



Population structure and genetic variability of mainland and insular populations of the Neotropical water rat, *Nectomys squamipes* (Rodentia, Sigmodontinae)

Francisca C. Almeida^{1,2}, Luana S. Maroja¹, Miguel A.M. Moreira², Héctor N. Seuánez^{1,2} and Rui Cerqueira³

¹Universidade Federal do Rio de Janeiro, Departamento de Genética, Rio de Janeiro, RJ, Brazil.

²Instituto Nacional de Câncer, Divisão de Genética, Rio de Janeiro, RJ, Brazil.

³Universidade Federal do Rio de Janeiro, Departamento de Ecologia, Rio de Janeiro, RJ, Brazil.

Abstract

Seven microsatellite loci were used to investigate the genetic variability and structure of six mainland and two island populations of the Neotropical water rat *Nectomys squamipes*, a South American semi-aquatic rodent species with a wide distribution. High levels of variability were found within mainland populations while island populations were less variable but the more differentiated in respect to allele number and frequency. The time of biological divergence between mainland and island populations coincided with geological data. A significant geographic structure was found in mainland populations ($\theta = 0.099$; $\rho = 0.086$) although the degree of differentiation was relatively low in respect to the distance between surveyed localities (24 to 740 km). Genetic and geographic distances were not positively correlated as previously found with random amplified polymorphic DNA (RAPD) markers. Significant but low genetic differentiation in the mainland and lack of isolation by distance can be explained by large population size and/or recent population expansion. Additionally, the agreement between the age of geologic events (sea level fluctuations) and divergence times for insular populations points to a good reference for molecular clock calibration to associate recent environmental changes and the distribution pattern of small mammals in the Brazilian Atlantic Forest.

Key words: microsatellites, population genetics, islands, population expansion, population size, divergence time.

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Introduction

The Neotropical water rat, *Nectomys squamipes* (Brants, 1827), is a semi-aquatic rodent species widely distributed along rivers and streams of the Atlantic Forest and Cerrado biomes in Eastern Brazil. Ecological studies showed that these rodents rarely move farther than 20 m from streams, their home range being restricted to a range of from 200 to 14,808 m² and 90% of displacements occurring only in the water or along riverbanks (Bergallo, 1995, MV Vieira, personal communication). These rodents rely on a generalist diet and the availability of riverbank habitats seems to be the only factor affecting their distribution that might also occur in the proximity of urban areas (Gentile, *et al.*, 2000), although their semi-aquatic habits may hinder migration between river basins and across mountain ranges.

Small non-flying mammals have been generally thought to show limited dispersal ability resulting in a pattern of structured genetic variability (Koenig *et al.*, 1996; Patton *et al.*, 1996). A six-year capture-recapture study of *N. squamipes* in a small area (3 km²) detected only 10 migrants (mostly males) out of 162 recaptured individuals with all the displacements occurring between adjacent sites (LS Maroja, personal observation). Microsatellite data, when analyzed in this population, showed significant, although minor, differentiation between collection sites (Maroja *et al.*, 2003a). Based on these results and theoretical models, wider areas would be expected to exhibit a higher degree of differentiation among sites. Contrary to these expectations, however, the morphology of *N. squamipes* was found to be fairly homogeneous across its wide geographic range (Bonvicino, 1994) and this low degree of differentiation between populations was confirmed by random amplified polymorphic DNA (RAPD) studies (Almeida *et al.*, 2000a). Since morphology may be subject to balancing selection and RAPD markers have well known

Send correspondence to Héctor N. Seuánez. Instituto Nacional de Câncer, Seção de Genética, Rua André Cavalcante 37, 4º andar, 20231-050 Rio de Janeiro, RJ, Brazil. E-mail: genetics@inca.gov.br.

limitations and are less efficient in detecting differentiation than microsatellite markers (Lynch and Milligan, 1994; Ross *et al.*, 1999; Loughheed *et al.*, 2000), we decided to use microsatellite markers to re-analyze the population samples previously studied using RAPD.

Although sigmodontine rodents are ecologically important and represent a significant portion of all South American mammalian species, only a few molecular studies have been conducted on the geographic variation of these rodents (*e.g.* Patton *et al.*, 1996; Loxterman *et al.*, 1998). To date, the only published report of a population study using microsatellites is that of Maroja *et al.* (2003a) who studied *N. squamipes* in a restricted area. The lack of taxonomic problems with this rodent along with its well-researched ecology and wide and well-defined geographic range make *N. squamipes* a good candidate for studies of population dynamics. Another important reason for studying the population genetics of *N. squamipes* is that it is a natural primary host for *Schistosoma mansoni*. Although it is apparently unaffected by this parasite, infected *N. squamipes* can potentially spread *S. mansoni* to uninfected areas (D'Andrea *et al.*, 2000; Maroja *et al.* 2003a).

In this paper we report an analysis of microsatellite data from five *N. squamipes* populations previously studied with RAPD along with data for one additional mainland and two insular *N. squamipes* populations. These islands were expected to have isolated populations which might be useful as comparisons for evaluating the role of dispersal in determining the genetic structure of mainland populations. To check whether the island populations were true isolates we calculated genetic distances and compared them with geological data on Holocene sea level fluctuations.

Materials and Methods

Population samples

One hundred and ten *Nectomys squamipes* were captured with Sherman or Tomahawk live traps in eight Brazilian localities (Figure 1), six mainland sites at Glicério (GL; $n = 26$); Fazenda União (FU; $n = 8$); Sumidouro (SU; $n = 18$), Tarituba (TA; $n = 21$) in Rio de Janeiro State; Pedreiras (PD; $n = 10$) in São Paulo State and Fazenda Canoas (FC; $n = 6$) in Minas Gerais State and two insular sites Ilha da Marambaia (IM; $n = 8$) and Ilha Grande (IG; $n = 13$) in Rio de Janeiro State. The number of specimens is shown in the parentheses. Voucher specimens were deposited in the Museu Nacional, Rio de Janeiro, Brazil. Samples from GL, FU, SU, PD and FC had previously been analysed using RAPD (Almeida *et al.*, 2000a). Geographic distances between mainland localities ranged from 23.7 to 741.5 km. Ilha Grande (IG) is 3.3 km from the mainland and has an area of 193 km² while Ilha de Marambaia (IM) is about four times smaller but is presently connected to the mainland by a small sand strip that probably appeared after the separa-

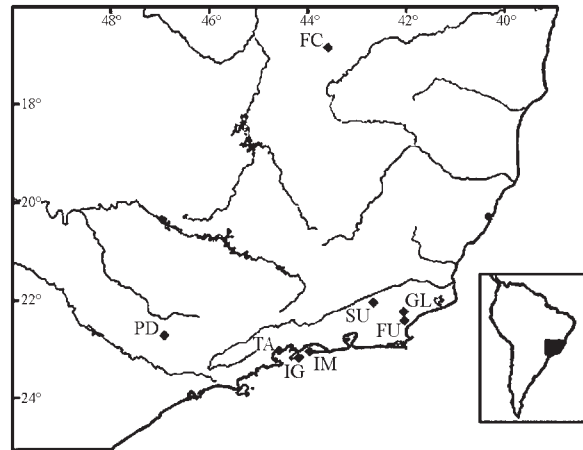


Figure 1 - Collection sites of *N. squamipes* in eastern Brazil.

tion of the island from the mainland some 7,000 years ago (Martins and Villwock, 1987; Cerqueira, 2000).

Most animals were sacrificed and their liver tissue aliquots were preserved in absolute ethanol. Blood was collected from the hind foot of several live Ilha Grande animals and kept frozen with EDTA until extraction. Total DNA was extracted from the liver samples following Smith *et al.* (1987) and from blood samples following Sambrook *et al.* (1989).

Microsatellite polymorphism analysis

Seven microsatellite markers (*Nec08*, *Nec12*, *Nec14*, *Nec15*, *Nec18*, *Nec23* and *Nec28*) were PCR amplified as previously described (Almeida *et al.*, 2000b; Maroja *et al.*, 2003b). The forward primer of each locus was labeled with a fluorescent dye (NED, HEX or 6-FAM) and the PCR products run in an automated ABI 377 sequencer with GeneScan 500-ROX (Applied Biosystems) as internal size standard and analyzed with the GeneScan program version 1.2.2 (Applied Biosystems).

Linkage disequilibrium between loci was tested with Fisher's exact test and the Markov chain procedure with 500,000 iterations and 1,000 de-memorization steps using the Genepop program version 3.4 (Raymond and Rousset, 1995a).

Microsatellite variation

For each population, the number of alleles (A), allelic richness (A_R ; El Mousadik and Petit, 1996), observed direct-count heterozygosity (H_{DC}) and unbiased expected heterozygosity (H_E ; Nei, 1978) were computed for each locus using the Fstat program version 2.9.3 (Goudet, 2001). Hardy-Weinberg equilibrium (HWE) was tested with Fisher's exact test and the Markov chain procedure (Guo and Thompson, 1992) with 1,000 de-memorization steps and 100,000 iterations using the Arlequin program version

2.0 (Schneider *et al.*, 2000). This test was performed separately for each population and each locus.

Population differentiation and isolation by distance

Genic (allelic frequency) differentiation between mainland populations was assessed with Fisher's exact test (Raymond and Rousset, 1995b) and genotypic differentiation between these populations using the log-likelihood G test (Goudet *et al.*, 1996), both statistics being calculated using the Genepop program version 3.4 (Raymond and Rousset, 1995a) and 1,000 iterations. Quantitative assessments of differentiation between the same populations were calculated with the F_{ST} and R_{ST} estimators. The R_{ST} statistic takes into account the stepwise mutation model (SMM) and for this reason should be more sensitive in detecting differentiation with microsatellite data (Slatkin, 1995). The F_{ST} estimator (θ) was obtained following Weir and Cockerham (1984) using Fstat 2.9.3, with mean, standard error and 95% confidence intervals (CI) calculated with permutations over loci. The R_{ST} (Slatkin, 1995) estimator (ρ) was calculated using significance values established with the RstCalc program version 2.2 (Goodman, 1997) using 1,000 permutations. Pairwise population estimates were calculated for both θ and ρ .

To estimate the contribution of the island populations to the global diversity of *N. squamipes* we carried out a three-level AMOVA (Analysis of Molecular Variance) using the Arlequin program version 2.0. Partitions of microsatellite data variance within populations, between populations and between the mainland group and the island group were calculated according to Excoffier *et al.* (1992). Differences in genetic diversity between mainland populations on one side, and insular populations on the other were verified by comparing allelic richness and observed heterozygosity with a permutation test using Fstat 2.9.3.

Following Rousset (1997), isolation by distance (IBD) between mainland populations was tested by comparing $\theta/1 - \theta$ with linear geographic distances between populations with the Mantel test using Genepop 3.4 (2,000 permutations).

Divergence time of insular populations

The divergence time of the IG and IM populations was estimated as $T = (\delta\mu)^2/2\nu$ (Goldstein *et al.*, 1995) where T is the divergence time in number of generations, ν is the mutation rate and $(\delta\mu)^2$ is a genetic distance estimate for microsatellite data that takes into account the SMM (calculated with RstCalc 2.2). We calculated $(\delta\mu)^2$ as the average of pairwise estimates between mainland and island populations and considered the mutation rate to be the same as for the *Mus musculus* microsatellite loci, *i.e.* 4.5×10^{-5} per generation (Dietrich *et al.*, 1992). A generation time of 110 days was assumed based on data from captive populations (D'Andrea *et al.*, 1996).

Sequential Bonferroni correction (Rice, 1989) was used for assessing statistical significance in all multiple tests.

Results

All loci analyzed in this study were highly polymorphic, with the total allele number ranging from 12 to 32 (Table 1). The *Nec23* locus had the largest number of alleles in the total count and within every population except one (TA). No significant linkage disequilibrium was found between any pair of loci either when pooling all populations or when considering each population separately. Genetic diversity within each population was found to be high, with allele number per locus varying from 1 to 17, and roughly related to sample size (Table 1). Private alleles were observed in all populations: seven in GL (in *Nec12*, *Nec14*, *Nec15*, *Nec18*, *Nec23*), five in PD (in *Nec12*, *Nec15*, *Nec18*), four in FU (in *Nec14*, *Nec15*, *Nec23*), six in FC (in *Nec08*, *Nec14*, *Nec15*, *Nec23*), six in SU (in *Nec08*, *Nec15*, *Nec18*, *Nec23*), four in TA (in *Nec12*, *Nec14*, *Nec15*), two in IM (in *Nec23*), and two in IG (in *Nec08*). The highest number of private alleles (10) was found at *Nec23* while *Nec28* did not show any private allele.

Mean H_E was very high in all mainland populations, averaging 0.82 with a range of 0.625 to 0.970 (Table 1). The highest H_E value was found in the FC population despite it having the smallest sample size. The FC population was excluded from estimates of allelic richness so that this parameter could be calculated using a minimum of eight individuals instead of six, increasing its confidence (Table 1).

Departures from HWE were detected in three cases (GL, *Nec14*; FC, *Nec08*; IG, *Nec15*) but these results were not statistically significant following the Bonferroni correction; for the remaining analyses we assumed HWE within populations. These slight departures in specific loci can be accounted for by the presence of population specific null alleles. Since PCR amplifications were generally successful, null alleles were probably rare. An exception to this occurred in the *Nec15* locus in the IG population for which three individual DNA samples could not be amplified and two different homozygous genotypes were found but heterozygotes were completely absent.

Both genic and genotypic differentiation between mainland populations were highly significant in each locus and across loci ($p < 0.0005$). The *Nec23* locus has a gap of approximately 40 bp between two consecutive alleles and therefore is not suitable for analysis assuming SMM so we excluded this locus from the R_{ST} and F_{ST} estimates in order to make them comparable. The θ estimator of F_{ST} was 0.100 ± 0.012 (SE) with a 95% CI = 0.082 - 0.123, while the ρ estimator of R_{ST} was 0.086 ± 0.003 (SE) with a 95% CI of 0.074 - 0.172 (both at $p < 0.0005$). No

Table 1 - Genetic diversity of *Nectomys squamipes* populations.

Population (number of specimens)		Microsatellite locus								Mean	SD
		<i>Nec08</i>	<i>Nec12</i>	<i>Nec14</i>	<i>Nec15</i>	<i>Nec18</i>	<i>Nec28</i>	<i>Nec23</i>			
Glicério (26)	A (A_R)	8 (6.20)	14 (8.61)	10 (6.89)	10 (7.23)	14 (8.97)	9 (6.87)	17 (8.99)	11.7 (7.68)	0.5	
	H_{DC}	0.769	0.923	0.654	0.846	0.846	0.885	0.846	0.824	0.013	
	H_E	0.814	0.883	0.811	0.827	0.911	0.848	0.903	0.857	0.006	
Fazenda União (8)	A (A_R)	3 (2.80)	5 (4.94)	8 (7.53)	6 (5.73)	10 (8.56)	7 (6.00)	11 (10.21)	7.1 (6.54)	0.4	
	H_{DC}	0.40	0.800	0.900	0.600	0.900	0.700	0.778	0.725	0.026	
	H_E	0.574	0.794	0.889	0.778	0.828	0.633	0.928	0.775	0.018	
Pedreiras (10)	A (A_R)	5 (5.00)	8 (8.00)	3 (3.00)	7 (7.00)	5 (5.00)	6 (6.00)	9 (9.00)	6.1 (6.1)	0.3	
	H_{DC}	0.625	0.750	0.625	1.000	0.500	0.750	1.000	0.750	0.107	
	H_E	0.808	0.813	0.625	0.848	0.679	0.821	0.917	0.787	0.014	
Fazenda Canoas (6)	A	4	7	6	8	7	7	10	7	0.2	
	H_{DC}	0.167	1.000	1.000	0.833	0.833	0.833	1.000	0.809	0.042	
	H_E	0.682	0.917	0.800	0.933	0.900	0.833	0.970	0.862	0.014	
Sumidouro (18)	A (A_R)	6 (4.72)	10 (7.76)	9 (7.02)	7 (5.99)	11 (8.05)	6 (4.65)	13 (8.45)	8.9 (6.66)	0.4	
	H_{DC}	0.778	0.889	0.833	0.722	0.889	0.778	0.722	0.802	0.010	
	H_E	0.732	0.882	0.837	0.832	0.874	0.706	0.870	0.819	0.010	
Tarituba (21)	A (A_R)	5 (4.46)	8 (6.30)	8 (7.11)	9 (6.91)	12 (8.78)	6 (5.44)	8 (5.59)	8.0 (6.37)	0.3	
	H_{DC}	0.571	0.809	0.952	0.857	0.857	0.905	0.762	0.816	0.018	
	H_E	0.726	0.837	0.870	0.858	0.911	0.812	0.757	0.824	0.009	
Ilha da Marambaia (8)	A (A_R)	3 (3.00)	1 (1.00)	1 (1.00)	5 (5.00)	2 (2.00)	5 (5.00)	10 (10.00)	3.9 (3.9)	0.5	
	H_{DC}	0.750	0.000	0.000	0.625	0.125	0.875	1.000	0.482	0.061	
	H_E	0.625	0.000	0.000	0.777	0.125	0.759	0.942	0.461	0.058	
Ilha Grande (13)	A (A_R)	2 (1.86)	3 (2.72)	5 (4.56)	2 (2.00)	4 (3.95)	5 (4.52)	7 (6.03)	4.0 (3.66)	0.3	
	H_{DC}	0.154	0.308	0.615	0.000	0.923	0.692	0.692	0.483	0.048	
	H_E	0.148	0.282	0.763	0.356	0.728	0.734	0.800	0.544	0.039	
All	A	13	25	16	18	21	12	32	19.4	7.3	

A = number of alleles; A_R = allelic richness; H_{DC} = direct-count heterozygosity; H_E = expected heterozygosity; SD = standard deviation.

correlation between genetic and geographic distance was apparent ($p = 0.54$).

The island populations showed significantly lower genetic diversity when compared to the mainland populations, both in terms of A_R and H_{DC} ($p = 0.032$ and $p = 0.027$, one-sided probability). Although we grouped both islands when comparing them with the mainland group, these results are valid for both insular populations because they showed very similar estimates of allelic richness and proportion of heterozygotes. The lower variability of island populations reached an extreme value in the IM population, which was monomorphic at two loci (*Nec12* and *Nec14*) and was the only case of monomorphism. Nevertheless, the genetic diversity found in the two island populations accounted for 6.4% of the total diversity. Variation between populations within each group, mainland and islands, accounted for 14.03% of the total variation. Pairwise estimates of F_{ST} also confirmed the high degree of differentia-

tion of the island populations (Table 2). While pairwise θ values between mainland populations ranged from 0.045 to 0.145, pairwise values involving the island populations ranged from 0.224 to 0.469. The ρ estimator, although lower than θ in mainland comparisons, indicated even higher differentiation in the islands, with values ranging from 0.340 to 0.623 in pairwise comparisons including insular populations. This result is in accordance with the proposition that R_{ST} indexes are more sensitive in comparisons between populations that have been isolated from each other for a longer time (Slatkin, 1995).

Mean genetic distance ($\delta\mu^2$) between IG and the mainland populations was 1.907 ± 0.175 (\pm standard deviation) corresponding to $21,196 \pm 1,941$ generations and to a divergence time of $7,065 \pm 647$ years. Similar estimates between IM and mainland populations showed a mean $(\delta\mu)^2 = 0.896 \pm 0.192$ corresponding to $9,955 \pm 2,134$ generations and to $3,319 \pm 711$ years.

Table 2 - Pairwise estimates of θ (above diagonal) and ρ (below diagonal) between *N. squamipes* populations. Values in bold are not significant (Bonferroni correction).

Population	GL	FU	SU	TA	PD	FC	IM	IG
GL		0.086	0.090	0.098	0.089	0.046	0.277	0.237
FU	0.169		0.095	0.142	0.141	0.089	0.333	0.278
SU	0.079	0.077		0.099	0.119	0.071	0.282	0.266
TA	0.108	0.208	0.062		0.127	0.106	0.254	0.260
PD	-0.011	0.238	0.124	0.104		0.109	0.333	0.306
FC	0.046	0.027	0.027	0.021	0.038		0.304	0.287
IM	0.340	0.340	0.435	0.511	0.425	0.406		0.472
IG	0.560	0.536	0.583	0.534	0.606	0.520	0.623	

Population key: GL= Glicério; FU = Fazenda União; SU = Sumidouro; TA = Tarituba; PD = Pedreiras; FC = Fazenda Canoas IM = Ilha da Marambaia; IG = Ilha Grande.

Discussion

Microsatellite data indicate little differentiation between mainland populations of *Nectomys squamipes* located between 23 to 740 km apart. Differentiation estimates were very low even when compared to data of other rodent species (Stewart *et al.*, 1999; Loxterman *et al.*, 1998; Wlasiuk *et al.*, 2003). A likely explanation for our results is that gene flow might be higher than suggested by field studies. The greater differentiation of island populations, in view that they are isolated from any other, may support this hypothesis. However, an alternative explanation that does not conflict with predictions and field estimates of *N. squamipes* dispersal ability is also possible, and low estimates of differentiation might be a consequence of a recent rapid range expansion and/or very large local populations resulting in the absence of migration-drift equilibrium (Kimura and Weiss, 1964; Slatkin, 1989; Slatkin, 1993; Neigel, 1997; Whilock and McCauley, 1999). Lack of isolation by distance, as found in our data, is typical of populations that have not yet attained equilibrium (Hutchinson and Templeton, 1999).

It has been theoretically demonstrated that populations with a large size will attain equilibrium (basically determined by drift) in a very long time, proportional to the size of the population (Slatkin, 1993; Neigel, 1997; Whilock and McCauley, 1999). The high degree of variability observed by us indicates fairly large population sizes on the mainland (Estoup *et al.*, 1995). Another reason for populations not being in equilibrium could be a recent population expansion. The isolation by distance trend (although not significant) found in a considerably smaller area (Maroja *et al.*, 2003a) is in accordance with this hypothesis because equilibrium is first reached between nearby populations (Crow and Aoki, 1984). Although it has been suggested that population expansions result in low genetic

variability (Slatkin, 1993), there is empirical evidence that fast expansions may occur without significant loss of variability (Zenger *et al.*, 2003). Tests for population expansion are available for analyzing microsatellite data, but due to the limitations that will be discussed in the next paragraphs, we chose to not use them in the present study. The results presented here, however, provide a preliminary indication of population expansion of a widespread rodent species in eastern South America and should encourage further investigations on the subject.

The microsatellite loci used in this study showed differentiation indexes ($\theta = 0.100$ and $\rho = 0.086$) smaller than the index ($F_{ST} = 0.17$) obtained in the previous RAPD analysis that included most of the mainland populations studied here (Almeida *et al.*, 2000a). Differentiation estimates related to the F -statistics are negatively correlated with the product of the effective population size (N_e), and the mutation rate (μ) plus the migration rate (m) (Wright, 1943). This implies that, if μ is low and N_e and m are high, differentiation will be underestimated even in the extreme case of complete isolation (Jin and Chakraborty, 1995; Hedrick, 1999; Balloux *et al.*, 2000). This is especially critical in the case of microsatellites that are susceptible to a high degree of homoplasy due to constraints in allele size (Slatkin, 1995). Microsatellite markers can also be problematic when the number of alleles per locus is relatively high as is the case for some of the loci with more than 20 alleles which were used in this study (Table 1). When a loci has a large number of alleles, very large samples are required for accurately estimating allelic frequency within populations (Leberg, 2002). Simulations by Leberg (2002), suggest that our samples may not be large enough to provide reliable estimates of population differentiation in view of the large number of alleles in most loci. The high variability of each mainland population is, however, a valid finding, indicating large population sizes and relative stability on a recent time scale.

This is the first report of higher differentiation detected with RAPD markers as opposed to microsatellites. Although the F_{ST} estimate based on RAPD markers is only slightly higher, it is probably more reliable in view of the limitations of some microsatellite loci as discussed above. Nevertheless, we can reasonably propose that the degree of differentiation between mainland populations is low despite predictions based on the ecology of *N. squamipes*. Sampling a larger number of individuals per population can make differentiation estimates more accurate, although other less variable molecular markers like mtDNA might be more suitable for the study of rodent populations (Matocq *et al.*, 2000).

Despite the small sample sizes we were able to detect a higher differentiation in insular than mainland populations, agreeing with the geographic isolation of the island populations. The divergence time of the IG population estimated from genetic distance ($7,065 \pm 647$ years) was coin-

cident with the minimum time of geographic separation predicted by geological data (7,000 years before the present (YBP); Martins and Villwock, 1987), while estimates of divergence time of the IM population were considerably lower ($3,319 \pm 711$ years). One explanation for this discrepancy is a greater migration rate of the IM population because the Ilha da Marambaia is presently connected and closer to the mainland. This connection, however, consists of a very narrow sand-bridge (at some points only 2-meters wide) making water-rat migration rather unlikely. Dispersion by rafting (Loxterman *et al.*, 1998; Patton *et al.*, 1996) and sea level fluctuations might provide a better explanation because two low-sea-level events at *ca.* 3,900 and 2,700 YBP coincide temporally with the above cited divergence time for the IM population and would have reduced the distance between Marambaia Island and the mainland (Martins and Villwock, 1986).

It is important to remember that most of the divergence of the insular populations is due to their low genetic variability. The existence of fewer alleles than in the mainland populations leads to differences in allelic frequency, which greatly affects differentiation estimates. Reduced diversity is expected to be found in island populations because these populations are more subject to the effects of geographic isolation, smaller population size and founder effect (Grant, 1998). Even in the case of an extreme founder effect, however, the high mutation rates of microsatellites should be enough to restore the genetic variability in these islands given the large number of generations after isolation. Nevertheless, this would not be true if the islands had very small populations and/or went through a recent population bottleneck. At least Ilha Grande seems to be large enough to support a large population. Currently, both islands are relatively well preserved. Ilha Grande has most of its inland area protected and the occupation of the remaining areas is under government control. Ilha da Marambaia is a military area of restricted access and is also largely forested. Both islands, however, were subjected to sugar cane and coffee monoculture from the middle of the XVIII century to the end of the XIX century. Although the human population of these islands has never been large, and *N. squamipes* is capable of living in populated areas, it is difficult to assess whether past activities in these two islands have affected the population of this rodent on these islands. A larger survey of these islands, sampling more sites and using different markers, would be necessary to test for population bottlenecks in the recent history of these populations.

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