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# Genetic Diversity Analysis of the Only White-Red Deer Population in China

**Ruobing Han, Shanshan Gui, Xunwu Zhao and Heping Li***

*College of Wildlife and Protected Area, Northeast Forestry University, Harbin 150040, China.*

## ABSTRACT

The white-red deer population in China is composed of 28 individuals introduced from New Zealand. However, the genetic relationships between these individuals is unknown. The aim of the present study was to determine the genetic diversity and genetic relationship in the white-red deer population. We used ten microsatellite loci to genotype all 28 white-red deer individuals in China, and constructed a Neighbor-joining (NJ) tree based on genetic distances. In addition, genetic structure analysis was carried out. The results showed that a total of 67 alleles were detected, and the average number of alleles at each locus was 6.7. There was high genetic diversity but severe inbreeding in the white-red deer population, and there is no father-mother-offspring relationship or father-offspring relationship in white-red deer population. Our study provides the essential data support and reference for the conservation and sustainable development of the domestic white-red deer population in China.

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**HPL designed the experiment. SSG performed the experiment. RBH write the manuscript. HPL and XWZ revised the manuscript.**

**Key words**
**White-red deer, Genetic diversity, Microsatellite DNA, Genetic relationship, China**

## INTRODUCTION

White-red deer, also known as Danish White-red deer in Europe, is a "subspecies" of red deer genetic variants (Bartos, 1992) that is only bred in very low numbers worldwide. Prior to 28 white-red deer introduced from New Zealand by Liaoning Deer Breeding Base of State Administration in April 2015, white-red deer did not occur in China. Additionally, it is well known that the white-red deer is a rare wild animal. It is not only a precious natural resource but also an essential economic resource, showing that the protection and research of white-red deer population are particularly crucial and necessary.

Microsatellite DNA marker is widely used in research of animal genetic diversity due to its unique characteristics (wide distribution, rich polymorphism, codominant inheritance, and rapid detection). At present, the application of microsatellites in cervid animals mainly includes sika deer, red deer, and black tail deer (Brinkman *et al*., 2010; Hu *et al*., 2018; Lv *et al*., 2014; Talbot *et al*., 1996). However, the research on white-red deer is still limited to describing its location and apparent characteristics, which have not been reported at the molecular level, such as the research on the genetic relationships and genetic diversity among 28 individuals in the white-red deer population.

The current study aims to explore the genetic relationship and genetic diversity of the only white-red deer population in China by microsatellite DNA marker based on feces DNA. It is of great practical significance for the conservation of wild animals to collect white-red deer fecal by non-invasive sampling method. Their genetic relationship was consequently identified. The genetic diversity analysis of the domestic white-red deer population provides novel insight into genetic information and reference for the genetic management of the white-red deer population in China.

## MATERIALS AND METHODS

*Sample collection*

All individuals (28) in the white-red deer population were collected from Liaoning deer breeding base of the state forestry administration in China, including 16 males and 12 females. This is the only stable white-red deer population in China until now, introduced from three regions in New Zealand. To avoid repeated sampling of the same individual, we collected freshly shed excrements and recorded the collected samples immediately. Twenty-eight

* Corresponding author: lihepinghrb2002@nefu.edu.cn
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fecal samples were stored at -80°C until DNA extraction.

*DNA extraction*

DNA was extracted in accordance with the manufacturer instructions of the TIANamp Stool DNA Kit DP328. Concentration and purity of extracted DNA were evaluated on a Nano Drop 2000 spectrophotometer and by gel electrophoresis. The extracted DNA was stored at -20°C until use.

*Screening of microsatellite loci and amplification*

Based on the previous study (Steffen *et al*., 1993; Dewoody *et al*., 1995; Jones *et al*., 2000; Buchanan *et al*., 1994; Zhang *et al*., 2010), we first selected the twenty pair microsatellite primers with good polymorphism, and the information of specific primers is shown in Table I. For ensure the accuracy and reliability of the screening results, 10 μL and 25 μL PCR amplification systems were applied, respectively. The 10 μL reaction mixture included 1 μl buffer, 0.8 μl dNTP, 0.05 μl rTaq DNA polymerase, 0.25 μl of each primer, 50 ng DNA template, and adding sterilized deionized water up to a total volume of 10 μl. The 25 μL reaction mixture included 2 μl buffer, 2 μl dNTP, 0.2 μl rTaq DNA polymerase, 1 μl of each primer, 100 ng DNA template, and finally adding sterilized deionized water up to a total volume of 25 μl. Amplification program was pre-denaturation at 94°C for 5min, 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C-60°C for 30 s, and extension at 72°C for 1 min. Finally, the amplification program was extension at 72 °C for 10 min. PCR amplified products were stored at 4°C.

*Identification of genetic relationship*

Because potential parent-offspring relationships among the 28 introduced white-red deer individuals was unknown, all individuals were used as offspring and candidate parents separately to analyze simulation of parentage 1000 times and the strict confidence was 95% as well as relaxed confidence was 80%. The higher the LOD value of candidate parents, the more likely they are to be real parents. Consequently, parentage analysis wizard was performed on 28 white-red deer individuals by Cervus 3.0 (Slate *et al*., 2000; Kalinowski, 2007).

*Analysis of clustering*

The genetic structure of white-red deer population was analyzed using STRUCTURE (Pritchard *et al*., 2000). Ten iterations were performed for each K value from 2 to 10. The Length of Burnin Period was 10000, followed by 10000 repeats of the MCMC algorithm. In addition, Structure Harverster was used to identify the most probably K in the white-red deer population.

**Table I. Twenty microsatellite primer sequences.**

| Locus | Forward Primer | Reverse Primer | TM/°C |
|---|---|---|---|
| N | TCCAGAGAAGCAACCAATAG | GTGTGCCTTAAACAACCTGT | 56 |
| BM203 | GGGTGTGACATTTTGTTCCC | CTGCTCGCCACTAGTCCTTC | 54 |
| BM848 | TGGTTGGAAGGAAAACTTGG | CCTCTGCTCCTCAAGACAC | 55 |
| BM6506 | GCACGTGGTAAAGAGATGGC | AGCAACTTGAGCATGGCAC | 63 |
| BM6438 | TTGAGCACAGACACAGACTGG | ACTGAATGCCTCCTTTGTGC | 58 |
| BM4208 | TCAGTACACTGGCCACCATG | CACTGCATGCTTTTCCAAAC | 56 |
| BM5004 | TCTGGAGTGAATGTTTCTGAGG | TTGTGATGAGCACCTGAAGG | 49 |
| T123 | GTTTCCTTGGCACATCTCT | CTGTCGTTGTTGTTCTGTTG | 53 |
| T156 | TCTTCCTGACCTGTGTCTTG | GATGAATACCCAGTCTTGTCTG | 55 |
| T501 | CTCCTCATTATTACCCTGTGAA | ACATGCTTTGACCAAGACC | 53 |
| T507 | AGGCAGATGCTTCACCATC | TGTGGAGCACCTCACACAT | 55 |
| T530 | GTCCTCACAGCAGCTCTATG | GCATTCTTTAGAACTCCAACTG | 56 |
| C217 | GCAGGAAGGAGGAGACAGTA | GCTGGTTCGTTATCATTTAGC | 56 |
| C180 | GGTGGGCATTCAGTAGA | AGGCAGAGAAGGCATTG | 52 |
| C143 | AAGGAGTCTTTCAGTTTTGAGA | GGTTCTGTCTTTGCTTGTTG | 53 |
| C229 | CCCTGTGGTCTAGCAAA | ATAGGCACATGCTCATAAG | 52 |
| T172 | AGCATCTCCCCTTTCAACA | CTTCCCAACCCAAGTATCG | 54 |
| T193 | AGTCCAAGCCTGCTAAATAA | CTGCTGTTGTCATCATTACC | 53 |
| T108 | CATGTGGAGATAGGTAGACAGA | CCATTCTGAGTAGCTGATTCA | 54 |
| BM888 | AGGCCATATAGGAGGCAAGCTT | CTCGGTCAGCTCAAAAGCAG | 57 |

*Statistical analysis*

We used Excel, GeneMarkerV1.91 (Hulce *et al*., 2011), and MicroChecker to statistics of alleles size for each microsatellite locus. The analysis of Allele Frequency was performed using Cervus 3.0, including the calculation of many important parameters, such as the number of alleles (Na), polymorphism information content (PIC), observed heterozygosity (Ho), and expected heterozygosity (He). Meantime, the analysis of paternity inference was performed. The effective number of alleles (Ne), Allele frequency, genotype frequency, Shannon information index, Fis, Ewen-Watterson test, and Hardy-Weinberg equilibrium were calculated by POPGEN32 (Yeh *et al*., 1997). Finally, Population 1.2.32 was used to construct the NJ tree.

## RESULTS

*Genotyping*

In the current study, 12 pair microsatellite primers (BM203, BM6506, C217, T123, C180, BM5004, C143, T172, T156, T501, T507, and T193) that can amplify specific products were screened from 20 pair microsatellite primers. The results of genotyping showed that the amplification of two microsatellite loci (T501 and BM6506) was unstable and the polymorphism was low. Therefore, 10 pair microsatellite primers were screened due to their high polymorphism.

*Allele frequency and genetic diversity*

A total of 67 alleles were detected with allele frequency analysis, Na at each locus was 4-10, and the average number of alleles at each locus was 6.7 (Table II). The average Ne of 10 microsatellite loci in the introduced white-red deer population ranged from 3.0312 to 5.9268. The PIC of all microsatellite loci was more than 0.5, which was highly polymorphic. He of microsatellite locus in this population varied from 0.863 to 0.847, and the Ho varied from 0.071 to 0.37. The Shannon information index varied from 1.2103 to 1.9046, and the average Shannon information index was 1.6331. In addition, the average of Fis was 0.7818 (Table III).

Chi-square test was used to determine whether the genotype frequency of each microsatellite loci was consistent with Hardy-Weinberg equilibrium. Additionally, the Ewen-Watterson test was carried out. The p-value of 10 microsatellite loci was less than 0.01. It showed that the microsatellite loci of the introduced white-red deer population deviated significantly from Hardy-Weinberg equilibrium ($0.01<P<0.05$) (Supplementary Table SI).

The Ewen-Watterson test for neutrality equilibrium indicated that the three microsatellite loci were non-neutral sites (BM5004, T507, C180), the observed F values were below the lower limit of 95% confidence interval. The results suggested that these three loci may be affected by migration, genetic drift or selection. In addition, the remaining 7 microsatellites were neutral sites, indicating that these microsatellites loci could not be affected by the genetic structure of the white-red deer population (Supplementary Table SII).

**Table II. The major genetic diversity parameters of the 10 loci.**

| Loci | Na | Ne | I | He | Ho | PIC |
|---|---|---|---|---|---|---|
| BM203 | 8 | 4.0165 | 1.6438 | 0.762 | 0.370 | 0.713 |
| BM5004 | 7 | 5.6201 | 1.8253 | 0.837 | 0.179 | 0.798 |
| T123 | 8 | 5.0744 | 1.7820 | 0.818 | 0.286 | 0.776 |
| T156 | 8 | 4.5982 | 1.7515 | 0.797 | 0.071 | 0.755 |
| T172 | 10 | 5.0744 | 1.9046 | 0.818 | 0.071 | 0.780 |
| T193 | 5 | 3.5395 | 1.4140 | 0.731 | 0.143 | 0.675 |
| T507 | 7 | 5.9268 | 1.8610 | 0.847 | 0.148 | 0.810 |
| C143 | 4 | 3.3433 | 1.2922 | 0.714 | 0.143 | 0.650 |
| C180 | 6 | 4.8986 | 1.6468 | 0.811 | 0.115 | 0.765 |
| C217 | 4 | 3.0312 | 1.2103 | 0.683 | 0.111 | 0.608 |
| Mean | 6.7 | 4.5123 | 1.6331 | 0.7816 | 0.164 | 0.7331 |

No, number of alles; Ne, effective number of alleles; I, Shannon information index; Ho, observed heterozygosity; He, expected heterozygosity; PIC, polymorphism information content.

**Table III. The result of Fis.**

| Locus | Sample size | Fis |
|---|---|---|
| BM203 | 54 | 0.4575 |
| BM5004 | 56 | 0.7828 |
| T123 | 56 | 0.6442 |
| T156 | 56 | 0.9087 |
| T172 | 56 | 0.9110 |
| T193 | 56 | 0.8009 |
| T507 | 54 | 0.8218 |
| C143 | 56 | 0.7962 |
| C180 | 52 | 0.8550 |
| C217 | 54 | 0.8342 |
| Mean | 55 | 0.7818 |

*Genetic distance and phylogenetic analysis*

It can be concluded that the pairwise genetic distance among 28 white-red deer individuals ranged from 0.5289 to 2.9444, among which the distance between D33 and D26 was the farthest (2.9444), and the distance between D29 and D24 was the closest (0.5289) (Supplementary Table SIII).

The N-J tree revealed three clusters, namely A, B, and C. The number of white-red deer in A was the most (12), followed by C (10), and the number of while-red deer in B was the least (6) (Fig. 1).

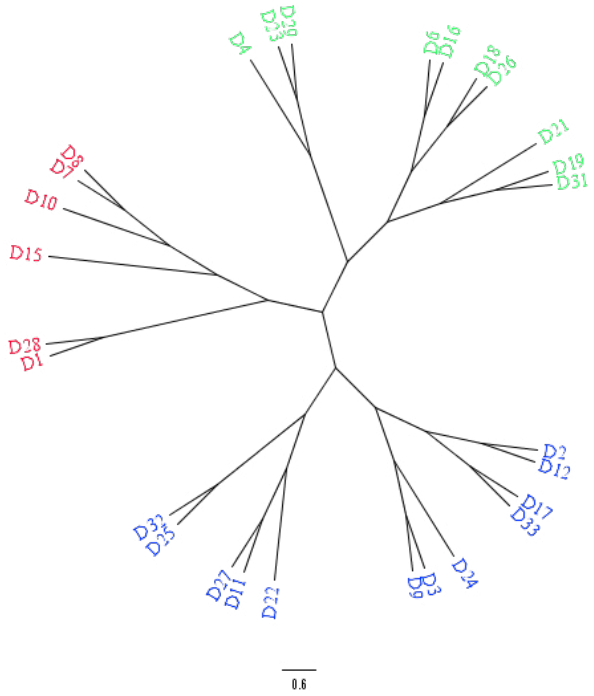


Fig. 1. Construction of NJ tree.

*Genetic relationship of white-red deer population*

There were 12 females and 16 males in the white-red deer population. The LOD and critical Delta were calculated separately after 1000 simulations. The results showed that all LOD value were less than 0. According to the identification results of LOD and Delta, indicating that there was no father mother offspring relationship or father offspring relationship among 28 white red deer individuals.

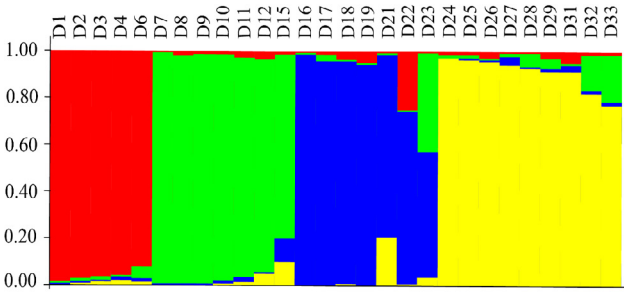


Fig. 2. STRUCTURE analysis of white red deer using 10 microsatellite loci.

*Clustering in white-red deer population*

We used Bayesian clustering to carry out the clustering analysis for the white-red deer population. The results of STRUCTURE showed that the most probably K was k=4, suggesting that there were four distinct groups (Fig. 2). D1, D2, D3, and D4 may have similar mixed ancestors. D7, D8, D9, and D10 may have the similar genetic structure. In addition, D24, D25, and D26 may also have similar mixture ancestors.

## DISCUSSION

The introduced white-red deer population from New Zealand to China has undoubtedly increased the species diversity of cervid animals in China. Unfortunately, the genetic relationship among 28 individuals and the genetic diversity of the white-red deer population remains unsolved. The study of genetic diversity and genetic relationships between different individuals provides a basic reference for the conservation and sustainable development of the white-red deer population.

In the present study, 10 pairs of microsatellite primers with good polymorphism were determined. There were many parameters that can be used to measure the genetic diversity of this population, including Na, Ne, Ho, He, and PIC. In addition, allele frequency can be used to estimate genetic variation. The allele difference of most significant was T172, which differs from 124 to 168 by 44bp. The gene frequency of 216 bp in the BM2003 locus is 0.3889, and that of 214 bp is only 0.0185. Meanwhile, the closer the Ne was to the Na, the more evenly alleles were distributed in this population. The average of Na (6.7) in the white-red deer population was higher than that of Ne (4.5123), proving that the alleles were unevenly distributed. This phenomenon may be caused by artificial selection or drift or new mutation.

The previous study showed that the loss of alleles could be roughly estimated by the difference between Na and Ne (Tian *et al.*, 2010). He of 10 microsatellite loci in the white-red deer population was higher than that of Ho, and the average He (0.7816) was also much higher than that of Ho (0.164), indicating that the inbreeding of the population was serious. On the other hand, the average Fis of this white-red deer population was 0.7818. This vital parameter Fis also proved the inbreeding of this population was serious. It is well known that inbreeding was not conducive to development of white-red deer population. In summary, the results of our study showed that the inbreeding of domestic white-red deer populations was severe.

The PIC can reflect the degree of genetic variation of this population at multiple microsatellite loci. A

microsatellite loci with PIC>0.5 was highly polymorphic, 0.25<PIC<0.5 was moderately polymorphic, while the microsatellite loci with PIC<0.25 was low polymorphic (Botstein *et al*., 1980). The PIC of most microsatellite loci in the current study was highly polymorphic (80%). Among them, the PIC of C217 and T507 were the highest and lowest, respectively. Furthermore, previous studies considered PIC>0.7 as the optimal genetic markers (Purvis and Franklin, 2015). Seven out of ten microsatellite loci in our study were optimal genetic markers, showing that the microsatellite loci screened was reasonable for studying the genetic diversity of white-red deer population. Moreover, the white-red deer population has the potential for conservation and sustainable development because of its high genetic diversity.

It is concluded that the introduced white-red deer population was deviated significantly from the Hardy-Weinberg equilibrium, which is consistent with the conclusion of previous studies. The phenomenon that rare endangered populations do not conform to the mendelian inheritance was common (Ardren, 1999). It may be artificial breeding selection has broken the Hardy-Weinberg equilibrium.

Twenty-eight white-red deer can be clustering three branches (A, B and C). This can be proved by the fact that the individuals in the white-red deer population were selected from deer farms in three regions when they were introduced from New Zealand. Besides, there was no father-mother-offspring relationships or father-offspring relationships among 28 white-red deer individuals. This is undoubted positive significance to the sustainable development of the white-red deer population in China. The results of STRUCTURE showed that the white-red deer population in China could be divided into four separate groups. We should pay attention to avoiding mating between individuals with the close relationship in the future breeding process.

In conclusion, genetic diversity plays an essential role during the species survival and evolution. The genetic diversity of the white-red deer population in China was high through microsatellite DNA marker analysis, which has a positive significance for the further sustainable development of the white-red deer population in China. Furthermore, the inbreeding in the white-red deer population was serious, and this reminds us that avoiding inbreeding could be contributed to the conservation and breeding of rare species. On the other hand, scientific feeding and management should be formulated to create conditions for the white-red deer populations to adapt the new environment. Moreover, human awareness of conserving the endangered species should be raised and excessive human interference should be avoided.

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*Supplementary material*

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*Statement of conflict of interest*

The authors have declared no conflict of interest.

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