, we tested the hypothesis that the continental *C. magna* is *C.*
107 *intermedia* sister species, estimated their minimal divergence time, and contrasted their
108 genetic diversities.

109

110 **Material and Methods**

111 *Sampling, DNA extraction and PCR* – *C. intermedia* tissue samples were collected
112 from ear tips from 70 individuals between March 2004 and June 2005 in Moleques do Sul
113 Archipelago covering three generations in a capture-mark-recapture program described in
114 Salvador & Fernandez (2008a). Nineteen *C. magna* tissue samples from northern Uruguay
115 were obtained by Kraus *et al.* (2005) and were kindly provided by the authors. Two additional
116 samples of *C. magna* from Santa Catarina state were captured by bait trapping in September
117 2007 in the district of Pinheira/SC, just in front of the Moleques do Sul islands (Figure 1)
118 using the same approach applied in Salvador & Fernandez (2008a, 2008b) and observing the
119 ethical guidelines in Gannon *et al.* (2007). Four other *C. magna* samples from Rio Grande do
120 Sul state used in the mtDNA analyses were obtained by our group. The ear tissue samples
121 were maintained in a solution with 70% ethanol and kept in our laboratory. DNA was
122 extracted using a salt-precipitation protocol (Medrano *et al.* 1990) and the extractions were
123 verified and quantified in 1% agarose gel stained with GelRed®. For standardization
124 purposes, all DNA samples were diluted to ~5ng/μL in water Milli-Q®. The microsatellite
125 loci, as well as the primers and PCR amplification condition used here were detailed
126 elsewhere (Kanitz *et al.* in preparation). In summary, 12 STR loci were developed to be
127 highly informative using the draft genomic sequence of *C. porcellus* and tested in *C. magna*
128 and *C. aperea* (*Cavy1*, *Cavy2*, *Cavy4*, *Cavy6*, *Cavy7*, *Cavy8*, *Cavy9*, *Cavy10*, *Cavy12*,
129 *Cavy13*, *Cavy14*, and *Cavy16*). Amplification of the mtDNA HVSI control region was done
130 using the following primers: ‘CCCAAARCTGRWATTCTWATTAAACT’ as forward; and
131 ‘ATGGCCCTGAAGWAAGAACC’ as reverse. These primers were designed based on the *C.*
132 *porcellus* complete mitochondrial genome sequence (accession number AJ222767) as well as
133 other rodent species mtDNA sequences available in GenBank looking for conserved regions.

134 The primers we used for the *cytochrome b* amplifications were
135 ‘ATTCCTACATGGAGTTTAACCATGAC’ and ‘CCCATCTCTGGCTTACAAGACCAG’.
136 All mtDNA reactions were performed in a 20 μ L reaction using 10 ng of genomic DNA, with
137 100 μ M of all dNTPs, 0.25 μ M of each the reverse and the forward primers, 1.5 mM MgCl₂,
138 1X PCR Buffer and 1 U of *Taq* DNA polymerase *Platinum* (Invitrogen). The thermal profile
139 used for this locus was as follows: initial denaturing for 3 min at 94 °C, followed by 35 cycles
140 of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min. After this, there were more 5 min for
141 final extension at 72 °C and cooling at 4 °C.

142 *Genotyping and sequencing* – The microsatellite amplified products were separated in
143 an automated sequencer MegaBACE1000 (GE Healthcare[®]) and genotyped in the software
144 Genetic Profiler v.3.1 (GE Healthcare[®]) using different dyes for each locus. The mtDNA
145 segments were sequenced after enzymatic purification with Exonuclease-I and Shrimp
146 Alkaline Phosphatase. The sequencing reactions were performed using the ET Terminator
147 cycle sequencing kit and run in a MegaBACE1000 (GE Healthcare[®]). The chromatogram
148 reads (forward and reverse) were assembled and visualized using the Phred-Phrap-Consed
149 package (Ewing *et al.* 1998; Gordon *et al.* 1998).

150 *Genetic diversity* – The mtDNA segments were aligned using muscle3.6 (Edgar 2004)
151 which were visually checked in BioEdit7 (Hall 1999). Summary statistics were calculated
152 using DnaSP4.5 (Rozas *et al.* 2003) and Arlequin 3.1 (Excoffier *et al.* 2005). We also
153 constructed haplotype networks using median-joining (Bandelt *et al.* 1999) as implemented in
154 Network 4.5.1 (<http://www.fluxus-engineering.com>). For the STR markers, we performed the
155 calculation of the genetic summary statistics and Hardy-Weinberg equilibrium test in
156 Arlequin 3.1 (Excoffier *et al.* 2005). Moreover, we calculated the fixation index between the
157 two species using the STR loci (with F_{ST} and R_{ST}).

158 *Divergence time* – The divergence time between *C. intermedia* and *C. magna* was
159 calculated using the two mtDNA regions (HVSI and *cytochrome b*) independently. We used
160 the Bayesian approach implemented in BEAST 1.4.8 (Drummond & Rambaut. 2007) setting a
161 calibration point for the Hystricomorpha (formerly Caviomorpha) diversification as estimated
162 in Wyss *et al.* (1993) and applied in Huchon & Douzery (2001) of ~32 million years ago
163 (MYA). The *C. intermedia* and *C. magna* sequences were aligned – using muscle3.6 (Edgar
164 2004) – with other sequences of species belonging to the Hystricomorpha group: *C. porcellus*
165 (GenBank accession numbers AF491746 for HVSI; DQ017047 for *cytochrome b*),
166 *Hydrochaerus hydrochaeris* (EU149776 and FJ430787), *Agouti paca* (three individuals for
167 each region, AY206599 and AY206574, AY206598 and AY206573, and AY206597 and
168 AY206572), *Ctenomys torquatus* (EU530577 and EU519318), *C. talarum* (EF531750 and
169 AF144283), *C. rionegrensis* (AY755461 and AF538377), *Octodon degus* (three individuals
170 for each region, AY007365 and AM407929, AY007364 and AF422914, and AY007363 and
171 AF007059), *Echimys didelphoides* (EU313280 and EU302705), *E. chrysurus* (EU313262 and
172 EU313213), and *E. macrurus* (EU313289 and EU302703). We applied the Uncorrelated
173 Lognormal Relaxed clock with the HKY mutational model using four categories of gamma
174 correction with no fixed mutation rate. We set the root age as our calibration point with a
175 uniform prior from 31 to 33 MYA. Each HVSI and *cytochrome b* runs took 10 million
176 MCMC iterations sampled every 1,000 steps after a 10% initial burn-in. We checked the
177 results in the software Tracer1.4 (<http://beast.bio.ed.ac.uk>).

178 *N_e estimations* – Two approaches were used for estimating N_e for *C. intermedia*: the
179 variance based N_e (N_{eV}) and the inbreeding based N_e (N_{eI}) (see Leberg 2005 for a review). For
180 the first approach, we divided our *C. intermedia* sample in two temporal separated sets. The
181 first generation comprised 23 individuals collected in April 2004; the second, 17 individuals

182 in March 2005. The time between these two samples (one year) is enough for fitting three new
183 offsprings according to previous studies (Salvador & Fernandez 2008a), but also may contain
184 individuals from previous generations, so we assumed to have in average two separated
185 generations (i.e. a generation time of six months). The N_{eV} calculations were made using three
186 approaches: the Nei & Tajima (1981) moment-based and the maximum-likelihood (ML)
187 estimates with the marginal-probability method (Wang 2001), both implemented in the
188 program MLNE (Wang & Whitlock 2003), and a Monte Carlo coalescent-based search
189 (Berthier *et al.* 2003) using CoNe program (Anderson 2005). We ran the ML MLNE analysis
190 assuming no migration from any source population, maximum N_e of 10,000, and the number
191 of generations between samples of two. For CoNe, we assumed a uniform prior for N_e
192 between 4 and 10,000, and we ran 100,000 Monte Carlo replicates which should be enough
193 for acquiring confident results according to the program manual (Anderson 2005).

194 The N_{eI} estimation consisted of two search strategies, the first being a ML method for
195 estimating θ implemented in the program Lamarc 2.1.3 (Kuhner 2006). After trial searches,
196 Lamarc was run with 10 short chains and two long chains of 10,000 and 200,000 iterations,
197 respectively, using the stepwise mutational model (SMM) (Kimura & Ohta 1978) and the
198 variable loci only. Estimates of N_e were made using the mutation rate, $\mu=5 \times 10^{-4}$ in the
199 equation $N_e = \theta/4\mu$.

200 The second approach used for estimating N_{eI} was the recently developed approximate
201 Bayesian computation (ABC) as implemented in the programs DIYABC (Cornuet *et al.* 2008)
202 and ONeSAMP (Tallmon *et al.* 2008). In DIYABC, we tested three scenarios consisting of
203 one population with different priors for N_e . Scenario 1 had N_e between 4 and 100 individuals;
204 Scenario 2, between 100 and 1,000; and Scenario 3, between 1,000 and 10,000 individuals.
205 We assumed the 12 STR loci to evolve under the more realistic generalized stepwise mutation

206 model (GSM) (Estoup *et al.* 2002) which is a simplified version of the two-phase mutation
207 model (TPM) (Di Rienzo *et al.* 1994), with mutation rate free to vary from 1×10^{-4} to 1×10^{-2}
208 and the coefficients of geometric distribution (P) from 0.1 to 1. Motif sizes and prior allele
209 ranges were adjusted to fit our real loci. The summary statistics recorded for each simulation
210 were the mean (i) number of alleles (A), (ii) genetic diversity (H_e), (iii) allelic size range
211 (AR), and (iv) Garza-Williamson's index (M) (Garza & Williamson 2001). After preliminary
212 simulations for adjusting the priors, we ran 17,000,000 simulations for testing between the
213 alternative models and estimation of the posterior probability distribution of the parameters.
214 We assessed the posterior probability of each scenario using both direct estimate and logistic
215 regression approaches using the 500 and 5,000 best simulations respectively. The best
216 simulations under an ABC approach are the ones with the closest values of summary statistics
217 to the observed data's summary statistics. For the best supported scenario, we estimated the
218 posterior distribution of the parameters N_e , θ , μ and P using the logit transformation for the
219 5000 best simulations. In addition to the DIYABC estimations, we performed a N_e estimation
220 in ONeSAMP assuming the N_e prior limits (4-100) according to the most supported scenario
221 in DIYABC. We used only the variable loci in this analysis because ONeSAMP cannot deal
222 with monomorphic data.

223 Additionally to the historical and current N_e estimations, we tested for recent ($t < 4N_e$
224 generations) bottlenecks in *C. intermedia* using the program Bottleneck version 1.2.02 (Piry *et*
225 *al.* 1999). Based on the observation that recently occurring population declines cause rarer
226 alleles to disappear more rapidly than others, this approach is designed to test for the excess of
227 expected heterozygosity in the population following the rationale presented in Luikart &
228 Cornuet (1999). We applied these tests for the two different generations used in the N_{ev}
229 estimations as well as the whole sample across the generations used in the N_{el} estimations. For

230 both sampling strategies, we ran 1000 replications assuming different mutational models
231 [SMM, TPM and IAM (Kimura & Crow 1964)].

232

233 **Results**

234 *Genetic Diversity* – We obtained HVSI sequences for 28 *C. intermedia* and 25 *C.*
235 *magna* individuals. In the 368 nucleotide positions sequenced, we found a single haplotype
236 for *C. intermedia* and five for *C. magna* with 13 variable sites in the whole. This and other
237 summary statistics are presented in the Table 1. For the 12 STR loci, we genotyped all 70 *C.*
238 *intermedia* individuals, focused in individuals from at least two separated generations, plus 21
239 *C. magna*, including the two specimens from the probably closest population to the insular
240 species (from Pinheira/SC). The average missing data was 3.5% per locus. There were only
241 four variable loci in *C. intermedia* while all 12 loci were variable to *C. magna*. Both the STR
242 and HVSI summary statistics show a remarkable discrepancy between the genetic diversity of
243 the two species in which the insular cavy is far less variable than its continental sibling in all
244 studied loci (Table 1).

245 For the fixation indices between the two species using the STR loci, we have found an
246 average F_{ST} of 0.704 and an R_{ST} value of 0.935 where both were highly significant ($p < 0.001$).
247 Furthermore, a large number of private alleles (88%) were found between the species. For the
248 HVSI and *cytochrome b* mtDNA regions, the mean uncorrected divergences between the two
249 species are 6.7% and 1.2%, respectively. In the HVSI haplotype network (Figure 2), there are
250 11 mutational events separating the closest haplotypes of the two species, indicating a
251 complete isolation and a strong divergence between the two species using both kinds of
252 markers. *C. magna* presents a much higher diversity with very divergent haplotypes that are
253 geographically structured, where the northern samples (from SC in front of Moleques do Sul

254 Islands) are genetically closest to *C. intermedia* (Figure 2). These results support the
255 hypothesis of *C. intermedia* isolation from the nearly distributed populations of *C. magna*.

256 *Divergence Time* – Assuming a calibration point for the Hystricomorpha radiation as
257 32 MYA (Wyss *et al.* 1993, Huchon & Douzery 2001), we were able to estimate the
258 divergence time between the *C. intermedia* and the *C. magna* mtDNA lineages. Using both
259 the HVSI and the *cytochrome b* partial sequences independently, the separation time between
260 both species were 1.73 and 1.41MYA respectively. The 95%HPD intervals were from 0.72 to
261 3.08 MYA for the HVSI; and 0.38 to 3.15 MYA for the *cytochrome b*. In Figure 3, we show
262 the *cytochrome b* Bayesian phylogenetic tree with the above time estimates. Both the
263 *cytochrome b* and HVSI (not shown) phylogenies recovered virtually the same topology
264 except for the positioning of the capybara (*H. hydrochaeris*) which grouped with the genus
265 *Cavia* in the HVSI reconstruction. Furthermore, using the 32 MYA calibration point, the
266 posterior estimates for the mean mutation rates for each region were very similar: 0.77% per
267 million years for the *cytochrome b*, and 1.13% per million years for the HVSI. Also, these
268 estimates – especially for the *cytochrome b* – are very close to the conventional value of 1%
269 per million years for the mtDNA mutation rate (Avice 2000).

270 *Effective Population Size* – The estimation of the *C. intermedia* current effective
271 population size (N_{eV}) were very consistent among the different methods and provided values
272 (Table 2) very close to the observed N_c of 42 individuals (Salvador & Fernandez 2008a).
273 Furthermore, the comparison of the three tested scenarios for N_{eI} strongly supports the
274 scenario with the smallest population size (Figure 4). In this scenario, the median posterior
275 estimate for N_e is 29 (Table 2, Figure 5). The estimated historical population sizes (N_{eI}) are
276 only slightly smaller than the N_{eV} (Table 2) suggesting that no substantial variation in the
277 species' population size might have happened in its recent evolutionary past. This hypothesis

278 is supported by the tests in the program Bottleneck in which none of the Wilcoxon signed
279 tests were significant independently of the different mutation models and sampling strategies
280 used.

281

282 **Discussion**

283 Our mtDNA divergence time estimations have shown that the *C. intermedia* haplotype
284 diverged from the *C. magna* lineage at least ~0.4 million years in the past. Although this
285 mtDNA divergence likely predates *C. intermedia* speciation for an unknown period of time,
286 the very high divergence found in the microsatellite data, the clear morphological and
287 cytological differences all strongly suggest that the insular species diverged from its
288 continental sister-species very long ago, tens of thousands of years before the present.

289 *C. intermedia* population size was quite stable as observed during 15 months in
290 Salvador & Fernandez (2008a). All our results corroborate this scenario of high population
291 size stability, very likely for a long period, since both the historical and current N_e estimations
292 (N_{eI} with the N_{eV} , Table 2) provided very similar results and all genetic results supported
293 demographic stability. In special, there is no evidence for a recent population bottleneck, that
294 is, that the population size of the species was larger anytime in the past than its present size.
295 Although the absence of variability in the mtDNA of this species may be explained by a
296 selective sweep, all results from the nuclear microsatellites strongly corroborate that its low
297 diversity is a consequence of the population demography. Moreover, *C. intermedia* seems to
298 maximize its N_e . The N_{eV} assessments are not different from N_e estimated in Salvador &
299 Fernandez (2008a), around 40-45 individuals. This maximization is probably due to the
300 demographic characteristics noticed in Salvador & Fernandez (2008b) as part of the island
301 syndrome (Adler & Levins 1994). The population tends to keep its sex ratio always close to

302 1:1 and most of the population is composed by sexually mature individuals, increasing the
303 effective breeding number and making the N_{eV} to closely approach the N_c . Most of these
304 features could have been fixed by selection and are common among other rodent island
305 endemic species (Glivitz 1980, Crowel 1983, Adler 1996).

306 Considering these results, one question that rises is for how long this species
307 maintained this extremely reduced population size? The island itself is very small (10.5 ha)
308 and has a very limited resources supply (the area actually occupied by the grasses that are the
309 main food of the species is only 0.77 ha) and hardly could support a much bigger population
310 than it actually does. In fact, it has been shown that the *C. intermedia* population size is
311 regulated by density-dependent mortality (Salvador & Fernandez 2008a). Besides, there is no
312 support for a any significant change in the area of the island in the recent past, considering the
313 island morphology and the bathymetric data (Cherem *et al.* 1999), and given that the sea level
314 have not changed more than about 2 m in at least the last 7,000 years (Pirazzoli & Pluet 1991,
315 Milne *et al.* 2005). This is also very likely valid for the vegetation coverage of the Island.
316 Migration is also very unlikely; the closest *C. magna* population is in the continent 14 km
317 over sea away from Moleques do Sul. Additionally, there is no evidence of occurrence of any
318 *Cavia* species in the few other islands in the nearby area. This includes the larger island of
319 Florianopolis (Figure 1) where several rodent species currently occur (Graipel *et al.* 2001) or
320 were found to occur before modern human occupation in the last centuries as found in
321 zooarcheological studies (Castilho & Simões-Lopes 2001). Furthermore, *C. intermedia* itself
322 was found only in Moleques do Sul to date, and it is very unlikely to be found anywhere else
323 in the continent, since it was never observed in any study in the nearby region (Cherem &
324 Perez 1996, Castilho & Simões-Lopes 2001, Graipel *et al.* 2001, Cherem *et al.* 2004, Graipel
325 *et al.* 2006) and the coastal region is dominated by the sister *C. magna*. All this suggests that

326 the *C. intermedia* likely originated in the Moleques do Sul Island as a peripheral population of
327 *C. magna* and that has remained isolated there for at least 8,000 years. In summary, our
328 results suggest that this single population of the species may have maintained an extremely
329 small size (<100 individuals) for several thousand years.

330 It also seems that inbreeding depression is not taking place in *C. intermedia*. Most
331 individuals look healthy and there is no evidence of high young mortality (Salvador &
332 Fernandez 2008a). This might be due to a long term purging of the species gene pool through
333 purifying selection given its very low population size and long time in isolation (Frankham *et*
334 *al.* 2002). The maximization of its N_e described above should have helped it to survive for a
335 longer time, although an effective size of ~40 for an isolated population is still extremely low,
336 making this uniquely lucky species still at serious risk of extinction.

337 Along with its extremely reduced population size, *C. intermedia* shows particularly
338 low indices of genetic diversity. Its sister taxon, *C. magna*, is at least seven times more
339 diverse (H_e) for the microsatellite loci. Furthermore, *C. intermedia* shows a very limited
340 genetic diversity even if we compare to other mammals with naturally reduced diversity as the
341 African cheetah (Marker *et al.* 2008), the South-American maned wolf (SLB and
342 collaborators, unpublished results), or the brown bears (Paetkau *et al.* 1998). However, these
343 species have population sizes of reasonable sizes, having recovered from ancient (thousands
344 of years ago) population bottlenecks. Reduced diversities as that we found here for the
345 Moleques do Sul cavy were found to date in species that suffered extreme decline caused by
346 recent (tens to a few hundreds years) anthropic impacts, such the Hawaiian monk seal
347 (Schultz *et al.* 2009) and the northern elephant seal (Hoelzel *et al.* 1993, Weber *et al.* 2000).
348 However, the Madagascar fish-eagle (Johnson *et al.* 2009) is perhaps the only species so far in
349 which there is convincing evidence for a long period of very reduced genetic diversity

350 coupled with naturally very small population abundance. Its present abundance is at least 220
351 individuals over a relatively large range in northwestern Madagascar coast, and this number is
352 certainly an underestimate, given that there are regions that were not yet thoroughly surveyed
353 (www.unep-wcmc.org/species/data/species_sheets/fisheagl.htm,
354 www.iucnredlist.org/details/144338). Anyway, our results suggest that *C. intermedia*
355 underwent a similar history of long-term reduced size, although at much more extreme
356 conditions.

357 All the above results led to the relevant question of how *C. intermedia* could have
358 survived for so long considering the *a priori* prediction of high probability of extinction? We
359 hypothesize that *C. intermedia* overcame deleterious inbreeding effects a long time ago and
360 genetic purging is probably the answer. This purifying selective force acts on inbred
361 populations by sieving the individuals expressing the recessive deleterious genes present in
362 the population gene pool (Frankham *et al.* 2002, Crnokrak & Barrett 2002, Frankham 2005).
363 So, the Moleques do Sul cavy does not suffer with inbreeding depression because, very
364 probably, very few or no deleterious genes continue to exist in this population. However,
365 genetic diversity is also important for keeping evolutionary potential (Frankham *et al.* 2002).
366 A changing environment poses new selective pressures to the populations over time. For
367 better adapting to these changes, a population should have a reservoir of genetic possibilities
368 (Franklin & Frankham 1998). However, *C. intermedia* seems to face a very stable
369 environment in the last ~6,000 years (Scheel-Ybert 2000) Therefore, it probably did not have
370 to deal with changing selective pressures during most part of its isolation, making ineffective
371 the need for evolutionary potential in its case.

372 In summary, we found that *C. intermedia* presents an extremely reduced neutral
373 genetic diversity that is very likely a consequence of thousands of years of an extremely small

374 population size and that it likely survived given the relative environmental stability and got
375 over inbreeding depression through purging. This scenario brings *C. intermedia* as a very
376 special case in the evolutionary literature as possibly the only known living mammal species
377 with a long history with such an extremely reduced population size, making it an excellent
378 textbook case for studying the consequences of very long reduced population sizes in the
379 natural habitat. It is important to further study this extraordinary species, such as to better
380 characterize its genetic diversity at the genomic level and in special at non-neutral loci, such
381 as the MHC loci (and other loci subject to balancing selection) to better understand its
382 extreme evolutionary history. Also, we would like to reinforce the need for greater care
383 against the threats this population might be subject to, like irregular human visits to the island
384 and introduction of predators or competitors which, in spite of any possible genetic
385 adaptation, might suddenly lead this remarkable species to extinction.

386

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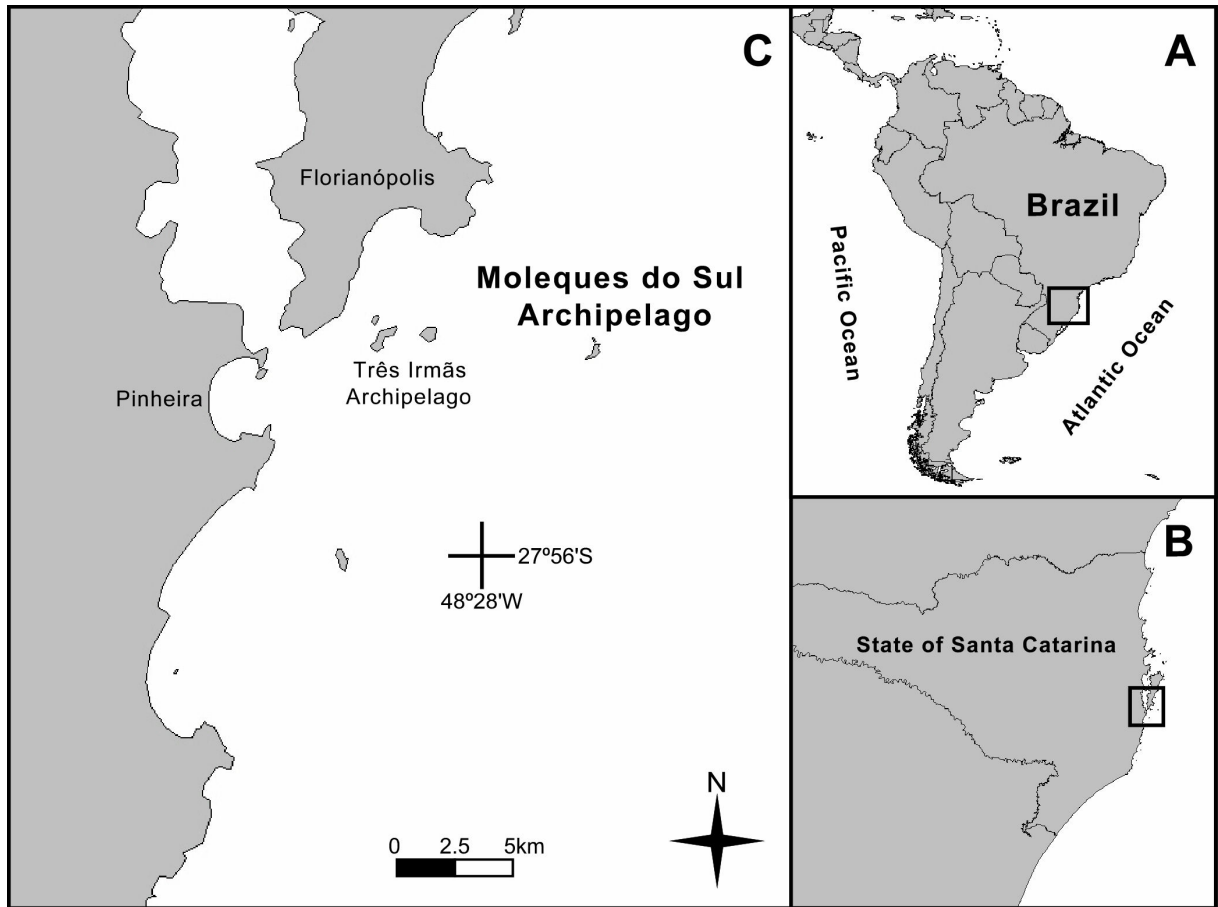


Figure 1: Localization of the Moleques do Sul Archipelago in Southern Brazil. The map in **A** shows a wider continental view with a square augmented in map **B**. Map **C** is the magnified square shown in **B**. All maps have the same orientation.

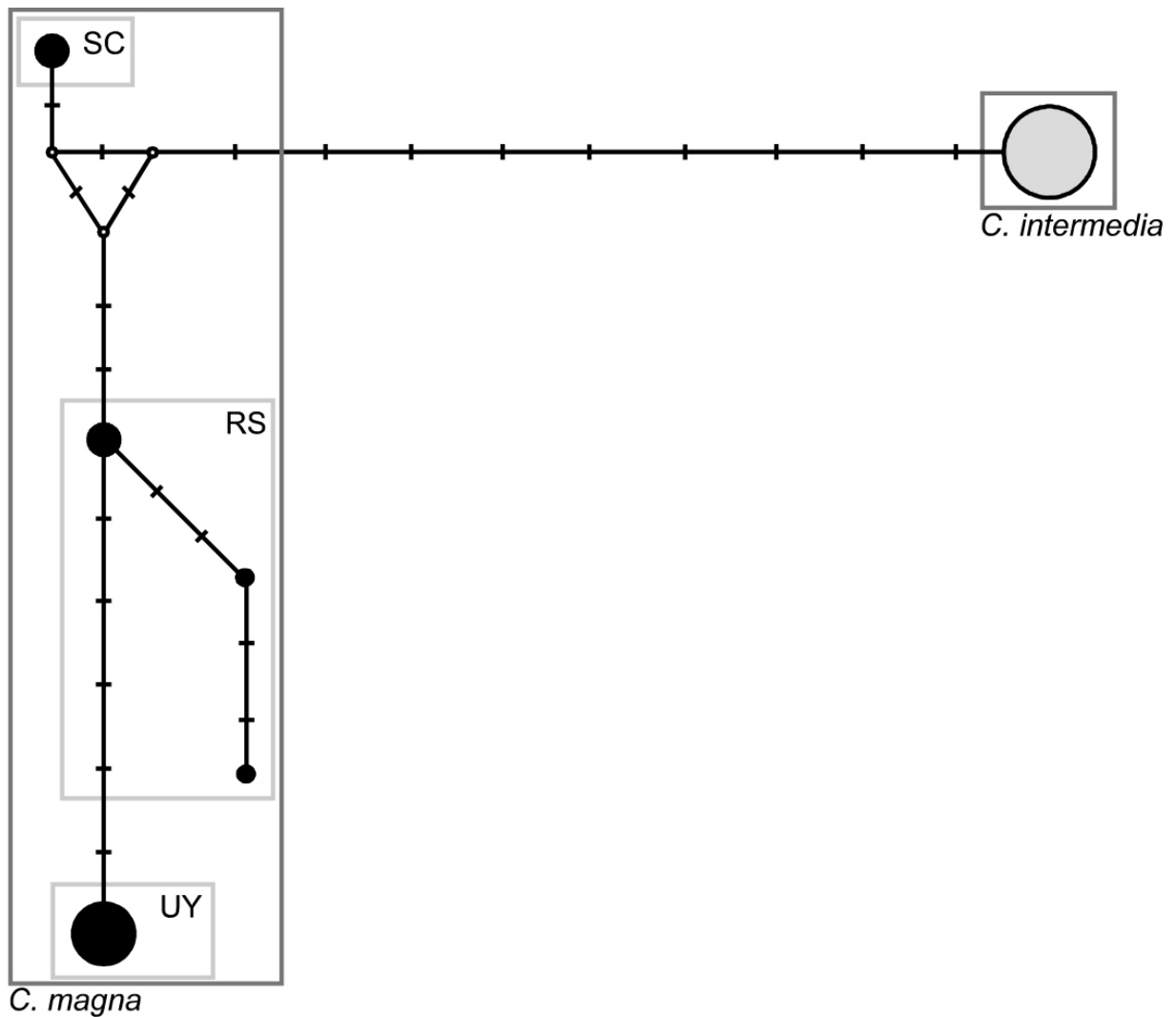


Figure 2: Median-Joining Network tree showing the relationship among the *C. intermedia* (in gray) and *C. magna* (black) HVSI haplotypes. Nodes are proportional to number of individuals, except for the empty nodes which represent missing haplotypes. Slashes represent mutational events. *C. magna* (SC) are from the State of Santa Catarina closest to Moleques do Sul; *C. magna* (RS) are from the State of Rio Grande do Sul; and *C. magna* (UY) are from northern Uruguay.

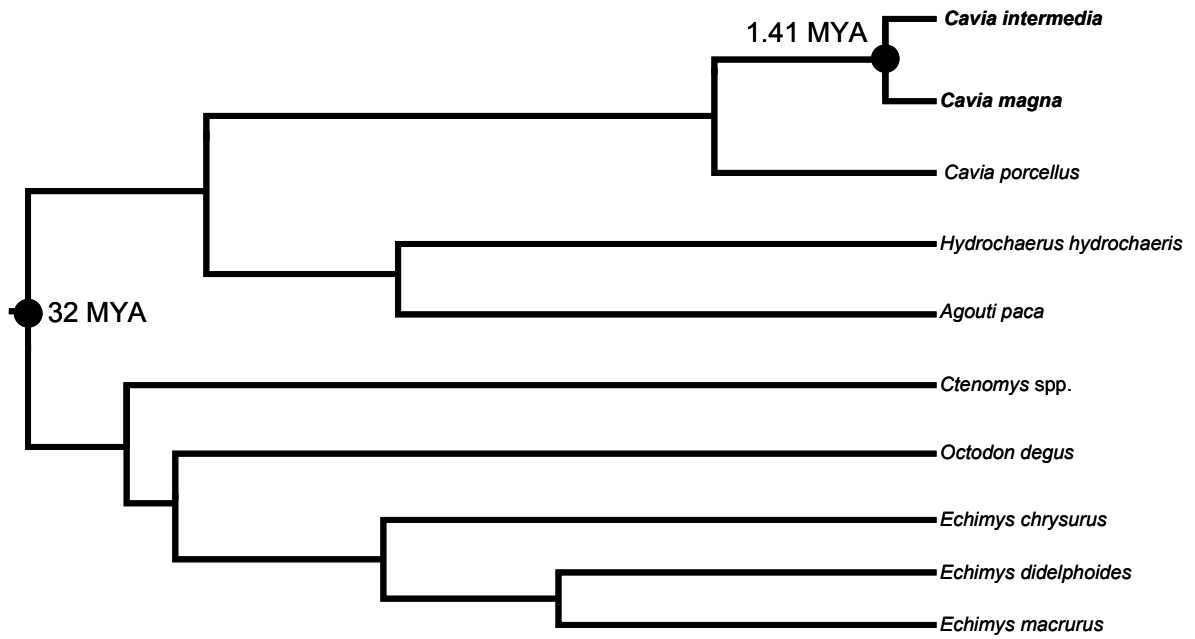


Figure 3: *Cytochrome b* Bayesian phylogenetic tree with the Hystricomorpha diversification date (32 MYA) in Wyss *et al.* (1993) and Huchon & Douzery (2001) as a calibration point.

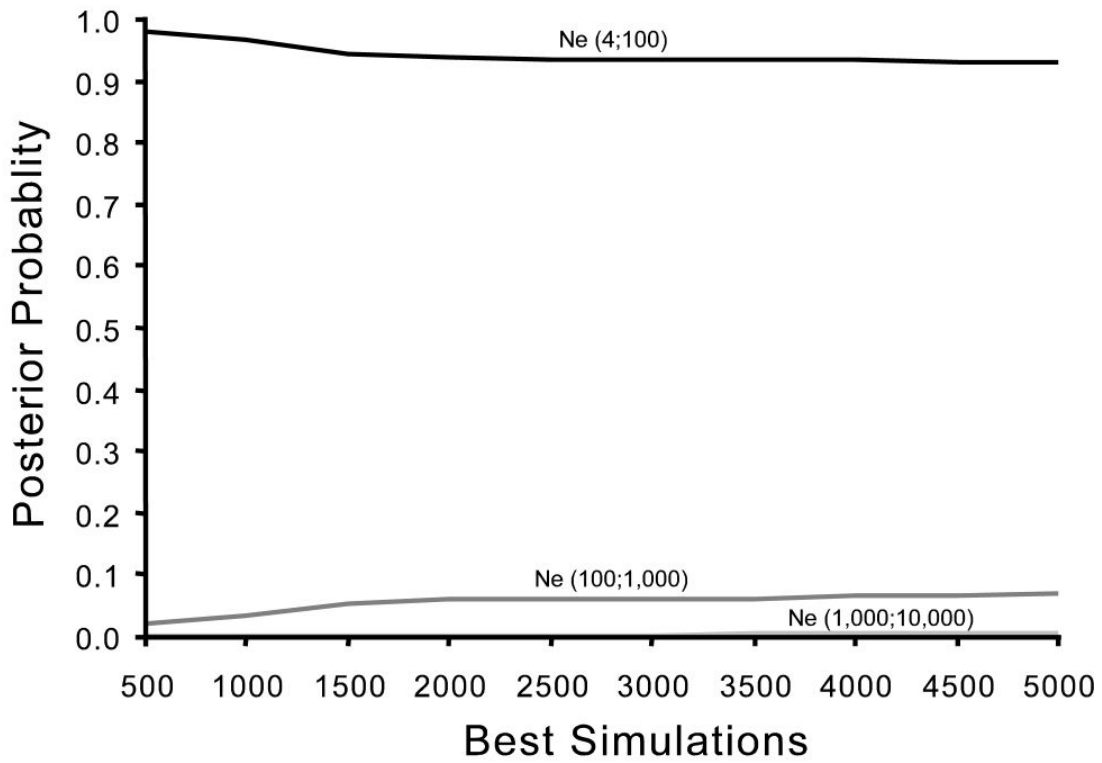


Figure 4: Posterior probability of alternative scenarios in different sets of closest-to-observed simulations based on the logistic regression. The black upper line refers to Scenario 1 (N_e between 4 and 100), the middle dark-gray line to the Scenario 2 ($N_e = 100-1,000$), and the lower light-gray line (very close to the X axis) to the Scenario 3 ($N_e = 1,000-10,000$).

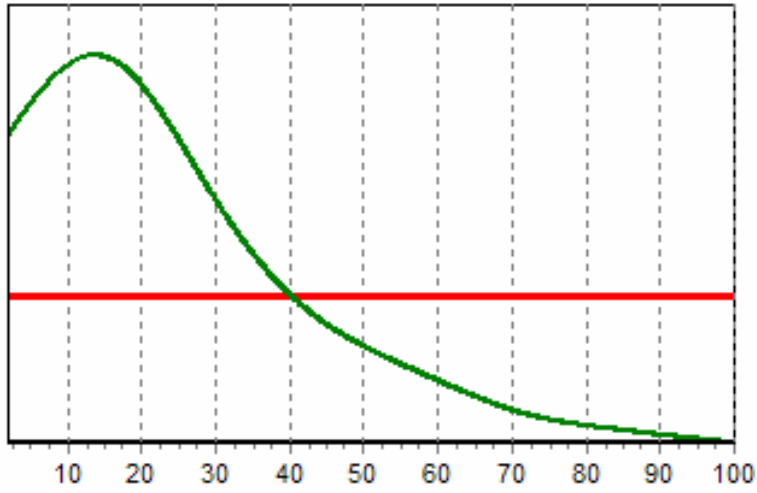


Figure 5: Posterior distribution (in green) of the parameter N_e for *C. intermedia* from the best supported scenario as estimated in the program DIYABC. The red line is the prior distribution for N_e .

Table 1: Comparative summary statistics for *C. intermedia* (*C.i.*) and *C. magna* (*C.m.*) for the 12 STR loci and the HVSI mtDNA region where loci named *Cavy#* are the STR loci from Kanitz *et al.* (in prep). “Mean” refers to the STR loci as well as the standard deviation (S.D.) below it. The summary statistics for the STR loci are number of alleles (**A**), allelic range (**AR**), expected heterozygosity (**H_e**), observed heterozygosity (**H_o**), Garza-Williamson index (**M**), and pair-wise fixation index (**F_{ST}**). For the mtDNA region, the statistics are the number of haplotypes (**h**), haplotype diversity (**H_d**), nucleotide diversity (**π**), Rho statistics (**ρ**), Tajima’s neutrality test (**Tajima’s D**) and distance-based fixation index (**Φ_{ST}**). Hardy-Weinberg Equilibrium significance are coded as * for $p < 0.05$ and ** for $p < 0.01$ in the H_0 statistics. NS in the Tajima’s D results means not significant ($\alpha = 0.05$). All **F_{ST}** and **Φ_{ST}** results were significant ($p < 0.01$).

Locus	A		AR		H _e		H _o		M		F _{ST}	
	C. i.	C. m.	C. i.	C. m.	C. i.	C. m.	C. i.	C. m.	C. i.	C. m.	C. i.	C. m.
Cavy1	1	10	0	35	0	0.721	0	0.444**	-	0.278	-	0.809
Cavy2	1	4	0	61	0	0.576	0	0.571	-	0.065	-	0.845
Cavy4	1	2	0	2	0	0.251	0	0.286	-	0.667	-	0.927
Cavy6	7	11	9	59	0.590	0.860	0.529*	0.800	0.700	0.183	0.700	0.279
Cavy7	1	3	0	2	0	0.286	0	0.316	-	1.000	-	0.929
Cavy8	1	5	0	14	0	0.747	0	0.533*	-	0.333	-	0.740
Cavy9	2	9	2	22	0.305	0.724	0.257	0.714	0.667	0.391	0.667	0.550
Cavy10	1	7	0	22	0	0.832	0	0.476**	-	0.304	-	0.756
Cavy12	1	3	0	7	0	0.535	0	0.714	-	0.375	-	0.683
Cavy13	1	5	0	11	0	0.670	0	0.571	-	0.417	-	0.652
Cavy14	4	10	3	29	0.155	0.782	0.149	0.700**	1.000	0.333	1.000	0.643
Cavy16	4	16	10	24	0.058	0.882	0.059	0.619**	0.364	0.640	0.364	0.639
Mean	2.08	7.08	2.00	24.00	0.0923	0.6554	0.0423	0.5676	0.6826	0.4155	0.6826	0.704
S.D.	1.93	4.19	3.64	19.71	0.1824	0.2086	0.0850	0.2000	0.2602	0.2485	0.2602	0.179
	h		Hd		π		ρ		Tajima's D		Φ_{ST}	
hvs1	1	5	0	0.4380	0	0.0122	0	4.3334	0 ^{NS}	-0.4002 ^{NS}	0	0.902
S.D.	-	-	0	0.0147	0	0.0034	0	1.6905	-	-	-	-

Table 2: Current (N_{eV}) and historical (N_{eI}) effective population size estimated for *C. intermedia*. N_{eV} assessments are based on two generations between samples. N_{eI} is calculated over all 70 individuals. “ N_e ” represents a direct estimation (for moment-based calculation), a central tendency measure (such as maximum-likelihood estimation in MLNE, CoNe and Lamarc), or a central tendency measure such the median for the DIYABC and ONeSAMP results. The “**lower**” and “**upper**” estimations are based on the 95% percentiles for maximum-likelihood methods and 95% posterior probability distribution for Bayesian inferences.

	N_{eV}			N_{eI}		
	Moment	MLNE	CoNe	Lamarc	DIYABC	ONeSAMP
Ne	38	51	47	30	29	27
lower	-	13	8	28	6	17
upper	-	∞	∞	34	94	52
Mean		45			29	

CONCLUSÃO GERAL

Obtivemos êxito na caracterização das regiões de microssatélites para o seu uso como marcadores moleculares nas espécies continentais *Cavia magna* e *C. aperea*. Esses marcadores também podem ser usados com boa chance de sucesso em outras espécies do gênero. Aplicando esses mesmo marcadores juntamente com duas regiões do DNA mitocondrial, nós pudemos mensurar a diversidade genética neutra de *C. intermedia* que era o principal objetivo deste estudo.

Além disso, obtivemos a confirmação da relação de táxons-irmãos entre *C. magna* e *C. intermedia*. Também calculamos o tempo de divergência entre essas duas espécies e pudemos concluir que *Cavia intermedia* parece realmente estar isolada em Moleques do Sul há pelo menos cerca de 8.000 anos desde seu mais recente isolamento em relação ao continente, sendo muito provavelmente uma separação bastante mais antiga.

O preá de Moleques do Sul também apresentou níveis baixíssimos de diversidade. Estes sem precedentes em outras espécies que não tenham sido recentemente perturbadas pela ação humana. Através dessa diversidade, também pudemos estimar os tamanhos efetivos populacionais histórico e atual da espécie, o que nos mostrou que ela parece manter esse ínfimo tamanho populacional há bastante tempo. Provavelmente, desde o seu isolamento na ilha em que hoje habita. Isso pode ter sido possível por diversas razões. *C. intermedia* não parece sofrer efeitos de depressão por endocruzamento por possivelmente ter expurgado alelos deletérios durante as fases iniciais de sua evolução em Moleques do Sul, além de aparentemente maximizar seu tamanho efetivo em relação ao tamanho de censo expressando características demográficas típicas de uma síndrome de ilhas (Adler & Levins 1994, Salvador & Fernandez 2008b).

Chamamos também à atenção o fato de que *C. intermedia* habita Moleques do Sul por período de relativa estabilidade ambiental na região (Scheel-Ybert 2000). Fato esse que deve diminuir a necessidade do chamado potencial evolutivo, comumente ausente em populações cuja diversidade é reduzida (Franklin & Frankham 1998). Entretanto, as atuais perturbações humanas

podem alterar radicalmente este cenário. Ações como caça (comum em espécies continentais), queimadas e introdução de competidores ou predadores podem causar sérios efeitos na diminuta população de *C. intermedia*. Portanto, ações educação da população e de turistas (Moleques do Sul é um ponto bastante conhecido por mergulhadores), assim como uma fiscalização constante do local que já pertence ao Parque Estadual da Serra do Tabuleiro podem permitir que essa curiosa espécie siga sua história evolutiva sem maiores danos diretos.

Estudos mais aprofundados sobre a diversidade genética desta espécie deverão ser feitos para melhor caracterizar a sua diversidade neutra e também sujeita à seleção (em loci de MHC, por exemplo). Além de um aprofundamento do estudo da diversidade da própria espécie insular, a ampliação da amostragem da espécie-irmã continental – *C. magna* – pode trazer ainda mais informação sobre a origem de *C. intermedia*.

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