中国三种实验用小型猪线粒体 DNA D-loop 多态性分析

刘中禄[1],魏 泓[1],曾养志[2],王爱德[3],甘世祥[4]

(1.第三军医大学实验动物中心,重庆 400038;2.云南农业大学动物资源学院,昆明 650201;
3.广西大学动物科学系,南宁 530005;4.贵阳中医学院实验动物中心,贵阳 550002)

摘 要 分析中国三种实验用小型猪线粒体 DNA (mtDNA) D-loop 的多态性,建立各品种品系猪的遗传标记,为各品种、品系猪的鉴别提供依据。应用 PCR 技术分别对西双版纳近交系小耳猪、广西巴马小型猪、贵州小型香猪和长白猪的血液总 DNA 样品中 mtDNA D-loop 进行扩增,用 23 种限制性内切酶消化,观察其酶切多态。PCR 扩增其 mtDNA D-loop 5′端 227bp 高变区域,应用 PCR SSCP 和 PCR 直接测序分析,观察其单链构象多态和序列多态。结果显示:三种小型猪之间未见酶切长度多态、单链构象多态和序列多态。与长白猪之间表现出单链构象多态和序列多态。本研究认为:三种实验用小型猪之间 mtDNA 多态性贫乏,证明其亲源关系很近,在母系起源和进化上有一致性,应用 PCR RFLP、PCR SSCP 和 PCR 直接测序分析,尚不能作为三种实验用小型猪品种、品系鉴定的依据,但与长白猪等欧系猪比较有一定差异。

关键词 小型猪 聚合酶链反应 线粒体 DNA 控制区 多态性

中图分类号:Q343.3 **文献标识码**:A **文章编号**:0253-9772(2001)02-0123-05

Analysis of the Polymorphism of mtDNA D-loop in Three Breeds of Laboratory Miniature Pig in China

LIU Zhong-lu[1], WEI Hong[1], ZENG Yang-zi[2], WANG Ai-de[3], GAN Shi-xiang[4]

(1. *Laboratory Animal Center, Third Military Medical University, Chongqing* 400038, *China*;
2. *Yunna Agriculture University, Kunming* 650201, *China*; 3. *Guangxi University, Nanning* 530005 *China*;
4. *Guiyang College of Traditional Chinese Medicine, Guiyang* 550002, *China*)

Abstract: The present study is to analyze the polymorphism of the mtDNA D-loop in three breeds of laboratory miniature pigs in China, and to establish its cytoplasmic DNA markers to distinguish among them. The polymorphism of mtDNA D-loop and its 5′-end high variable regions were detected by PCR-RFLP, PCR-SSCP and PCR-direct sequencing on Xishuangbanna Small-ear inbred pig, Guizhou miniature Xiang pig, Guangxi Bama miniature pig and Landrace. There was no polymorphism obtained among or within three breeds of Chinese laboratory miniature pigs besides Landrace. It is concluded that the polymorphism of mtDNA D-loop within the three breeds of Chinese laboratory miniature pigs is poor, These methods cannot be used to distinguish among them, but it can be used to distinguish them from Landrace.

Key words: Miniature pig; PCR; mtDNA D-loop; polymorphism

收稿日期:2000-04-21 **修回日期**:2000-12-21

基金项目:The project supported by National "973" Key Project Program (G200016106) and National "9,5" Key Program (96-A23-06-01) and National Natural Science Foundation of China (NSFC) (No.39830190, 39770398)

作者简介:LIU zhong-lu, male, 34 years old, master, associate profess, Genetic control of laboratory animal) **现工作单位**:第三军医大学高原军事医学系, Tel:(023)68752330.

联 系 人 魏 泓 E-mail:Weihong@mail.tmmu.com.cn

Animal mitochondrial DNA (mtDNA) is a closed circular molecule of about 16500 base pairs containing the genes of 13 proteins, 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA). The studies on the genetic function of mtDNA attracted the interest of many investigators just after its discovery occurred about 30 years ago. Over 20 species of animal mtDNA were sequenced including pig[1], horse, mouse, rat, cow, chicken as well as human being. There is a single noncoding region in pigs, which is spans between the structural genes for $tRNA^{Phe}$ and $tRNA^{Pro}$. It has been shown to contain sequences essential for the initiation of transcription and replication and therefor has been designated the control region or D-loop. The D-loop rich in A/T and is the most variable part of the genome, diverging both in length and base composition. its nucleotide substitution rate is 5 ~ 10 times higher than the rate found in the remainder of the mitochondrial genome, making it useful for studies of genetic variability among populations and phylogenetic analysis. Up to now, the sequences of porcine mtDNA D-loop 5′-end[2], 3′-end[3] and whole region[4] were sequenced, and their variations were studied by mtDNA-RFLP[7,8,10], mtDNA D-loop PCR (polymerase chain reaction)-RFLP[5,6] and mtDNA D-loop 5′-end PCR-SSCP[4], but there were no report concerning Guangxi Bama miniature pig, Guizhou miniature Xing pig and Xishuangbanna small-ear inbred strain pig which were used as Chinese laboratory animal in medical biology. Thus our aims are to analyze their polymorphism and to establish its cytoplasmic marker for distinguish them by mtDNA D-loop RFLP, mtDNA D-loop 5′-end SSCP and PCR directing sequences.

1 MATERIALS AND METHODS

1.1 Animals

Three different breeds of Chinese laboratory miniature pigs, namely, Guangxi Bama miniature pig(6), Guizhou miniature Xiang pig(12) and Xishuangbanna small-ear inbred strain pig (12), and Landrace(6), were collectd from Yunan Agriculture University, Guiyang College of Traditional Chinese Medicine, Guangxi University and Chongqing Pig Farm.

1.2 Reagent and equipment

Taq DNA polymarase, 10 × buffer, $MgCL_2$ dNTP were purchased from Boringman co. Other reagents were commercial preparations of the highest purity available. $GeneAmp^R$ PCR System 2400 and ABI PRISM™ sequencer.

1.3 Total DNA preparation

The blood template collection and the total DNA preparation according to the procedures of Liuzhonglu[9]. 1ml blood samples were collected from cervical vein, added with 1ml 2 × ST solution (0.64mol/L sucrose, 0.02mol/L Tris-HCl pH7.6, 0.01 mol/L $MgCl_2$, 2% Triton X-100), after it was mixed by vortexing and incubated for 15 min in ice water. The mixture was centrifuged for 5 min at 1000 × g to remove the cellar debris, and this step was repeated 1 ~ 2 times, digestive solution STE (10mmol/L Tris-HCl pH8.0, 0.25mmol/L $EDTANa_2$, 100m mol/L NaCl) 0.5ml and SDS、protease K (final concentration was 1% and 0.1μg/ml separately) was added to each tube. Mixture, storage and transportation were conducted at room temperature, according to phenol/chloroform/isopentanol extraction, ethanol precipitation. After it was centrifuged at 12 000 × g for 10 min, the total DNA was dissolved in an appropriate volume of TE solution (10mmol/L Tris-HCl, 1mmol/L EDTA, pH 8.0) and stored at 4℃。

1.4 Primer design and synthesis

In accordance with the porcine mtDNA whole sequence (16 680bp) was sequenced by Ursing[1] to design primer P_1 5′-AGACTAACTCCGCCATCAG-3′ (15 380 ~ 15 398bp) and P_2 5′-CGCGGATACTTGCATGTGT-3′ (114 ~ 132bp) to amplify mtDNA D-loop, which is about 1 480bp, primer P_3 5′-GTACATAGCACATATCATGTC-3′ AND P_4 5′-TAAGGGGAAAGAGTGGGCGAT-3′ were designed by Vrsing's Mechocl to amplify the porcine mtDNA D-loop 5′-end which is about 227bp, and the primers were synthesized in Shanghai Institution of Cell Biology[1].

1.5 PCP amplification mixture and procedure

200ng of total DNA was used for amplification in 50μl of PCR mixture containing 0.5μmol/L each of primers, 0.2mmol/L dNTP, 1.5 mmol/L Mg^{++} and 2 units *Taq* DNA polymerse. The reaction conditions were 94 ℃5 min, 30 cycles of 94℃ (1min), 58℃ (1min), and 72 ℃ (2min), in the last cycles the extention (72℃) time was 7 min, then it was stored at 4℃.

1.6 The porcine mtDNA D-loop RFLP

The porcine mtDNA D-loop amplification product was completely digested in a volume of 20μl by incubating it at 37℃ for 4 hr with an appropriate amount of restriction endonuclease. After digestion, it was electrophoresed in an agarose gel (2%). Electrophoresis was performed in a horizontal slab gel in TBE buffer (89mmol/L Tris, 89 mmol/L boric acid, 2.5 mmol/L EDTA, pH 8.3) at 5V/cm, Ethidium bromide (0.5μg/μl) has been dissolved in the gel.

1.7 The porcine mtDNA D-Loop 5′-end PCR-SSCP

5μl of porcine mtDNA D-Loop amplification products were diluted 10 times in TE pH 8.0 buffer. 2μl of each dilution was mixed with 12μl of denaturation solution (95% de-ionized formamide solution, 20mmol/L NaOH, 0.025% bromide phenol bluend 0.025% bromophenol blue). and incubated at 100℃ for 5 min. After chilling on ice for 10min, 5μl of each sample was analyses by 8% polyacrylamide gel electrophoresis (acrylamide : bisacrylamide = 39:1, containing 5% glycerol in 0.5 TBE). The electrophoresis was per-

formed at a constant voltage of 30V under the temperature of 25℃. The results were visualized by silver staining.

1.8 The porcine mtDNA D-Loop 5'-end PCR direct sequencing

The porcine mtDNA D-Loop 5′-end PCR products were purified by 1.2% low-melting agarose gel electrophoresis. The same primers (P3, P4) were used for the PCR-direct sequencing primers. the sequencing method was followed according to the manual of ABI PRISMTM.

2 RESULTS

Clear amplification bands of the porcine mtDNA D-loop were obtained, and the fragment size was the same as that of early reports. Among the 36 pigs examined by 23 restriction enzymes, there is no variation within each of the breeds showing monomorphism(tab.1).

The results of the porcine mtDNA D-loop 5′-end PCR-SSCP analyses were showed as fig. 1, there was no variation among or within three breeds of Chinese laboratory miniature pigs besides Landrace.

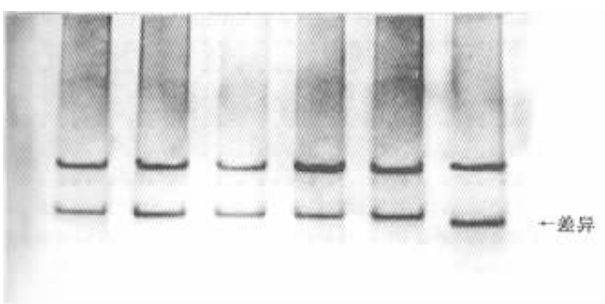

Fig.1 The porcine mtDNA D-loop 5′-end PCR-SSCP

1. Xishuangbanna Small-ear inbred pig JS strain; 2. Xishuangbanna Small-ear inbred pig JB strain; 3. Guangxi Bama miniature pig; 4. Guizhou miniature Xiang pig, experimental pig; 5. Guizhou miniature Xiang pig, civilian pig; 6. Landrace.

By comparison of the sequences of the porcine mtDNA D-loop 5′-end (fig.2), no difference was detected among the three breeds of Chinese laboratory miniature pigs, but 1 ~ 2 base variations were detected with other native pig, Meishan pig, while 4 base variation with Landrace.

Table 1 Restriction patterns of porcine mtDNA D-loop from six different breeds by 23 restriction enzymes

Restriction endonuclease	Recognition sequence	Molecular sizes of fragment	Number of cleavage sites
Acc Ⅰ	GT/(A,C)(T,G)AC	1480	0
Alu Ⅰ	AG/CT	360,340,300,120...	6
Apa Ⅰ	GGGCC/C	1050,430	1
Ava Ⅰ	C/(T,C)CG(A,G)G	1480	0
Bam HI	G/GATCC	990,490	1
Bgl Ⅰ	GCC(N)/NGGC	1480	0
Bgl Ⅱ	A/GATCT	1480	0
Dra Ⅰ	TTT/AAA	1380,100	1
Eco R Ⅰ	G/AATTC	1480	0
Eco RV	GAT/ATC	1480	0
Hae Ⅲ	(A,G)GCGC/(T,C)	1480	0
*Hin*c Ⅱ	GT(T/C)/(A/G)AC	1100,380	1
*Hin*d Ⅲ	A/AGCTT	1480	0
*Hin*f Ⅰ	G/ANTC	790,690	1
Hpa Ⅰ	GTT/AAC	1480	0
Nde Ⅱ	/GATC	800,300...	4
Pst Ⅰ	CTGCA/G	1480	0
Pvu Ⅱ	G/AATTC	1480	0
Rsa Ⅰ	GT/AC	990,480,100	2
Sca Ⅰ	AGT/ACT	1480	0
Stu Ⅰ	AGG/CCT	1480	0
Xba Ⅰ	T/CTAGA	1480	0
Xho Ⅰ	T/CTAGA	1480	0

3 DISCUSSION

The porcine mtDNA is about 16.5kb, which is in the scope of restriction endonuclease analysis, making it the subject of intense study using mtDNA-RFLP in recent years. Preparation of pure mtDNA represents a key step in these investigation. Purification of mtDNA is time-consumping and requires expensive equipment. In this report, total DNA was used to amplify mtDNA D-loop regions, which are highly variable regions in mtDNA. The procedures for preparation and purification of mtDNA should be simplified, but special attention must be paid to the step of total DNA centrifuge, which follows after ethanol precipitation, it should be centrifuged at 12 000 × g for 10min in case of the loss of mtDNA.

In the analysis of the porcine mtDNA D-loop RFLP patterns generated by 23 restriction enzymes revealed no difference among or within four different breeds. The PCR-RFLP showed good results, but it can test the mutation or difference on the recognition sites of restriction enzymes (RE) only, as mtDNA D-loop regions just 1480bp, its RE cleave sites is little, probably that is the reason for the poor of the polymorphism.

SSCP analysis is a useful technique to detect sequence differ-

ences . However , the results are strongly affected by the experimental conditions , and usually it requires optimization of precise gel concentration and temperature to get good band separations . In this study , we used constant 30 voltage at 25℃ for electrophoresis , satisfactory results was obtained . There is no difference among three breeds of Chinese laboratory miniature pigs besides Landrace . This result is in agreement with the suggestion that pigs were of two different maternal origins , from European and Asian wild boars . The porcine mtDNA D-loop PCR-directing sequencing analyses verified the results of PCR-RFLP and PCR-SSCP . The sequence data of three Chinese laboratory miniature pigs showed no difference among them . The base substitution of miniature pig comparison with other native Meishan pig is smaller (1 ~ 2base) than Landrace(4base) , which is belong to European pigs , the results also agree with that the three breeds of Chinese laboratory miniature pigs reared in southwest China.

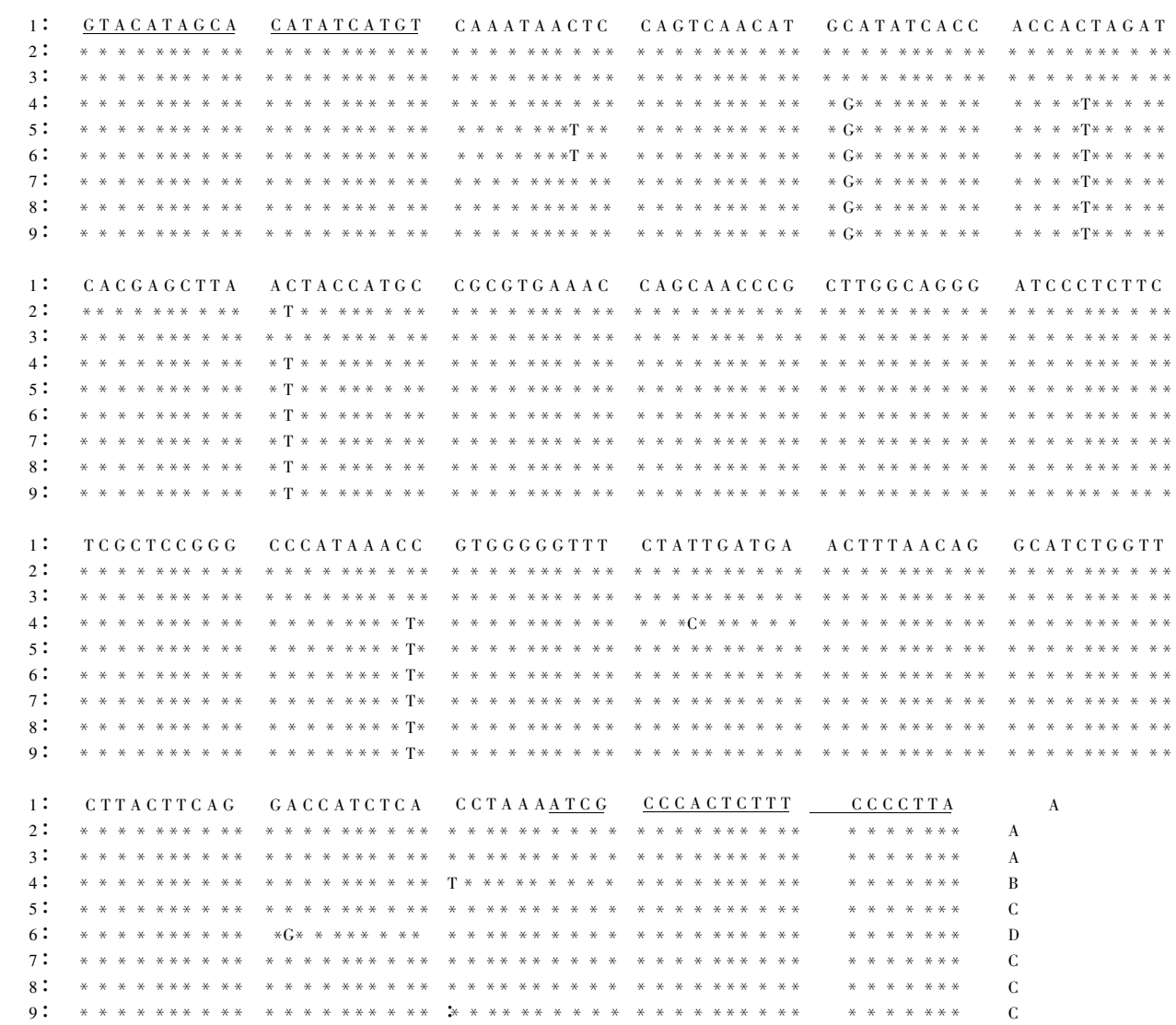

Fig.2 Comparison of the sequences of porcine mtDNA D-loop 5′-end

1. Landrce ; 2. Duroc ; 3. Large White ; 4. Gottingen Miniature pig ; 5.6. Meishang pig ;
7. Guangxi Bama miniature pig ; 8. Guizhou miniature Xiang pig ; 9. Xishuangbanna Small-ear inbred pig.
1 ~ 6 cite from Takeda K. primers regions are boxed and the types are indicated at the end of the panel.

In summary，useful cytoplasmic DNA markers for distinguishing among the three Chinese laboratory miniature pigs can not be obtained by the methods of PCR-RFLP、PCR-SSCP and PCR-directing sequence to analysis the polymorphism of the porcine mtDNA D-loop，the attention should be paid to the study of the nDNA.

参考文献（References）：

[1] Ursing B M，Arnason U . The complete mitochondrial DNA sequence of the pig[J] . J Mol Evol，1998，47(3)：302 ~ 306.

[2] Mackay S L D，Oliver P D，Laipis P J . Template-directed arrest of mammalian mitochondrial DNA synthesis[J] . Mol Cell Biol，1986，6(4)：1261 ~ 1267.

[3] Ghivizzani S C，Mackay S L，Madsen C S . Transcribed heteroplasmic repeated sequences in the porcine mitochondrial DNA D-loop region [J] . J Mol Evol，1993，37(1)：36 ~ 47.

[4] Takeda K，Onishi A，Ishida N . SSCP analysis of pig mitochondrial DNA D-loop region polymorphism[J] . Animal Genetics，1995，26(5)：321 ~ 326.

[5] Davoli R，Costosi E，Costs N . *Hinc* Ⅱ and *Hae* Ⅲ RFLPs in the porcine mtDNA D-loop region[J] . Animal Genetics，1995，26(3)：201 ~ 213.

[6] Zhao X B，Li N，Wu C X . The analysis of the porcine mtDNA D-loop polymorphism[J] . Hereditas (Beijing)，1997，19(suppl.)：23 ~ 26[赵兴波，李宁，吴常信 . 猪线粒体 DNA D-loop 的遗传变异分析 . 遗传，1997，19(增刊)：23 ~ 26.]

[7] Lan H，Shi L M . The origin and genetic differentiation of native breeds of pigs in southwest China approach from mtDNA polymorphism[J] . Biochem . Genet，1993，31：51 ~ 60.

[8] Lao W D . Qu S D，Cheng S Y . Study on mtDNA：The patterns of the porcine liver mtDNA[J] . ChineseJournal of Biochemistry and Molecular Biology，1981，13(2)：175 ~ 181 . [劳为德，曲善乐，陈受宜 . 线粒体基因组的研究 猪肝线粒体基因组的限制性内切酶图谱 . 生物化学与生物物理学报，1981，13(2)：175 ~ 181.]

[9] Liu Z L，Wang Y，Wei H . Effects of three sample preservation methods on total DNA preparation of porcine whole blood[J] . Acta Academiae Medicinae Militaris Tertiae，1999，21(1)：25 ~ 27 . [刘中绿，王勇，朱富旭 . 3 种保存血样方法对猪总 DNA 制备的影响 . 第三军医大学学报，1999，21(1)：25 ~ 27.]