Sequence Divergence of Mitochondrial 12S rRNA Gene and Phylogenetic Relationship Between *Rattus rattus* Sladeni and *Rattus rattus* Hainanicus*

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Abstract: The appearance and hair color between these two subspecies *Rattus rattus* Sladeni and *R. r.* Hainanicus are similar to each other. Their most major distinctive characteristic is the length ratio of tail to body. However, this characteristic was unstable in some measuring records. In Guangdong, *R. r.* Hainanicus is restrictedly distributed in the west region, and *R. r.* Sladeni is widely distributed in the other regions of this province. In this study, we detected the sequences of mitochondrial 12S rRNA gene fragments of 9 samples from *R. r.* Hainanicus and *R. r.* Sladeni (Longmen and Hong Kong populations). These 385 nucleotide positions of 12S rRNA gene fragment include 26 variable sites and 14 parsimony-informative sites. 3 insertion/deletion sites are observed. The phylogenetic relationships among these samples were constructed by Neighbor-joining and Maximum parsimony methods. The analysis shows that *R. r.* Hainanicus is more closely relative to the Longmen population of *R. r.* Sladeni than to the Hong Kong population of *R. r.* Sladeni. The sequencing analysis of 12S rRNA gene fragments is not agreement on the classification of subspecies *R. r.* Hainanicus inferred from the morphology and geographical distribution. The morphological variation of *R. r.* Hainanicus should result from the natural selection, which causes local adaptation and geographic isolation.

Key words: *Rattus rattus* Sladeni; *R. r.* Hainannicus; mitochondrial DNA; sequence; divergence; phylogenetic relationships
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Rattus rattus is widely distributed in the world. It is one of the major murines in the southern of China and the interested species of rat for the studies of karyotype and mtDNA. Its polymorphism of chromosomes and mtDNA sequences were widely found[1][2]. Its two subspecies *R. r.* Sladeni and *R. r.* Hainanicus live in mountain areas of Guangdong. In Guangdong, *R. r.* Hainanicus is restrictedly distributed in the west region, but *R. r.* Sladeni is widely distributed in the other regions. The appearance and hair color between these two subspecies are similar to each other. Their most major distinctive characteristics are the length ratio of tail to body. However, this characteristic was unstable in some measuring records[3]. We analyzed the karyotypes of these two subspecies. Their diploid numberer (2n) is 42, but the fundamental number (NF) is different. It showed the chromosome polymorphism. These contradictory phenomena make us to be interested in the phylogenetic relationship between these two subspecies, because the karyotype diversity and interspecific differentiation are not parallel. Mitochondrial DNA is maternally inherited and provides a simple non-recombining system. It is susceptible to specific sequence analysis and provides a magnified view of the difference within species and the evolutionary process. mtDNA sequencing is straighforward for the analysis of phylogeny.

1 Materials and Methods

1.1 Preparation of total DNA

The specimens were live-trapped in Guangdong province and Hong Kong. The individuals of *R. r.* Sladeni were captured in Hong Kong (sample S1, S2 and S3) and Longmen (sample S4, S5 and S6), and the specimens of *R. r.* Hainanicus (sample H1, H2 and H3) were from Zhanjiang. The QIAamp Tissue Kit (QIAGEN) was used to extract total DNA from 25 mg of muscle tissue. The isolated muscle tissues and extracted DNAs were stored in −20 ℃ freezer.

1.2 Polymerase chain reaction

Based on the published sequences of rat (Gadaleta *et al*, 1989), The primer pair HRN (5'-TGACTGCAGAGGGTGACGGGCGGTGTGTGT-3') /LRN (5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-

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3′) was designed and used for PCR and sequencing. Every 100 μL of reaction mixture contained 1 ~ 10 ng of total DNA; 8 μL of each dNTP mix (10 mmol/L); 10 μL of 10X PCR buffer; 2.5 μL of Tfl DNA polymerase (5000 U/mL) and 6 μL of $MgCl_2$ (25 mmol/L). The PCR reactions were carried out for a total of 30 cycles using a PTC — 100 Programmable Thermal Controller (MJ Research Inc.). The cycle was as follows: denaturation, 15 seconds at 94 ℃; annealing, 15 seconds at 50 ℃ and primer extension, 120 seconds at 72 ℃. PCR products were purified by centrifugal dialysis in the Centricon—100 units.

1.3 Sequencing reaction of mtDNA

The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (PERKIN ELMER) was used in the sequencing reaction. 20 μL of reaction solution contained 8 μL of Terminator Ready Reaction Mix, 90 ng DNA and 3.2 pmoles of primer. The thermal cycle was as follows: 96 ℃, 10 seconds; 50 ℃, 5 seconds, and 60 ℃, 4 minunts. The reaction was carried out for 25 cycles in the Gene Amp PCR System 2400 (PERKIN ELMER). The CENTRI—SEP column was used to purify the sequencing reaction product. Amplified 12S rRNA gene fragments were sequenced in the ABI PRISM 310 Genetic Analyzer (PERKIN ELMER).

1.4 Reconstruction of phylogenetic relationships

The ABI SeqEd™ package was used for DNA sequence editing. The raw data of mtDNA sequences were arranged with the SeqEd program and multiple alignments of sequences were performed using the program ClustalW. The software Mega 2 was used to compute basic statistical quantities. Its Kimura 2-parameter model was used to estimate genetic distance and reconstruct phylogenetic relationships, including Neighbor-joining (NJ) tree and Maximum parsimony (PM) tree. Transition and transversion data was considered to compute pairwise. Confidence in the phylogenetic tree was assessed by the bootstrap method, with 1000 replication. Outgroup was *R. norvigicus*[4].

2 Results

2.1 Sequence variability of 12S rRNA gene fragments

The sequences of 12S rRNA gene fragments of 9 samples were detected. Nucleotide frequencies are not significantly different among these samples (Tab.1). These 385 nucleotide positions of 12S rRNA fragment include 26 variable sites and 14 parsimony-informative sites3 insertion/deletion sites are observed. The G + C content in these sequences is lower than 0.5. This average ratio of transitional pairs to transversional pairs (si/sv) among these samples is 1.5.

The ratio of nucleotide difference between the individuals from two populations of *R. r.* Sladeni ranges from 3.1% to 3.6% and 2 sites of insertion/deletion are observed. This ratio between the individuals of *R. r.* Sladeni (Longmen population) and *R. r.* Hananicus is from 0.8% to 2.6% and 3 sites of insertion/deletion are found. The nucleotide difference between the individuals of *R. r.* Sladeni (Hong Kong population) and *R. r.* Hananicus ranges from 3.1% to 5.7% and 1 site of insertion/deletion is recorded. The average ratio of nucleotide transvertion between *R. r.* Sladeni (Hong Kong population) and *R. r.* Hananicus is 1.3%, between *R. r.* Sladeni (Longmen population) and *R. r.* Hananicus is 0.5%, and between Hong Kong population and Longmen population of *R. r.* Sladeni is 0.8%. In the Hong Kong population of *R. r.* Sladeni, 2 haplotypes were found. Longmen population of *R. r.* Sladeni and *R. r.* Hananicus separately own 3 heplotypes.

Tab.1 Nucleotide frequences of 12S rRNA gene fragment

Sample	T	C	A	G	Total
S1	37.6	17.0	26.6	18.8	383
S2	37.6	17.0	25.6	19.8	383
S3	37.6	17.0	25.6	19.8	383
H1	37.9	16.4	25.6	20.1	383
H2	38.2	17.5	24.6	19.6	382
H3	37.9	17.0	25.1	20.1	383
S4	37.8	17.1	25.5	19.7	381
S5	37.8	17.1	25.5	19.7	381
S6	37.5	17.1	25.7	19.7	381
Average	37.8	17.0	25.5	19.7	382.2

Tab.2 Genetic distance and standard error based on 12S rRNA sequences[1)]

	1	2	3	4	5	6	7	8	9	10
1.	*R. norvigicus*		0.013	0.011	0.011	0.010	0.009	0.009	0.008	0.008	0.009
2.	S1	0.057		0.005	0.005	0.013	0.012	0.011	0.010	0.010	0.010
3.	S2	0.046	0.011		0.000	0.012	0.010	0.009	0.009	0.009	0.010
4.	S3	0.046	0.011	0.000		0.012	0.010	0.009	0.009	0.009	0.010
5.	H1	0.035	0.060	0.049	0.049		0.008	0.006	0.008	0.008	0.009
6.	H2	0.032	0.049	0.038	0.038	0.027		0.005	0.006	0.006	0.007
7.	H3	0.027	0.043	0.032	0.032	0.016	0.011		0.005	0.005	0.005
8.	S4	0.024	0.035	0.032	0.032	0.024	0.013	0.008		0.000	0.003
9.	S5	0.024	0.035	0.032	0.032	0.024	0.013	0.008	0.000		0.003
10.	S6	0.027	0.038	0.035	0.035	0.027	0.016	0.011	0.003	0.003	

1) Lower triangle is genetic distance and upper triangle is standard error

2.2 Phylogeny of reconstruction

Kimura 2-parameter model was used to estimate the interspecific values of genetic distance (Tab 2). The genetic distances between different populations of *R. r.* Sladeni ranges from 0.032 ± 0.009 to 0.038 ± 0.010, that between *R. r.* Sladeni (Longmen population) and *R. r*Hananicus is from 0.008 ± 0.005 to 0.027 ± 0.009, and between *R. r.* Sladeni (Hong Kong population) and *R. r.* Hananicus is from 0.032 ± 0.009 to 0.060 ± 0.012.

NJ tree and PM tree were constructed (Fig. 1). In the NJ tree, Hong Kong population and Longmen population of *R. r.* Sladeni are clustered into one major clade, another clade is formed by the individuals of *R. r.* Hananicus. In the MP tree, *R. r.* Hananicus and Hong Kong population of *R. r.* Sladeni are clustered into one major clade, another clade is constructed by the individuals of Longmen population of *R. r.* Sladeni.

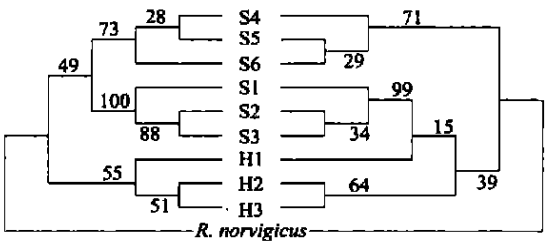

Fig. 1 Reconstruction of Phylogenetic trees inferred from 12S rRNA gene fragments by Kimura's 2-parameter distance and bootstrap confidence level (BCL)

Left: NJ tree; Right: MP tree; The number above/below the line is the value of BCL

3 Discussion

Morphological systematics and its phylogenetic relationships are based on the comparison of structures, history and geographical distribution. DNA sequencing can provide important data for historical biogeography because they permit direct measurement of genetic divergence within species and increased certainty in the inference of the history of genetic changes[5,6].

The wide spectrum of intraspecific divergence of mtDNA in *R. rattus* was reported[7]. In present study, the different haplotypes are found within population or subspecies of *R. rattus*. The nucleotide difference of 12S rRNA fragments among these samples is from 0.8% to 5.7%. This range is less than the record of intraspecific sequence divergence in other mitochodrial gene[2]. Comparing the data of substitution, transversion and genetic distance, these values between *R. r.* Sladeni (Hong Kong population) and *R. r.* Hainanicus are highest, that between *R. r.* Sladeni (HK) and *R. r.* Sladeni (Longmen population) are higher than that between *R. r.* Sladeni (Longmen) and *R. r.* Hainanicus. However, the ratio of insertion/delection site between them is inversing to that of substitution This analysis states that both nucleotide substitution and insertion/deletion are the evolutional ways of 12S rRNA gene within species *R. rattus*. *R. r.* Hainanicus from the west region of Guangdong is more closely relative to the Longmen population of *R. r. sladeni* from the east region than to the Hong Kong population of *R. r.* Sladeni from the central region. This phenomenon is supported by the report that East Asian R. rattus is more closely relate to American *R. rattus* than to the other in Asian[8]. The genetic distance between *R. r.* Hainanicus and the Longmen population of *R. r.* Sladeni is less than that between two different populations of *R. r.* Sladeni. Based on the sequencing data of 12S rRNA fragments, it is not agreement on the classification of subspecies *R. r.* Hainanicus inferred from the morphology and geographical distribution. The morphological variation and chromosomal polymorphism of *R. r.* Hainanicus resulted from the natural selection, which causes local adaptation and geographic isolation.

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施氏屋顶鼠和海南屋顶鼠的线粒体 12S rRNA 基因序列的歧异及其系统进化关系

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摘　要：屋顶鼠的两个亚种——施氏屋顶鼠和海南屋顶鼠的外形和毛色相似，在一些标本的测量记录中，它们间的主要区别特征之一的尾长与体长比却是不肯定的。在广东，海南屋顶鼠仅分布于粤西区，而施氏屋顶鼠则分布于其余区域。在我们的这项研究中，采用了 9 个分别来自于施氏屋顶鼠（龙门种群和香港种群）及海南屋顶鼠的样品进行了线粒体 12S rRNA 基因的测序，在所分析的 385 个核苷酸位点长度的片段中，包含有 26 个变异位点和 14 个简约信息点，观察到 3 个插入/缺失位点。通过邻接法和最大简约法重建这些鼠类样品的系统进化关系。相比于施氏屋顶鼠的香港种群，海南屋顶鼠与施氏屋顶鼠的龙门种群有着更紧密的亲缘关系。基于 12S rRNA 基因片段的序列数据，本项分析不赞同把海南屋顶鼠分类成为一个亚种的观点，认为海南屋顶鼠的形态变化是自然选择的结果，并由此导致当地适应和地理隔离。

关键词：施氏屋顶鼠；海南屋顶鼠；线粒体 DNA；序列；歧异；系统进化关系

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（上接第 81 页）

Synergism Between Low Concentration Buprofezin and *Metarhizium anisopliae* var. *acridum* on Controlling *Nilaparvata lugens* Stål (Homoptera: Delphacidae)

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Abstract: The synergism between low concentration buprofezin and *Metarhizium anisopliae* var. *acridum* to control brown planthopper (BPH) *Nilaparvata lugens* was tested in paddy field in Guang Zhou City, Guang Dong. Four treatments and one water control were included in the experiments: low application rates of buprofezin, high application rates of buprofezin, *Metarhizium anisopliae* var. *acridum* and low application rates of buprofezin + *Metarhizium anisopliae* var. *acridum* with each being replicated in three plots (4m×5m). Counts of the adults and nymphs of brown planthopper were made before the day of treatment, and 2nd, 6th, 11th, 16th and 21st day after treatment. The result showed that buprofezin synergizes *Metarhizium anisopliae* var. *acridum*. Both the density decrease rate and relative efficacy increased significantly from simultaneous use of *Metarhizium anisopliae* var. *acridum* and buprofezin comparing to those from lonely use. Thus the fungus and buprofezin combined formulation tested in this study are of great potential use in brown planthopper control.

Key words: *Metarhizium anisopliae* var. *acridum* Bosenberg et Strand; buprofezin; *Nilaparvata lugens* Stål; synergism