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褐马鸡圈养种群 ISSR 标记的遗传多样性*

武玉珍[1] 冯睿芝[2]

(1. 晋中学院生物科学与技术学院 晋中 030600; 2. 复旦大学生物医学研究院 上海 200032)

摘 要: 利用 ISSR 分子标记技术对褐马鸡庞泉沟国家自然保护区与太原市动物园 2 个种群的遗传多样性进行研究。从 20 个 ISSR 引物中筛选出 10 个引物对褐马鸡 2 个种群的 35 个样品进行扩增,得到 65 个清晰的扩增位点,多态带百分率(PPB)为 76.9%。2 个种群的多态带百分率和群体内遗传多样性指数分别为 54.10%,54.10%,0.302 5,0.205 6,前者遗传多样性略高于后者。2 个种群间的遗传分化系数(G_{st})、基因流估计值(N_m)、遗传相似性系数以及遗传距离均表明种群间的遗传分化较小。褐马鸡 UPGMA 系统树无明显的歧化,尚未表现出遗传趋异。褐马鸡 2 个种群间的遗传多样性指数比较接近,遗传多样性水平较低,核基因组与过去线粒体基因组遗传多样性的研究结果基本一致。

关键词: 褐马鸡; 濒危物种; 圈养; ISSR; 遗传多样性

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ISSR Analysis of Genetic Diversity of Two Brown-Eared Pheasant (*Crossoptilon mantchuricum*) Populations

Wu Yuzhen[1] Feng Ruizhi[2]

(1. *School of Biology Science and Technology, Jinzhong University Jingzhong* 030600; 2. *Institute of Biomedical Sciences, Fudan University Shanghai* 200032)

Abstract: Brown-eared Pheasant (*Crossoptilon mantchuricum*) is a critically endangered endemic species, which is listed in the national first-class protected birds in China. In this study, ISSR markers were used to investigate the genetic diversity of two populations of Brown-eared Pheasant in the Pangquangou Nature Reserve and Taiyuan Zoo. Ten primers out of 20 ISSR primers were selected for amplifying 35 samples of the two populations, and 65 clear amplification sites (PPB = 76.9%) were obtained. The percentage of polymorphic bands of the two populations was around 54.10%. Shannon's Information Index of the two populations was 0.302 5 and 0.205 6, respectively. The generic heterogeneity (G_{st}), genetic flow (N_m), genetic similarity index indicated that there was no significant genetic differentiation between the two populations. Statistical analysis and cluster tree diagram consistently illustrated that the genetic diversity between the two populations was at a low level. Individuals in each population had close genetic relationship. The result in this study were consistent to another study we did with the mitochondrial genome genetic diversity.

Key words: Brown-eared Pheasant; endangered species; populations; ISSR; genetic diversity

ISSRs (inter-simple sequence repeats) are DNA fragments amplified from PCR with microsatellite primers, and can be used as molecular markers for the different size of anchored primers (Zietkiewicz *et al.*, 1994; Gilbert *et al.*, 1999; Fang *et al.*, 1997). For the multi-locus fingerprinting profiles obtained, the applications of ISSR analysis widely spread to genetic identity (Aytekin *et al.*, 2011; Noroozi *et al.*, 2011), parentage, clone and strain identification, taxonomic studies on closely related species (Parsaeian *et al.*, 2011; Zhao *et al.*, 2008), gene mapping studies (Arcade *et al.*, 2000; Casasoli *et al.*, 2001; Sanker, 2001; Jin, 2007; Hizer *et al.*, 2002; Ostberg *et al.*, 2002) and genetic relationship judgments (Huang *et*

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al., 2000; Pharmawati *et al.*, 2005; Sica *et al.*, 2005). In the studies on animals, ISSRs have been successfully used in genetic diversity and genetic structure researches (Aytekin *et al.*, 2011; Abbot, 2001; Willis *et al.*, 2004; Kostia *et al.*, 2000). In China, ISSRs were applied for germplasm analysis of *Eriocheir sinensis* and *Antheraea pernyi* (Zheng *et al.*, 2007; Li *et al.*, 2007). Comparatively fewer studies using ISSRs for vertebrate have been reported, especially for critically endangered species. At present, Bai *et al.* (2001) and Bai (2004) have used ISSRs for the fingerprint analysis of captive populations of Manchurian Tiger and populations of chicken. In other countries, Aytekin *et al.* (2011) used ISSRs to assess genetic diversity in buffalo populations, Kostia *et al.* (2000) used ISSR to study the phylogenesis of 6 species of mammal, and Willis *et al.* (2004) used ISSRs and mitochondria DNA markers to study the natural hybridization of *Phocoenoides dalli* and *Phocoena phocoen*. In summary, the application of ISSRs in the study of higher animals, especially in the genetic diversity of endangered species is limited.

In this article, ISSRs together with the identification results of mitochondria DNA (we reported that in a separate paper) were used to study the genetic structure and genetic diversity of two populations of the Brown-eared Pheasant in Pangquangou Nature Reserve and Taiyuan Zoo. The degree concerning the genetic differentiation and gene flow between and within the two populations were also investigated. The molecular endangering mechanism was discussed and the genetic diversity was analyzed from the nuclear DNA level, hopefully that we can provide nuclear DNA proofs for the protection of the Brown-eared Pheasant and better understands the survival potential and the degree of endangering of this bird so as to assist the formulation and implementation of the Brown-eared Pheasant protection strategy.

1 Materials and methods

1.1 Samples

35 samples of the Brown-eared Pheasant from the two populations were collected, including 3 muscle samples and 15 blood samples from Pangquangou Nature Reserve and 17 blood samples from Taiyuan Zoo, respectively.

1.2 Genome DNA extraction

Genome DNA was extracted following the method described by Wu *et al.*(2010). Purity and concentration of DNA were tested by bio-photometer (Eppendorf). The DNA samples were diluted to 20 ng·mL^{-1} and stored at −20 ℃.

1.3 Primer synthesis

The synthesis of primers was conducted by Aoke Co, Ltd, Beijing following the ISSR primer sequences UBC set 9. According to the references of the study results of Abbot *et al.* (2001) and Bai *et al.*(2001), 20 primers were selected. 4 samples were randomly used as template and 10 primers with stable and clear PCR amplified bands were chosen to use in all the PCR with all of the DNA template samples.

1.4 ISSR-PCR and electrophoresis

PCR was performed in a 25 mL react system containing 2.5 mL of 10 × PCR Buffer (TaKaRa, China), 2 mL of 25 mmol·L^{-1} Mg^{2+} solution, 4 mL of 1.25 mmol·L^{-1} dNTP solution, 5.0 mL of 5 mmol·L^{-1} each primer solution, 0.5 mL of 2 U·mL^{-1} Taq DNA Polymerase (TaKaRa, China) and 1 mL of 10 − 150 ng·mL^{-1} DNA template solution. A PTC-150 thermal cycler was used and the thermal cycling profiles were as follows: an initial hot-start for 5 min at 94 ℃; 40 cycles amplification cycles of denaturation for 45 s at 94 ℃, annealing for 45 s at 38 − 54 ℃ and extension for 1 min at 72 ℃, and a final incubation for 7 min at 72 ℃ was performed to ensure complete extension of the PCR. The annealing temperatures of each primer are showed in Tab. 2. The PCR products were separated by the 1.5% agarose gel. DNA marker: DL2000 (TaKaRa, China).

1.5 Statistical analysis

The primary (0, 1) ISSR analysis matrix was formulated from the results of the experiments, 1 for products generated and 0 for no products generated. Percentage of polymorphic band (PPB), Shannon's Information Index (I), Nei's gene diversity (H_e), the total genetic diversity (H_t), the genetic diversity within a population (H_s), the coefficient of gene differentiation (G_{st}) and the estimate of gene flow (N_m) were calculated by POPGEN 1.31 (Yeh *et al.*, 1997). Nei's unbiased genetic distance and genetic similarity were calculated by PhyTool 6.0. According to Nei's

genetic distance, cluster analysis using Neighbor-Joining Method was performed using MEGA3.1 and clustering tree diagram was constructed. Based on these results, genetic diversity analysis between the two populations and genetic similarity analysis between each individual of the two populations were made.

2 Results and analysis

2.1 PCR-ISSR analysis

PCR for the 35 samples were performed using 10 selected primers (Tab.1). The results of agarose gel electrophoresis showed that TA, AG, CT, CA have high distribution frequency that they have been amplified from primers with these dinucleotide.

Tab.1 Distribution of polymorphism loci in the primers

Primer	Primer sequence	Number of bands	Number of polymorphic bands
806	$(TA)_8G$	2 – 8	7
807	$(TA)_8G$	3 – 8	6
808	$(AG)_8C$	2 – 6	5
809	$(AG)_8G$	3 – 9	7
811	$(GA)_8C$	2 – 8	7
812	$(GA)_8A$	2 – 5	3
815	$(CT)_8G$	1 – 5	4
816	$(CA)_8T$	2 – 6	4
817	$(CA)_8A$	1 – 5	4
818	$(CA)_8G$	2 – 5	3
Total		65	50

2.2 Percentage of polymorphic band

The results of amplification using 10 primers were showed in Tab.2. It was shown that 65 stable and clear bands were amplified and the sizes of these fragments were in the range of 300 – 2 000 bp. The numbers of amplified loci of each primer was 5 – 9, averaged 6.5. Of the total 65 loci 50 loci were polymorphic; the percentage of polymorphic band was 76.9%. Different primers have different numbers of amplified loci; the percentage of polymorphic band was 60.00% – 77.78%.

2.3 Genetic diversity of the two populations

Statistical analysis of 35 individuals of the two populations was performed using POPGRNE1.31. The results were showed in Tab.2. Effective numbers of alleles, H_e and I of the two populations of Pangquangou Nature Reserve and Taiyuan Zoo are 1.362 8, 0.205 6, 0.302 5 and 1.294 9, 0.175 2, 0.265 9, respectively. Observed numbers of alleles, number of polymorphic bands and percentage of polymorphic bands for the two populations are the same: 1.541 0, 33, and 54.10.

Tab.2 Statistical analysis of genetic diversity in two populations①

Population	Number of samples	N_a	N_e	H_e	I	Number of polymorphic bands	PPB
Pangquangou	18	1.541 0	1.362 8	0.205 6	0.302 5	33	54.10
Taiyuan Zoo	17	1.541 0	1.294 9	0.175 2	0.265 9	33	54.10
Average	17.5	1.541 0	1.328 8	0.190 4	0.284 2	33	54.10
Species	35	1.623 0	1.403 6	0.228 1	0.336 7	38	62.30

①N_a = Observed number of alleles; N_e = Effective number of alleles; H_e = Nei's (1973) gene diversity; I = Shannon's information index; PPB = Percentage of polymorphic loci.

At the population level, H_e, I and PPB are 0.190 4, 0.284 2 and 54.10%. At the species level, H_e, I and PPB are 0.228 1, 0.336 7 and 62.30%. Therefore, the genetic diversity at the species level is higher than that at the population level. The genetic diversity at population level of Taiyuan Zoo is a little different from that of Pangquangou Nature Reserve, but they did not reach the statistical significant level. Therefore, the genetic diversity of the two populations is close to each other.

2.4 Relationship of genetic diversity of each population

We used POPGRNE1.31 to calculate genetic diversity index between and within the two populations. The H_s, H_t, G_{st} and N_m are 0.227 7, 0.190 4, 0.164 0 and 2.549 3. On the whole, H_s are a little higher than H_t, and H_t is at a low level. G_{st} is small, 16.40% variations between the two populations, 83.60% variations within the populations and gene flows exist between them. From Tab.3, the Nei's gene identity of the two populations is 0.907 9 and the genetic distance is 0.096 6. It further illustrates that the genetic diversity of the two populations are nearly the same and the genetic variation between them are not evident.

Tab. 3 Nei's genetic identity and genetic distance between two populations①

Population	1	2
1		0.907 9
2	0.096 6	

① Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

2.5 Genetic distance and cluster analysis among individuals

Nei's genetic distance and genetic similarities of the two populations between each individual were calculated using PhylTools software. The average genetic similarity of the two populations is 0.506 1 and 0.759 1, and the average genetic distance is 0.493 9 and 0.240 9 respectively. The total of Nei's genetic distance and similarity coefficient of average of Brown-eared Pheasant 35 individuals of the two populations are 0.372 3 and 0.631 0 respectively. Using UPGMA (unweighted pair-group method with arithmetic means) Method to perform Nei's genetic distance cluster analysis achieved a result showing in Fig. 1, which demonstrates that the individuals of the two populations did not form 2 branches, but made cross correlations in each other's population.

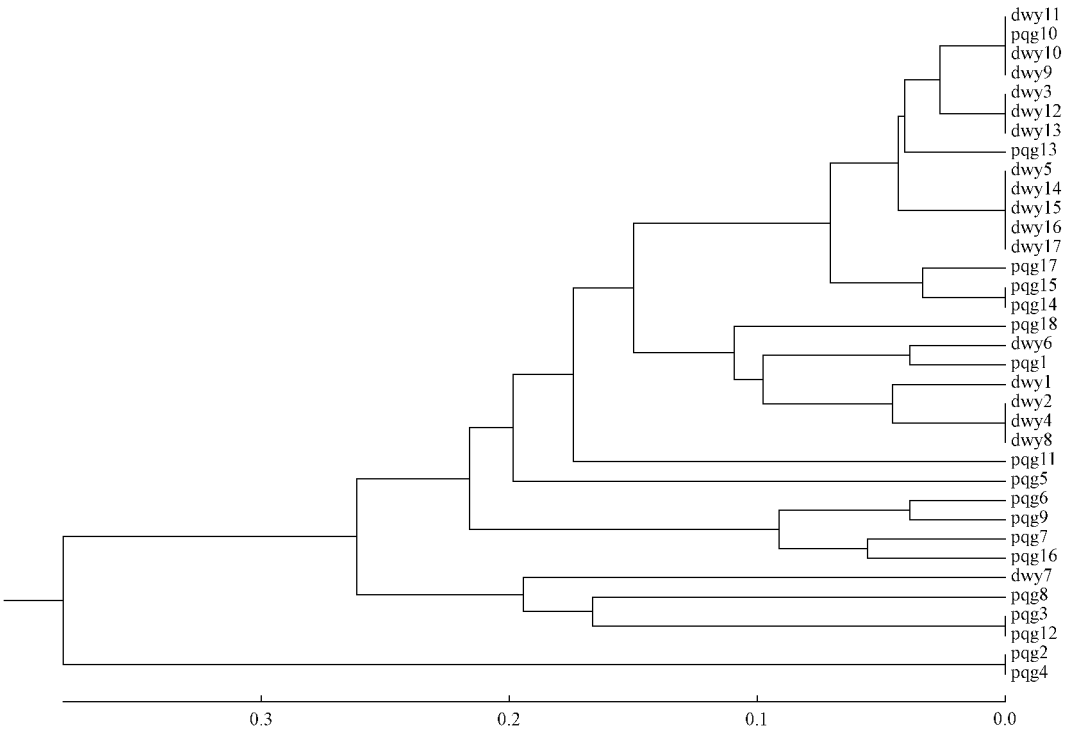

Fig. 1 Dendrogram of Brown-eared Pheasant based on Nei's distance of ISSR marker and clustered using UPGMA dendrogram using average linkage (between groups)
dwy: Taiyuan Zoo; pqg: Pangquangou

3 Discussions and conclusion

3.1 ISSR genetic diversity analysis of the Brown-eared Pheasant

The statistical result of the two population's shows that the diversity at species level is higher than that at population level, and the genetic diversity index of the two populations are close to each other, which means the genetic diversity of them is at a low level. At present, there are fewer reports in China using ISSR on birds and there is no comparison data for the study. However, the results are consistent with that using mitochondria control region as molecular marks in our previous publication (Wu *et al.*, 2010).

There is truly different between the genetic diversity of the two populations although not significant. The genetic diversity of the population of Pangquangou Nature Reserve is a little higher than that of Taiyuan Zoo (I of them are 0.302 5 and 0.205 6, and H_e of them are 0.265 9 and 0.175 2).

As ISSR is dominant marker, we cannot tell that related loci are homozygous or heterozygous. Therefore the ignorance of heterozygous gene will contribute to H_e, which leads to the underestimate to I for the total

genetic diversity and the genetic diversity within the population. The results in this research can be used to compare with other co-dominant molecular markers such as SSR and AFLP in further study, and enlarging sample range is another important aspect of better understanding of genetic diversity of the Brown-eared Pheasant.

3.2 Analysis of genetic variations between the two populations

The genetic diversity of the Brown-eared Pheasant populations is at a low level. The two populations have nearly the same genetic diversity within the population, which is higher than genetic diversity between the two populations. Genetic differentiation between the two populations is lower and there are gene flows. Professor Wright (Wright, 1931) thought that if gene flow between populations is >1, then homogenization will be made. Conversely, if gene flow is <1, then it will be the main reason for genetic differentiation. In our research, gene flow between the two populations is $>1.16.40\%$ variations are between the two populations and 83.60% variations are within the populations. Genetic similarity of the two populations is 0.907 9 and genetic distance is 0.096 6, demonstrating that the genetic diversity levels of the two populations are consistent, and genetic differentiation between them is not significant mainly due to the introduction of the species of Taiyuan Zoo from Pangquangou Nature Reserve.

3.3 Comparison between ISSR markers and mitochondria gene markers

The polymorphism of mitochondria DNA and nuclear DNA are sometimes inconsistent (Moritz, 1991; Curole *et al.*, 1999). Different genetic diversity indexes were obtained by using ISSR markers and mitochondria control region. However, both of them showed that the genetic diversity of the Brown-eared Pheasant of the two populations did not reach the statistical significant level and they are consistent.

For mitochondria control region, the average Nei's genetic distance of the individuals of the population of Pangquangou Nature Reserve and Taiyuan Zoo are 0.002 and 0.003, respectively. The average Nei's genetic distance of 20 individuals from Pangquangou Nature Reserve and Taiyuan Zoo is 0.002 (max: 0.009; min: 0.000). For ISSRs, the average Nei's genetic similarity of individuals from Pangquangou Nature Reserve and Taiyuan Zoo are 0.506 1 and 0.759 1, respectively. The average genetic distance of them is 0.493 9 and 0.240 9, respectively. The average Nei's genetic distance and genetic similarity of the whole 35 individuals from the two populations are 0.372 3 and 0.631 0, respectively (max: 1.000; min: 0.000). The average of Nei's gene diversity between the two populations is 0.907 9, and the average genetic distance is 0.372 3. Although above indexes are different, the genetic differentiation between the two populations of the two places is at a low level and the genetic variation between individuals of the two populations is not significant, suggesting that the individuals have close genetic relationship.

3.4 Conclusions

These data reflect the low level of genetic diversity of the Brown-eared Pheasant populations. The results of nuclear genome and mitochondria genome are consistent and both prove that the genetic differentiation between the two populations is not significant. Individuals between the two populations have close genetic distance and high degree of inbreeding.

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