



Microsatellite-based Genetic Diversity and Evolutionary Relationships of Six Dog Breeds

J. -H. Ye^{1, a}, D. -R. Ren^{2, a}, A. -F. Xie³, X. -P. Wu⁴, L. Xu¹, P. -F. Fu¹, H. -A. Zhao⁵ and Q. -Y. Yang^{1, *}

¹ Ministry of Public Security, Police Dog Base in Nanchang, 330100, Nanchang, China

ABSTRACT : The Tibetan Mastiff is one of the most archaic, ferocious and the largest dogs in the world. The Kunming dog is the chief working-dog breed in China. In this study, ten microsatellite loci were used to assess the genetic diversity and evolutionary relationships in six dog breeds, including Tibetan Mastiff, Kunming dog, Belgian Malinois, Labrador Retriever, English Springer Spaniel, and German Shepherd. The highest genetic diversity was exhibited by the Tibetan Mastiff, indicating useful protection and little inbreeding in the modern Tibetan Mastiff. Higher genetic diversity was observed in European breeds, supporting the hypotheses that breeders outcross their pure breed dogs occasionally to avoid deleterious effects in Europe. Evolutionary relationships showed that English Springer Spaniel and Labrador Retriever were clustered together, then with the Tibetan Mastiff, consistent with previous cluster results. German Shepherd and Kunming dog were grouped together, coinciding with the breeding history of Kunming dog. It is the first time that Tibetan Mastiff and Kunming dog have been analyzed with microsatellites. (**Key Words :** Dog, Genetic Diversity, Evolutionary Relationships, Microsatellite)

INTRODUCTION

Dogs (*Canis familiaris*) were domesticated from gray wolves (*Canis lupus*) at least 14,000 years ago (Vilà et al., 1997; Sablin et al., 2002; Savolainen et al., 2002). The various environmental conditions and selective breeding have generated a rich diversity of dog breeds. The World Canine Organization currently recognizes ~347 breeds of dogs across the world and some of them are very ancient breeds, such as the Chinese Shar-Pei, Pekingese and Chow Chow, and Tibetan Mastiff (Parker et al., 2004). Tibetan Mastiff, marked by the eastern deity dog, is also one of the oldest breeds in China. Tibetan mastiffs grow in China's Qingzang plateau at 3,000-5,000 meters altitude in cold districts, and have characteristics which were described by

Marco Polo, one of the most famous travelers, "as big as a donkey, when running they are as swift as the tiger, roaring as loud as the lion, with good looks". Uniquely bred by a China zoologist as a working-dog, Kunming dog has special adaptability to environment, longer stamina and fast speed, stronger disease-resistance and natural damage resistance, as well as feeding on coarse fodder, and it is the chief working-dog breed in China. Herein, the purpose of this study was to assess the genetic diversity of modern purebred Tibetan Mastiff and Kunming dog, and evaluate their evolutionary relationships with European breeds such as Belgian Malinois, Labrador Retriever, English Springer Spaniel, and German Shepherd by using microsatellite locus amplifications.

In recent years, microsatellite analysis has been widely used to determine population structure and genetic diversity, within and among animal populations, including dogs (Koskinen et al., 2000; Ichikawa et al., 2001; Nagamine et al., 2001; Kim et al., 2001; Sundqvist et al., 2001; Koskinen, 2003; Irion et al., 2003; DeNise et al., 2004; Puja et al., 2005; Chang et al., 2007; Wu et al., 2008). However, genetic diversity of Tibetan Mastiff and Kunming dog have not been studied by using microsatellites previously. Hence, in this study ten high polymorphic microsatellite loci were used to investigate genetic diversity in Tibetan Mastiff and Kunming dog for the first time, and to evaluate the

* Corresponding Author: Qianyong Yang. Tel: +86-791-6081096, Fax: +86-791-3702020, E-mail: yqyang100@sina.com

² College of Animal Science and Technology, Jiangxi Agricultural University, 330045, Nanchang, China.

³ Graduate School of Jiangxi Normal University, 330022, Nanchang, China.

⁴ Nanchang University, 330047, Nanchang, China.

⁵ Jiangxi Forensic Science Institute, Nanchang 330006, China.

^a Both authors contributed equally to this work and should be considered as first coauthors.

Received September 3, 2008; Accepted February 2, 2009

relationships with Belgian Malinois, Labrador Retriever, English Springer Spaniel, and German Shepherd. Allelic diversity, Nei's DA distance and a phylogenetic tree are presented.

MATERIALS AND METHODS

Sample preparation and genotyping

In this study, we examined 608 unrelated and purebred individuals representing six breeds: Tibetan Mastiff from Tibet of China, $n = 42$; Kunming dog from Yunnan Province of China, $n = 91$; German Shepherd from German, $n = 259$; Belgian Malinois from Belgium, $n = 85$; Labrador Retriever from U.K., $n = 82$; and English Springer Spaniel from U.K., $n = 49$. Genomic DNA used for polymerase chain reactions was extracted from blood by the conventional phenol/chloroform extraction method. Ten markers were selected from the commercial StockMarks[®] for Dogs Parentage Typing Kit from Applied Biosystems (ABI, Foster City, CA, USA). Markers were amplified in a 10- μ l PCR mixture as recommended by ABI (K-9 1 Version 3, 4307481C and a customized kit) in PTC-200 (rev: D.A, BIO-RAD, USA) and fragments were analyzed on ABI PRISM[®]3100 Genetic Analyzers. Microsatellite localizations, repeat names, dyes, allele ranges and annealing temperatures are shown in Table 1.

Genetic analysis

Allele number and frequencies per locus were obtained by direct counting of alleles from the individual samples for each breed. Observed heterozygosity (H_o) and expected heterozygosity (H_e) were assessed from allele frequencies (Nei, 1978). Polymorphic information content was calculated according to previous literature (Botstein et al., 1980). Genetic distances were computed according to DA genetic distances (Nei et al., 1983). A phylogenetic tree was estimated using the neighbor-joining (NJ) method in PAUP version 4.0b10 (Swofford, 2002). The reliability of the nodes was assessed with 1,000 bootstrap iterations (Felsenstein, 1985).

RESULTS AND DISCUSSION

Genetic diversity

Summaries of marker characteristics within each breed are presented in Table 2. Clearly the genetic variations of the ten microsatellite loci are considerable within the six breeds. The mean number of alleles per locus within breed ranged from 6.5 (Kunming dog) to 7.7 (Tibetan Mastiff). The average number of alleles per marker per breed was 7.1 and larger than 5.7 and 4.34 described by previous results (Kim et al., 2001; DeNise et al., 2004). Consistent with the previous result, *PEZ03* was also the most informative

Table 1. Characteristics of ten microsatellites in this study

Marker name	Chromosome location	Repeat name	DYE	Allele range (/bps)	Annealing temp. ($^{\circ}$ C)
PEZ1	CFA07	Di_repeat	FAM (blue)	92-136	58
FH2054	CFA12	(GATA)~16	FAM (blue)	140-183	58
FH2010	Synt05	(CAAA)10	FAM (blue)	210-260	58
PEZ5	CFA12	Tetra_repeat	JOE (green)	97-121	58
PEZ12	CFA03	Tetra_repeat	JOE (green)	250-320	58
PEZ3	CFA19	Tetra_repeat	NED (yellow)	95-154	55
PEZ6	CFA27	Tetra_repeat	NED (yellow)	164-214	56
PEZ8	CFA17	Tetra_repeat	NED (yellow)	222-260	58
FH2079	CFA24	Mix (GGAT)~47 scrambled	NED (yellow)	263-299	58
PEZ20	-	Tetra_repeat	JOE (green)	174-201	58

Table 2. Number of alleles (n), observed Heterozygosities (H_o), expected Heterozygosities (H_e), and polymorphism information content (PIC) at 10 canine microsatellite loci

Locus	Tibetan Mastiff				Kunming dog				German Shepherd				Belgian Malinois				Labrador Retriever				English Springer Spaniel			
	n	H_o	H_e	PIC	n	H_o	H_e	PIC	n	H_o	H_e	PIC	n	H_o	H_e	PIC	n	H_o	H_e	PIC	n	H_o	H_e	PIC
PEZ01	8	0.77	0.82	0.79	6	0.55	0.62	0.58	8	0.15	0.33	0.32	6	0.61	0.74	0.69	5	0.6	0.72	0.67	8	0.63	0.82	0.79
FHC2054	9	0.77	0.79	0.77	9	0.86	0.79	0.76	11	0.73	0.72	0.66	9	0.87	0.86	0.84	7	0.67	0.79	0.77	9	0.73	0.80	0.77
FHC2010	4	0.51	0.60	0.54	4	0.77	0.70	0.65	5	0.6	0.57	0.49	6	0.73	0.73	0.69	5	0.42	0.73	0.68	5	0.76	0.56	0.48
PEZ05	6	0.71	0.71	0.66	5	0.67	0.70	0.65	6	0.13	0.28	0.27	5	0.394	0.45	0.41	4	0.53	0.49	0.45	5	0.34	0.65	0.59
PEZ20	9	0.77	0.87	0.86	4	0.24	0.39	0.36	4	0.34	0.48	0.44	4	0.70	0.66	0.60	5	0.67	0.63	0.58	6	0.45	0.73	0.69
PEZ12	10	0.91	0.86	0.85	6	0.72	0.67	0.60	5	0.23	0.37	0.35	7	0.71	0.76	0.73	9	0.67	0.80	0.77	9	0.82	0.81	0.79
PEZ03	8	0.84	0.82	0.79	8	0.75	0.77	0.74	9	0.51	0.63	0.59	11	0.93	0.83	0.81	11	0.82	0.85	0.83	10	0.84	0.84	0.82
PEZ06	9	0.84	0.81	0.79	8	0.81	0.77	0.74	8	0.71	0.76	0.72	8	0.93	0.83	0.81	8	0.68	0.81	0.78	8	0.82	0.73	0.69
PEZ08	9	0.88	0.85	0.83	8	0.48	0.57	0.55	10	0.81	0.76	0.72	7	0.89	0.84	0.82	7	0.77	0.80	0.77	9	0.86	0.78	0.75
FHC2079	5	0.63	0.59	0.54	7	0.46	0.57	0.55	6	0.35	0.41	0.35	7	0.77	0.79	0.75	6	0.46	0.61	0.57	7	0.35	0.72	0.67
Mean	7.7	0.76	0.77	0.74	6.5	0.63	0.66	0.62	7.2	0.46	0.53	0.49	7	0.75	0.75	0.72	6.7	0.63	0.72	0.69	7.6	0.66	0.74	0.70

Table 3. Genetic distances between the six dog breeds

Breed	K	G	B	L	E.S.
Tibetan Mastiffs	0.194	0.239	0.159	0.148	0.165
Kunming dog		0.105	0.111	0.204	0.214
German Shepherd			0.162	0.254	0.212
Belgian Malinois				0.145	0.164
Labrador Retriever					0.099

K = Kunming dog; G = German Shepherd; B = Belgian Malinois; L = Labrador Retriever; E.S = English Springer Spaniel.

marker across breeds and higher than the average number of alleles per breed observed in previous study (DeNise et al., 2004). Observed and expected mean heterozygosities for the different dog breeds ranged from 0.46 to 0.76 and 0.53 to 0.77, respectively, which were higher than previous results (Kim et al., 2001). Note that the means of number of alleles, observed and expected heterozygosities, and polymorphism information in Tibetan Mastiff at all loci were higher than other breeds in this study. All Tibetan Mastiffs were collected in the remote villages of Qingzang plateau without good transport facilities, and there were very few crosses between different native breeds. Therefore, the results implied useful protection and little inbreeding in modern Tibetan Mastiff. It also reflected the successful protective plan for Tibetan Mastiff.

As expected, German Shepherd was observed to possess a low level of genetic diversity; rigorous phenotypic selection has likely resulted in loss of genetic information. Higher genetic diversity in other European dog breeds was observed in this study (Table 1). Most breeds have a recent origin, especially the breeds originated in Europe (Dennis-Bryan et al., 1988). The founding of stock of recent breeds was likely drawn from a previously well mixed and outbred pool of dogs. In addition, breeders outcross their pure breed dogs occasionally to avoid deleterious effects resulting from high levels of inbreeding or to eliminate specific genetic defects (Ubbink et al., 1992). Hybrid individuals and their progeny with heterosis may have been selected by breeders; hence foreign haplotypes derived from such crosses may increase in frequency. Average expected heterozygosity of Labrador Retriever (0.72) was higher than the previous result (0.64) (Irion et al., 2003).

The locus polymorphism was applied to estimate Nei's DA genetic distances. Genetic distances between the six breeds are showed in Table 3. Genetic divergence between the dog breeds varied from 0.099 (between the Labrador Retriever and the English Springer Spaniel) to 0.254 (between the German Shepherd and the Labrador Retriever). The genetic distances among Tibetan Mastiff, Kunming dog and German Shepherd were larger than those observed among Belgian Malinois, Labrador Retriever, and English Springer Spaniel. Previous results demonstrated that the large donkey-like Tibetan Mastiff was brought into the Himalayas and peninsular India by humans (Sharma et al., 2004). Alexander the Great is said to have received two

dogs from an Indian king, and he brought the breed back to Macedonia in 326 B.C. to the Molosses region. Since then, these dogs have been given the name of molasses (previously "Indian dog"). These molasses were used by the Romans for circus games, for combat and to guard their villas and estates (Guardamagna, 1995). Chinese chronicles related that Genghis Khan, a world-famous emperor of China, once organized an army corps of 30,000 Tibetan mastiffs in his Western march; afterwards some mastiffs were discarded in the region, becoming the ancestor of various dogs, such as French Saint Bernard, Spanish Great Pyrenees, British mastiff, Italian Neapolitan Mastiff and German Great Dane. Tibetan Mastiff thus enjoys the reputation of a World Breeding Dog. Therefore, the close genetic distance between Tibetan Mastiff and two European breeds such as Labrador Retriever and the English Springer spaniel are apprehensible.

Evolutionary relationship

PAUP software (version 4.0b10) was used to create an unrooted neighbor joining tree using DA distances with 1,000 bootstrap replications (Swofford, 2002) (Figure 1). Tibetan Mastiffs, English Springer Spaniel and Labrador Retriever are clustered together, separate from Belgian Malinois. European breeds appeared to stem from a single node without significant phylogenetic structure, indicating a recent origin and extensive hybridization between the breeds (Parker et al., 2004; Ostrander et al., 2005). Moreover, Springer Spaniel and Labrador Retriever appeared to have branched from Tibetan Mastiff. According to the domestication history of Tibetan Mastiff described above, Tibetan Mastiff or its offspring may have crossed with the ancestor of Springer Spaniel and Labrador Retriever. Previous research stated that the second cluster of canines was comprised of mastiff-type dogs including the Mastiff, Bullmastiff, German Shepherd, etc (Parker et al., 2004). Hence, German Shepherd, another ancient breed, should be closer to Tibetan Mastiff in the NJ tree because of the short time after domestication from gray wolves. However, the hypothesis conflicts with our result, which may be due to the interbreeding and violent phenotypic selection of German Shepherd, which was characteristic of the most famous working dog in the world. In contrast, Tibetan Mastiff has conserved its own characteristics because of its special living environment. Another cluster

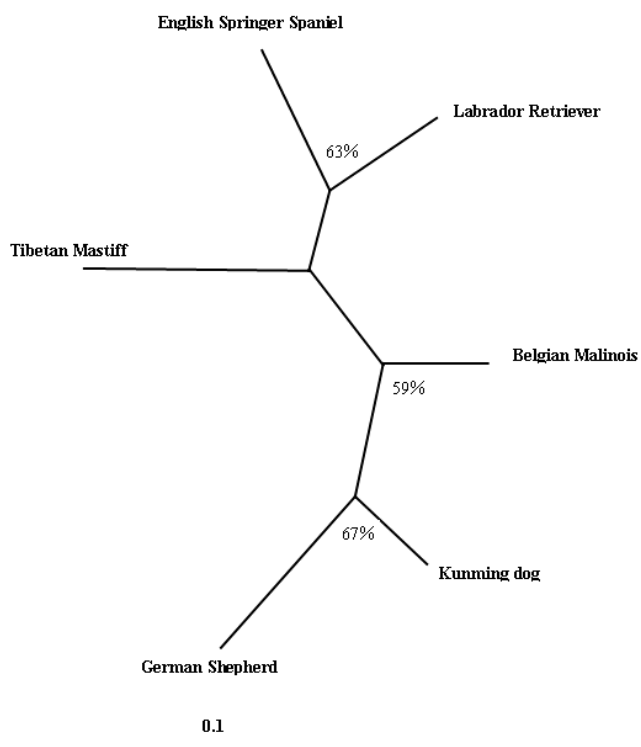


Figure 1. Unrooted neighbor-joining tree based on Nei's DA genetic distance at 10 nuclear microsatellites. Each tip represents a single population. Bootstrap values above 50% are shown.

was anchored by German Shepherd and Kunming dog. The Kunming dog, created in the early 1950's in Kunming Province of China and widely used as a working dog by the Chinese military and police presently, is an established breed of wolf-dog hybrid. It was established that dogs were derived from gray wolves (Vilà et al., 1997; Sablin et al., 2002; Savolainen et al., 2002), hence, it is understandable that Kunming dog, a breed of wolf-dog hybrid, together with German Shepherd, the oldest and well-known breed, were grouped together. Another important reason may be that Kunming dog, the predominant working dog in China, was possibly created by the use of stem from German Shepherd because of its outstanding properties as a working dog. As referred to by previous studies (Parker et al., 2004; Ostrander et al., 2005), the third cluster of dog breeds included working dogs, such as Belgian sheepdog and Belgian Tervuren. According to the records of the American Kennel Club, Belgian Malinois shares a common foundation with Belgian sheepdog and Belgian Tervuren. Our results showed that the genetic relationship of Belgian Malinois with other breeds is relatively distant.

In the Unrooted neighbor-joining tree based on Nei's DA genetic distance, nodes are all supported by bootstrap values but lower than 70%. The weak statistical support of the structure at the breed level is a classic problem and shows how breed-level cladograms can be of concern for analyses of domestic species (Bruford, 2004). Thus,

population genetic inferences should not be solely based on the topology of the NJ trees (Hollingsworth et al., 2004). In addition, a previous study used microsatellites to detect the relatedness of two breed pairs in a collection of 28 breeds but could not establish broader phylogenetic relationships among the breeds (Irion et al., 2003). Another study demonstrated large genetic distances among dog breeds (Koskinen, 2003). The two failed cases may reflect the complicated structure in purebred dog populations.

In conclusion, ten microsatellite loci were used to assess the genetic diversity and evolutionary relationships in six dog breeds, including Tibetan Mastiff, Kunming dog, Belgian Malinois, Labrador Retriever, English Springer Spaniel, and German Shepherd. The highest genetic diversity was exhibited by the Tibetan Mastiff and the lowest by the German Shepherd. Evolutionary relationships showed that Tibetan Mastiff, English Springer Spaniel and Labrador Retriever are clustered together, separate from Belgian Malinois. Another cluster was anchored by German Shepherd and Kunming dog. All results of this study will lead to better understanding of these dog breeds, especially for Tibetan Mastiff and Kunming dog.

ACKNOWLEDGMENTS

We appreciate the assistance of other colleagues in the policedog base of Nanchang for this study. The work was supported by the 10th five years key program for science and technology development of the Ministry of Public Security in China and Doctoral Starting Foundation of Jiangxi Agricultural University in China (2676).

REFERENCES

- Botstein, D., R. L. White, M. Skolnick and R. W. Davies. 1980. Construction of a genetic linkage map in man using restriction length polymorphism. *Am. J. Hum. Genetic.* 32:314-331.
- Bruford, M. W. 2004. Conservation genetics of UK livestock: from molecules to management. In: *Farm animal genetic resources* (Ed. G. Simm, B. Villanueva and S. Townsend). Nottingham University Press, UK. pp. 151-169.
- Chang, G. B., H. Chang, X. P. Liu, W. M. Zhao, D. J. Ji, Y. J. Mao, G. M. Song and X. K. Shi. 2007. Genetic diversity of wild quail in China ascertained with microsatellite DNA markers. *Asian-Aust. J. Anim. Sci.* 20:1783-1790.
- Dennis-Bryan, K. and J. Clutton-Brock. 1988. *Dogs of the last hundred years at the British Museum (Natural History)*. London: British Museum (Natural History).
- DeNise, S., E. Johnston, J. Halverson, K. Marshall, D. Rosenfeld, S. McKenna, T. Sharp and J. Edwards. 2004. Power of exclusion for parentage verification and probability of match for identity in American kennel club breeds using 17 canine microsatellite markers. *Anim. Genet.* 35:14-17.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

- Guardamagna, A. 1995. Le chien de Montagne des Pyrénées. Ed.de Vecchi, Paris. p. 161.
- Hollingsworth, P. M. and R. A. Ennos. 2004. Neighbour joining trees, dominant markers and population genetic structure. *Heredity* 92:490-498.
- Ichikawa, Y., K. Takagi, S. Tsumagari, K. Ishihama, M. Morita, M. Kanemaki, M. Takeishi and H. Takahashi. 2001. Canine parentage testing based on microsatellite polymorphisms. *J. Vet. Med. Sci.* 63:1209-1213.
- Irion, D. N., A. L. Schaffer, T. R. Famula, M. L. Eggleston, S. S. Hughes and N. C. Pedersen. 2003. Analysis of genetic variation in 28 dog breed populations with 100 microsatellite markers. *J. Hered.* 94:81-87.
- Kim, K. S., Y. Tanabe, C. K. Park and J. H. Ha. 2001. Genetic variability in east Asian dogs using microsatellite loci analysis. *J. Hered.* 92:398-403.
- Koskinen, M. T. 2003. Individual assignment using microsatellite DNA reveals unambiguous breed identification in the domestic dog. *Anim. Genet.* 34:297-301.
- Koskinen, M. T. and P. Bredbacka. 2000. Assessment of the population structure of five Finnish dog breeds with microsatellites. *Anim. Genet.* 31:310-317.
- Nagamine, Y. and M. Higuchi. 2001. Genetic distance and classification of domestic animals using genetic markers. *J. Anim. Breed Genet.* 118:101-109.
- Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89:583-590.
- Nei, M., F. Tajima and Y. Tatenno. 1983. Accuracy of estimation phylogenetic trees from molecular data. *J. Mol. Evol.* 19:153-170.
- Ostrander, E. A. and R. K. Wayne. 2005. The canine genome. *Genome Res.* 15:1706-1716.
- Parker, H. G., L. V. Kim, N. B. Sutter, S. Carlson, T. D. Lorentzen, T. B. Malek, G. S. Johnson, H. B. DeFrance, E. A. Ostrander and L. Kruglyak. 2004. Genetic structure of the purebred domestic dog. *Science* 304:1160-1164.
- Puja, I. K., D. N. Irion, A. L. Schaffer and N. C. Pedersen. 2005. The Kintamani dog: Genetic profile of an emerging breed from Bali, Indonesia. *J. Hered.* 96:854-859.
- Sablin, M. V. and G. A. Khlopachev. 2002. The earliest ice age dogs: evidence from Eliseevichi. *Curr Anthropol.* 43:795-799.
- Savolainen, P., Y. P. Zhang, J. Luo, J. Lundeberg and T. Leitner. 2002. Genetic evidence for an East Asian origin of domestic dogs. *Science* 298:1610-1613.
- Sharma, D. K., J. E. Maldonado, Y. V. Jhala and R. C. Fleischer. 2004. Ancient wolf lineages in India. *Proc. Biol. Sci. (Suppl 3)* 271:S1-S4.
- Sundqvist, A.-K., S. Björnerfeldt, J. A. Leonard, F. Hailer, A. Hedhammar, H. Ellegren and C. Vilà. 2006. Unequal contribution of sexes in the origin of dog breeds. *Genetics* 172:1121-1128.
- Swofford, D. L. 2002. Phylogenetic analysis using parsimony (and Other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
- Ubbink, G. J., B. W. Knol and J. Bouw. 1992. The relationship between homozygosity and the occurrence of specific diseases in the Bouvier Belge Flanders dogs in the Netherlands-inbreeding and disease in the Bluvier dog. *Vet Q.* 14:137-140.
- Vilà, C., P. Savolainen, J. E. Maldonado, I. R. Amorim, J. E. Rice, R. L. Honeycutt, K. A. Crandall, J. Lundeberg and R. K. Wayne. 1997. Multiple and ancient origins of the domestic dog. *Science* 276:1687-1689.
- Wu Yan, Xiao-Lin Liu, Shui-Sheng Hou and Wei Huang. 2008. Study on genetic diversity of six duck populations with microsatellite DNA. *Asian-Aust. J. Anim. Sci.* 21:776-783.