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Characterization of six rat strains (*Rattus norvegicus*) by mitochondrial DNA restriction fragment length polymorphism

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Abstract

Restriction fragment length polymorphism (RFLP) was used to examine the extent of mtDNA polymorphism among six strains of rats (*Rattus norvegicus*) - Wistar, Wistar Munich, Brown Norway, Wistar Kyoto, SHR and SHR-SP. A survey of 26 restriction enzymes has revealed a low level of genetic divergence among strains. The sites of cleavage by *Eco*RI, *Nco*I and *Xmn*I were shown to be polymorphic. The use of these three enzymes allows the 6 strains to be classified into 4 haplotypes and identifies specific markers for each one. The percentage of sequence divergence among all pairs of haplotypes ranged from 0.035 to 0.33%, which is the result of a severe population constriction undergone by the strains. These haplotypes are easily demonstrable and therefore RFLP analysis can be employed for genetic monitoring of rats within animal facilities or among different laboratories.

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- Restriction fragment length polymorphism (RFLP)
- *Rattus norvegicus*
- mtDNA
- Laboratory rats
- Genetic monitoring

Introduction

Rats are useful models employed to study cardiovascular physiology and to understand complex pathological states such as hypertension and diabetes mellitus (1). *Rattus norvegicus* is the rodent species that gave rise to all types of laboratory rat strains currently in use. In the late fifties and early sixties several strains of rats were developed for high blood pressure studies by selective intercross to fix a phenotype among individuals of outbred colonies. For instance, the spontaneously hypertensive rat (SHR) was obtained by intercrossing individuals of an outbred colony of Wistar Kyoto (WKY) rats with the highest blood pressure values (2). This process was repeated in other laboratories and today several strains of hypertensive rats with slightly different characteristics exist (3).

Several studies on genetic variability and phylogeny in rodents based on mitochondrial and nuclear DNA have been published (4-6). At present, anonymous markers throughout the rat genome are available for DNA "fingerprinting" (7,8). Restriction fragment length polymorphism (RFLP) analysis of the nuclear genome was used to measure the level of genetic divergence between SHR and the Wistar-Kyoto rats (9). Also, arbitrarily primed polymerase chain reaction (AP-PCR) was performed to develop nuclear genetic markers and to perform genetic linkage

analysis in rat strains (10). More recently, Jacob et al. (11) used a large panel of markers to develop a genetic linkage map for rats. The power of these methods is highlighted by their use in studies to analyze complex traits such as high blood pressure and diabetes mellitus (12-14). However, these techniques are complex and are not available in most animal facilities where genetic monitoring is undertaken.

Mitochondrial DNA diversity is known to exist within the same species in different taxa such as primates (15), fishes (16), and birds (17). Intraspecific mtDNA polymorphism in *Rattus norvegicus* strains was observed using the endonuclease *Eco*RI. The fragment pattern produced by *Eco*RI permitted the classification of different rat strains into two categories, type A and type B (18,19). Brown and Simpson (20) and Hayashi et al. (21) found an extensive intra- and interspecific mtDNA polymorphism using not only *Eco*RI but also *Hha*I, *Hin*dII, *Hin*f III, *Hin*fI and *Hae*III restriction endonucleases.

Maternal mode of inheritance and lack of recombination of mtDNA are features that can be used to obtain information about the female genealogy and to organize the individuals into matriarchal lineages. Due to the ease of genotyping, mtDNA polymorphism may be a useful genetic marker to monitor inbred lines in a variety of laboratories (22,23).

In the present study, a broad mtDNA survey of 6 strains of *Rattus norvegicus* was carried out by RFLP. In addition, we established the pattern of genetic divergence among these commonly used rat strains within haplotypes that can be easily employed for genetic monitoring of animal facilities utilizing three restriction endonucleases.

Material and Methods

Experimental animals

Six strains of *Rattus norvegicus* commonly used in the laboratory were investigated: Wistar, Wistar Munich, Brown Norway, Wistar Kyoto, SHR, and SHR-SP (spontaneously hypertensive rat - stroke prone). Male and female rats of 200 g were obtained from the animal facility at the Federal University of São Paulo. Ten animals from each strain were sampled from different matings. All procedures followed the "Guide for the care and use of laboratory animals" (DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892) and the guidelines of the animal welfare act.

Mitochondrial DNA isolation

Mitochondrial DNA was extracted from 3-4 g of liver and heart from each rat after an overnight fast in order to decrease the glycogen content in the liver. The tissues were homogenized in TEK buffer (50 mM Tris, 10 mM EDTA, 0.2 M KCl, 0.25 M sucrose, pH 7.5) and the mtDNA was isolated according to the method of Chapman and Powers (24).

Restriction endonuclease digestion

The isolated mtDNA was digested with 26 restriction endonucleases: *Apa*I (GGGCC/C), *Ava*II (G/GWCC), *Bam*HI (G/GATCC), *Bgl*II (A/GATCT), *Bss*HII (G/CGCGC), *Cla*I (AT/CGAT), *Dra*I (TTT/AAA), *Eco*RI (G/AATTC), *Eco*RV (GAT/ATC), *Hin*cII (GTY/RAC), *Hin*dIII (A/AGCTT), *Hha*I (GCG/C), *Hpa*I (GTT/AAC), *Kpn*I (GGTAC/C), *Mlu*I (A/CGCGT), *Nco*I (C/CATGG), *Nde*I (CA/TATG), *Nhe*I (G/CTAGC), *Pst*I (CTGCA/G), *Pvu*II (CAG/CTG), *Sac*I (GAGCT/C), *Sal*I (G/TCGAC), *Sma*I (CCC/GGG), *Xba*I (T/CTAGA), *Xho*I (C/TCGAG), and *Xmn*I (GAANN/NNTTC). mtDNA was digested and incubated at 37°C for 3-4 h for complete digestion as specified by the manufacturer (Gibco, BRL, Gaithersburg, MD). The reaction products were separated by electrophoresis in 0.8% horizontal agarose gel and

visualized under UV light following ethidium bromide staining. The images were printed or stored for future analysis (Eagle Eye System II, Stratagene).

DNA size estimation

The mobility pattern of the mtDNA fragments was compared to that of the standard marker lambda *Hin*dIII and of the 100-bp DNA ladder (Gibco, BRL). Differences in fragment mobility were measured directly from the gel image and the DNA size was estimated using the DNAGEL software (25).

Data analysis

Percent sequence divergence among mtDNA haplotypes was calculated according to the following formula: $d = [-\log_e S]/r$. For a class of endonucleases, r is the number of nucleotides for a recognition sequence. $S = 2m_{xy}/(m_x + m_y)$ where m_x is the number of restriction sites for xth haplotypes, m_y the number of restriction sites observed for the yth haplotype, and m_{xy} the common number of restrictions between the xth and yth haplotype (26,27). A parsimony network was constructed connecting the different haplotypes. Gains and losses at the network site are depicted as a single mutational step by a slash in the phenogram.

Results

In order to develop a simple method to monitor and test the use of mitochondrial DNA as a genetic marker for laboratory rats, we performed a systematic survey using 26 restriction enzymes. Thirteen of them have not been tested for this objective in published studies. Three of the 26 endonucleases, *Cla*I, *Nhe*I and *Sac*I, were able to hydrolyze the mtDNA from the 6 *Rattus norvegicus* strains in only one site. Twenty endonucleases cut the DNA twice or more, but did not reveal polymorphism. Three enzymes, *Xmn*I, *Nco*I, and *Eco*RI, were successful in demonstrating differences in cleavage patterns which were considered to be polymorphic.

*Eco*RI proved to be the most informative enzyme with three different cleavage patterns found in the 6 strains studied, as previously shown. The Wistar strain mtDNA presented 6 fragments, which correspond to type A (α), and Wistar Munich showed 4 fragments, described as type B (ß) (18,19). Wistar Kyoto, SHR, SHR-SP, and Brown Norway rats showed a different pattern that is cited as type III by Brown and Simpson (20) (Figure 1).

Two new polymorphic sites were detected during the present survey. First, *Nco*I digestion revealed two restriction patterns. Wistar Kyoto, SHR, and SHR-SP mtDNA was cleaved into two similar size fragments, whereas Wistar Munich, Wistar, and Brown Norway mtDNA samples showed a single site restriction fragment (Figure 2). The second restriction enzyme, *Xmn*I, also produced two restriction patterns. Wistar Kyoto, SHR, SHR-SP, Wistar, and Brown Norway mtDNA samples showed three fragments while Wistar Munich mtDNA produced only two (Figure 3).

The differences in cleavage patterns shown by *Hha*I (2 types) and *Hin*dII (2 types)

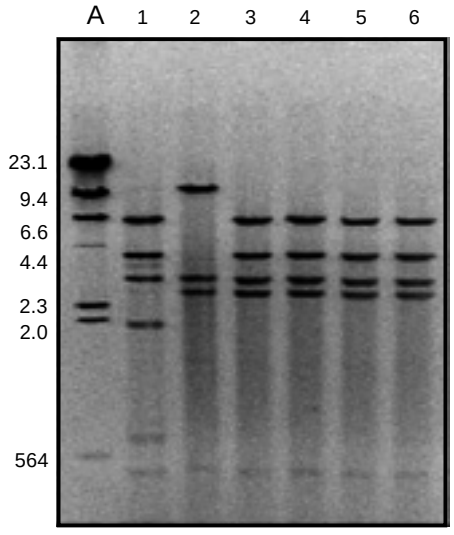

Figure 1 - *Eco*RI mtDNA cleavage pattern types of *Rattus norvegicus* strains. *Lane A*, λ *Hin*dIII fragment; *lane 1*, cleavage pattern I, Wistar; *lane 2*, cleavage pattern II, Wistar Munich; *lanes 3-6*, cleavage pattern III, Wistar Kyoto, SHR, SHR-SP, and Brown Norway, respectively.

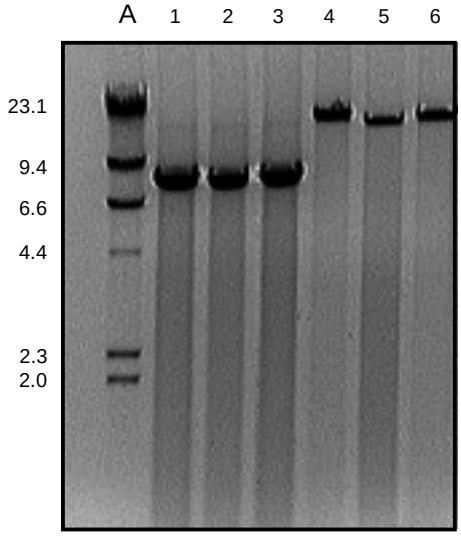

Figure 2 - *Nco*I mtDNA cleavage pattern types of *R. norvegicus* strains. *Lane A*, λ *Hin*dIII fragment; *lanes 1-3*, cleavage pattern I, Wistar Kyoto, SHR, SHR-SP, respectively; *lanes 4-6*, cleavage pattern II, Wistar, Wistar Munich, and Brown Norway, respectively.

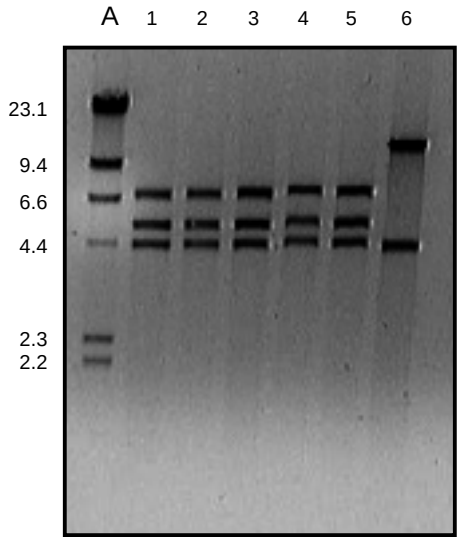

Figure 3 - *Xmn*I mtDNA cleavage pattern types of *R. norvegicus* strains. *Lane A*, λ *Hin*dIII fragment; *lanes 1-5*, cleavage pattern I, Wistar Kyoto, SHR, SHR-SP, Wistar, and Brown Norway, respectively; *lane 6*, cleavage pattern II, Wistar Munich.

Table 1 - Mitochondrial DNA clonal lineages of 6 strains of *Rattus norvegicus* based on restriction sites produced by *Eco*RI, *Nco*I, and *Xmn*I.

The Roman numerals indicate the cleavage pattern types for each enzyme.

Clone	*Eco*RI	*Nco*I	*Xmn*I	Strains
A	I	II	I	Wistar
B	II	II	II	Wistar Munich
C	III	I	I	Wistar Kyoto, SHR, SHR-SP
D	III	II	I	Brown Norway

among several strains of *R. norvegicus* (28) were not observed in the 6 strains presently studied. The informative enzymes *Hin*fII and *Hae*III utilized by Brown and Simpson (20) were not used in our study since the large numbers of small size fragments produced are difficult to visualize in ethidium bromide-stained gels.

The analyses of the 6 strains with the three polymorphic enzymes (*Eco*RI, *Xmn*I and *Nco*I) produced four haplotypes (Table 1). Wistar was designated as haplotype "A"; Wistar Munich as "B", Wistar Kyoto, SHR, and SHR-SP as "C", and Brown Norway as "D".

The estimated length of the mtDNA from the 6 *Rattus norvegicus* strains averaged 16,500 ± 500 bp, which is in close agreement with data previously published by Gadaleta et al. (29) and Clark-Walker (30). Table 2 shows the estimated mtDNA fragment sizes produced by each polymorphic enzyme.

A parsimony network was assembled connecting the various clonal lineages according to the pattern produced by the endonuclease digestions (Figure 4). In the phenogram one can estimate the relative genetic distance by counting the number of site differences among the haplotypes. Therefore, intraspecific nucleotide diversity between all pairs of haplotypes can be calculated according to their rate of nucleotide substitutions. Percent sequence divergence (or index of nucleotide diversity) ranged from 0.035 to 0.33% (Table 3).

The homogeneity of the strains studied was tested by analyzing the pattern of mtDNA fragments produced by *Eco*RI, *Nco*I and *Xmn*I in 30 Wistar Munich rats from different matings. No differences in restriction patterns were found, indicating that the animal colony tested is uniform.

Discussion

Laboratory rats undergo a severe process of selection and inbreeding in order to pro-

duce isogenic lineages and the genetic standardization required for experimental studies (31). Rats have been one of the most frequently used animals to study cardiovascular physiology. Nonetheless, genetic variability has been identified in several strains of rats from different commercial sources as well as from the same breeding facilities, a fact that may influence interpretation of the results (32).

Genetic variation in mtDNA is expected to be small or even null since the process of achieving inbred lines begins with a few animals which possess the genetic characteristics to be selected and fixed.

The various *R. norvegicus* types can be regarded as different lineages due to the intensive inbreeding methods and also to the absence of gene flow among strains to produce rats for different experimental purposes. Consequently, the different strains may be grouped into clonal lineages and their genetic variability assessed.

As expected, our findings showed no differences among Wistar Kyoto, SHR and SHR-SP strains upon analysis with the 26 enzymes tested, which is consistent with the results found by Johnson et al. (9). This lack of divergence was expected since SHR and SHR-SP were developed from Wistar Kyoto rats by selection from a common maternal lineage. The three polymorphic enzymes identified in this work can easily be used to classify Wistar Kyoto, SHR and SHR-SP into the same haplotype (C).

Mitochondrial DNA from the Brown Norway strain exhibited the same pattern as found in SHR, SHR-SP and Wistar Kyoto upon digestion with *Eco*RI and *Xmn*I. In contrast, *Nco*I produced a different cleavage pattern. Thus, Brown Norway rats were assigned to a different haplotype (D). Wistar and Wistar Munich rats were classified as clones A and B, which confirms the previously described types A and B (18). These 2 strains were classified differently upon digestion with the endonucleases *Eco*RI and *Xmn*I.

Table 2 - Fragment sizes (kb) produced by *Eco*RI, *Nco*I and *Xmn*I restriction enzymes in mtDNA from *Rattus norvegicus*.

The Roman numerals indicate the cleavage pattern types associated with each polymorphic enzyme.

*Eco*RI			*Nco*I		*Xmn*I	
I	II	III	I	II	I	II
6.49	10.4	6.49	16.43	8.83	7.11	12.27
4.11	3.05	4.11		7.60	5.06	4.04
3.05	2.54	3.05			4.04	
1.87	0.46	2.54				
0.64		0.46				
0.46						
16.6	16.4	16.6	16.4	16.4	16.2	16.3

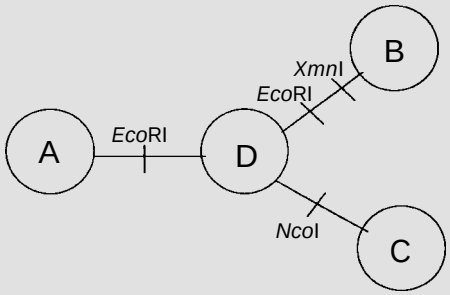

Figure 4 - Parsimony network showing restriction site gains or losses (slash in the diagram) in *Rattus norvegicus* mitochondrial DNA haplotypes. The DNA polymorphism is indicated on the phenogram by its restriction endonuclease.

Table 3 - Matrix of percent sequence divergence among the four haplotypes obtained by mtDNA digestion with *Eco*RI, *Nco*I, and *Xmn*I from 6 strains of *Rattus norvegicus*.

	A	B	C	D
A	-	0.33	0.14	0.1
B	0.33	-	0.25	0.22
C	0.14	0.25	-	0.035
D	0.1	0.22	0.035	-

The three informative enzymes, *Eco*RI and those identified in the present study, *Xmn*I and *Nco*I, can be used to separate the 6 rat strains into specific haplotypes with no need for sophisticated laboratory equipment.

Various statistical approaches have been used to estimate mtDNA sequence divergence (33-35) and a range of results has been obtained. In the present study the nucleotide diversity for *R. norvegicus* ranged from 0.035

to 0.33%, which is lower than the values found by Brown and Simpson (20), 0.4 to 1.8%, and Hayashi et al. (21), 1%. The lower values of genetic divergence among the four clonal lineages obtained in our study can be explained by the larger number of enzymes surveyed. It is important to note that the final result was dependent on the analysis of all the informative and non-informative enzymes. Additionally, the low divergence may be due to the severe population constriction undergone by the strains which were generated from a small number of females. The highest sequence divergence value of 0.33% was found between haplotypes A (Wistar) and B (Wistar Munich). This distance is illustrated in the parsimony network showing two restriction site losses for *Eco*RI and one for *Xmn*I. Our results suggest that the Wistar rat is the ancestral strain from which all the other strains studied originated.

It is interesting to note the low level of divergence between haplotype D (Brown Norway) and the others. This is consistent with a common and recent female origin of the Brown Norway strain compared to the others even though the different coat coloration of the Brown Norway strain indicates the contrary. A wild male rat probably introduced the dark coloration phenotype during crossbreeding.

The absence of differences in mitochondrial DNA pattern among offspring from Wistar Munich rats originating from distinct mating pairs indicated a common founder stock in the breeding facility studied. The origin of *R. norvegicus* plays an essential role in the genetic background of the various rat strains used in experimental studies. Therefore, the maintenance of genetic uniformity of laboratory rats depends on a careful monitoring of the strains.

The results obtained here suggest that the analysis of mtDNA with *Eco*RI, *Nco*I, and *Xmn*I may be a useful tool to monitor laboratory rats, preventing strain contamination and maintaining population uniformity in animal facilities. The use of specific mtDNA probes and of non-invasive techniques (36) further improved the use of mtDNA for the maintenance of the genetic quality of laboratory rats. It is important to note that sorting rat strain according to mtDNA clonal lineages does not preclude a certain level of nuclear DNA polymorphism, which may be secondary to an outbred male colony, or to incomplete generation of inbreeding.

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