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Genetic Variation and Divergence among Swamp Buffalo, River Buffalo and Cattle: A Microsatellite Survey on Five Populations in China

Yi Zhang, Dongxiao Sun, Ying Yu and Yuan Zhang*

Department of Animal Genetics and Breeding, College of Animal Science and Technology
China Agricultural University, Beijing 100193, China

ABSTRACT : Domestic buffalo and cattle are two extremely important livestock species in worldwide agricultural production. In this paper, to investigate genetic diversity and divergence among swamp buffalo, river buffalo and cattle, 30 microsatellite markers were screened on 168 individuals sampled from five populations. Substantial differences were observed among the three groups of animals with respect to allele frequency distribution, allele size and polymorphism. The cattle sample (Mongolian) showed significantly higher genetic variability (0.674 of gene diversity, $p<0.01$), and the swamp and river buffalo samples displayed similar degree of genetic variation (0.536 in swamp and 0.546 in river, $p = 0.92$). Results of both phylogenetic tree and multivariate analysis could distinguish three groups of animals, suggesting their deep evolutionary divergence. Additionally, using $(\delta\mu)^2$ genetic distance, we estimated a divergence time of 1.7 million years between swamp and river buffalo that strongly supported distinct genetic origins for the two buffalo types. (**Key Words :** Buffalo, Cattle, Microsatellite, Genetic Diversity, Divergence)

INTRODUCTION

During the past decade the microsatellite has been widely used in population genetic studies of livestock species. Inferences on the basis of microsatellite variation have been demonstrated to be extremely useful for population clustering, genetic divergence estimation, animal domestication analysis, parentage testing and individual identification, and genetic resources conservation. Microsatellites are informative markers useful for investigating livestock genetic diversity and population relationships (Hoffmann et al., 2004). To date, a large number of microsatellite variation studies of livestock animals have been carried out, principally on different breeds or populations within species (e.g. Baumung et al., 2004; Sukla et al., 2007; Sraphet et al., 2008). Limited investigations, however, have focused on the genetic diversity between species. Indeed, cross-species variation constitutes another rich resource of farm animal genetic diversity. Comparisons between closely related species enable us characterize the pattern of genetic variation and the genetic divergence in an evolutionary perspective.

* Corresponding Author: Yuan Zhang. Tel: +86-10-62733687, Fax: +86-10-62733687, E-mail: changy@cau.edu.cn
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Cattle and buffalo are two important livestock species worldwide raised in various agricultural systems furnishing mankind with meat, milk and work power. Despite some similarities with respect to morphologic and genetic characters, cattle and buffalo are divergent evolutionarily and are classified as different genera within the subfamily of *Bovinae* (*Bos* and *Bubalus*) (Scherf, 2000). The domestic buffalo are further divided into two major types, i.e. swamp and river, with considerable differentiation (Cockrill, 1974; Barker et al., 1997a, 1997b; Zhang et al., 2006, 2007; Lei et al., 2007; Kumar et al., 2007). Ritz et al. (2000), using microsatellite markers, analyzed the phylogenetic relationships among several cattle-like species, including cattle, yak, bison, Asian buffalo and African buffalo. However, little information concerning genetic diversity was presented. A recent study on major histocompatibilty complex (MHC) (Sena et al., 2003) uncovered different features in the functional gene between cattle and buffalo. In this paper, 30 microsatellite loci were surveyed to investigate the degree and pattern of genetic variation in five populations representing cattle (*Bos taurus*), swamp buffalo and river buffalo (*Bubalus bubalis*), and to assess genetic divergence among them.

MATERIALS AND METHODS

Animals

In total 168 blood or skin samples were collected from five populations representing swamp and river types of buffalo (*Bubalus bubalis*) and taurine cattle (*Bos taurus*) (Table 1). The indigenous swamp buffalo and a cattle breed, Mongolian, were sampled from local village populations. The river buffalo animals were selected from two exotic breeds, Murrah and Nili-Ravi, that were introduced from India and Pakistan, respectively, and sampling was based on the pedigree to avoid close relationships among individuals. Genomic DNA was extracted by a standard proteinase K digestion followed by phenol/chloroform extraction (Sambrook et al., 1989).

Microsatellite genotyping

Thirty microsatellite markers recommended by FAO and ISAG for domestic buffalo genetic diversity analysis (Hoffmann et al., 2004) were used (Table 2). The forward primer of each locus was end-labeled with fluorescent dye (6-FAM, TET or HEX). Nine multiplex PCR sets, including 4 triplex and 5 duplex, were developed to amplify 22 of 30 microsatellite loci (Table 2). Amplification of the remaining

Table 1. Locality and numbers of sampled populations

Species/type	Population/breed	Locality	Sample number	Total number
Swamp buffalo (*Bubalus bubalis*)	Guizhou	Fenggang, Zunyi, Guizhou province.	30	60
	Fuan	Fuan, Ningde, Fujian province.	30	
River buffalo (*Bubalus bubalis*)	Nili-Ravi	Institute of buffalo research, Nanning, Guangxi.	30	60
	Murrah	Institute of buffalo research, Nanning, Guangxi.	30	
Cattle (*Bos taurus*)	Mongolian	Xiligol, Inner Mongolia.	48	48

Table 2. Total numbers of alleles and the mean length of alleles for 30 microsatellites in swamp and river buffalo and Mongolian cattle[1]

	Number of alleles			Mean length of alleles		
	Swamp	River	Cattle	Swamp	River	Cattle
CSSM033	7	6	7	164.2	161.3	159.8
CSSM038[3]	4	5	13	174.5	172.2	170.7
CSSM043[1]	6	4	10	241.6	235.5	255.2
CSSM047[2]	5	12	6	136.3	144.7	156.0
CSSM036[1]	5	5	7	169.7	169.1	169.3
CSSM019[3]	9	6	7	152.5	138.9	153.9
CSRM060	4	5	11	96.2	117.2	102.2
CSSM029[2]	6	2	11	186.9	187.0	243.1
CSSM041[5]	4	6	6	139.9	141.7	134.3
CSSM057	8	5	6	118.6	125.3	110.5
BRN[1]	7	3	9	139.3	135.8	146.3
CSSM032[5]	5	5	7	217.1	212.0	217.9
CSSM008[4]	7	5	3	185.2	187.2	199.6
CSSM045	1	4	5	102.0	111.8	109.2
CSSM022[2]	3	5	6	207.8	209.9	223.0
CSSM046[4]	3	4	8	156.5	156.9	170.4
CSSM013[4]	5	2	2	167.2	166.3	160.7
ETH003	6	10	10	102.4	114.7	121.2
CSSM061	7	9	8	116.1	115.3	114.9
BMC1013[3]	7	6	3	243.2	235.9	220.5
DRB3[6]	13	9	16	182.1	173.8	189.4
CSSM062[6]	4	5	5	124.1	127.6	138.2
CSSME070[9]	3	3	6	125.8	131.3	139.6
ETH121	3	6	12	186.2	191.2	201.6
ILSTS033[7]	3	6	12	154.7	148.7	148.0
ILSTS005[7]	4	3	3	182.1	181.2	185.6
ILSTS030[8]	4	5	4	161.1	162.4	155.9
ILSTS008[8]	1	2	4	172.0	172.2	179.1
RM099	1	2	7	91.0	91.8	123.5
HMH1R[9]	1	1	6	169.0	169.0	182.1
Total	146	151	220	-	-	-

[1] The labels on the loci names denote multiplex PCR sets with the same numbers representing one combination.

Table 3. The mean number of alleles per locus (MNA), gene diversity and polymorphism information content (PIC) in three groups of animals[1]

	MNA	Gene diversity	PIC
Swamp buffalo	4.87 (2.64)	0.536 (0.047)	0.492 (0.191)
River buffalo	5.03 (2.48)	0.546 (0.036)	0.485 (0.243)
Cattle	7.33 (3.35)	0.674 (0.028)	0.627 (0.164)

[1] The standard deviation for each measure is given in parentheses.

8 loci was performed independently.

PCR reaction was carried out in a final volume of 20 μl containing 30-50 ng of genomic DNA, 2 mM $MgCl_2$, 200 μM each dNTP, 0.03-0.35 μM of each primer and 1 unit of Taq DNA polymerase. The mixture was incubated at 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 55-60°C for 30 s and 72°C for 30 s, and a final incubation at 72°C for 5 min. Genotypes on each marker were determined by a 4.5% denaturing polyacrylamide gel on an ABI 377 DNA Sequencer (Applied Biosystems) using the internal lane size standard GeneScan™-TAMARA 350 (Applied Biosystems).

Data analysis

Allele frequency, number of alleles per locus and gene diversity (i.e. expected heterozygosity) were calculated using the FSTAT program (Goudet, 2001). Polymorphic information content (PIC) values for each locus were computed according to Botstein et al. (1980). Significant differences in genetic variability measures between animal groups were tested after Estoup et al. (1995) using Wilcoxon's signed rank test. A neighbor-joining phylogenetic tree was constructed based on Nei's D_A genetic distance (Nei et al., 1983) using the DISPAN software (Ota, 1993). The robustness of the tree was evaluated with a bootstrap test of 1,000 resamplings of loci with replacement. Moreover, a correspondence analysis (Lebart et al., 1984) was performed in the GENETIX package (Belkhir et al., 1998) to explore the genetic relationship among populations. As a multivariate statistical method, correspondence analysis can convert the information from a large number of loci and alleles into a few synthetic variables. The factors, or axes, are ranked and independent of each other. The $(\delta\mu)^2$ genetic distance (Goldstein et al., 1995), which is linear with increasing evolutionary time, was computed using the MICROSAT program (http://hpgl.stanford.edu/projects/microsat/) to estimate the divergence time assuming a molecular clock.

RESULTS AND DISCUSSION

Microsatellite allele profile

All microsatellite markers surveyed here were successfully amplified and genotyped both in buffalo and in cattle. In aggregate, 146, 151 and 220 alleles were identified across 30 loci in swamp buffalo, river buffalo and cattle, respectively. Most significantly, the three groups of animals exhibited distinct patterns of genetic variation on many loci (Table 2). For example, Mongolian cattle displayed polymorphism in all loci; swamp buffalo and river buffalo, however, showed monomorphism at four (*CSSM045*, *ILSTS008*, *RM099*, and *HMH1R*) and one (*HMH1R*) loci, respectively. The locus, *CSSM047*, was observed with 12 alleles in river buffalo and only 5 alleles in swamp buffalo; *CSSM029* had 6 alleles in swamp buffalo and 2 alleles in river buffalo. Swamp buffalo totally shared with river buffalo 76 alleles throughout 30 loci; the alleles shared by all three groups were 28, on average only 0.93 per locus (data not shown). Pearson correlation coefficients (*r*) of the number of alleles (NA) for each locus were 0.485 ($p<0.01$) between swamp buffalo and river buffalo, 0.298 ($p = 0.11$) between swamp buffalo and cattle and 0.301 ($p = 0.11$) between river buffalo and cattle. The insignificant and relatively low *r* value is indicative that polymorphism of a particular locus varied between buffalo and cattle.

In addition, 18 of 30 loci revealed longer alleles in cattle than in buffalo (Table 2). This observation was to some extent consistent with a previously reported ascertainment bias that microsatellites isolated in one species tended to be greater than homologous loci in related species (Ellegren et al., 1997; Vowles and Amos, 2006).

Genetic variability

Table 3 summarized three widely used measures for genetic diversity, namely, the mean number of alleles per locus (MNA), gene diversity (GD) and polymorphism information content (PIC). Swamp and river buffalo had a similar degree of genetic variability (Wilcoxon's signed rank test, $p = 0.82$ for MNA, $p = 0.92$ for GD, and $p = 0.91$ for PIC). Mongolian cattle, however, indicated significantly higher variability than the two types of domestic buffalo (Wilcoxon's signed rank test, $p<0.01$ for all three measures).

The low genetic variation of buffalo might result from species-specific demographic history. However, it should be noted that, since all microsatellite loci examined here were derived from cattle, and cross-species amplification in buffalo could possibly lead to ascertainment bias (Ellegren et al., 1997), it might somehow affect the interpretation of results. Therefore, future examinations on buffalo-isolated loci and reciprocal analyses are expected to test and confirm the findings in this study.

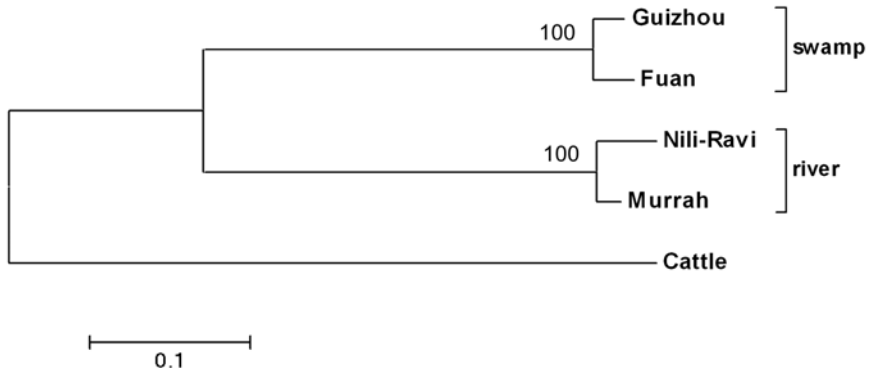

Figure 1. Neighbor-joining tree of five populations based on D_A genetic distance.

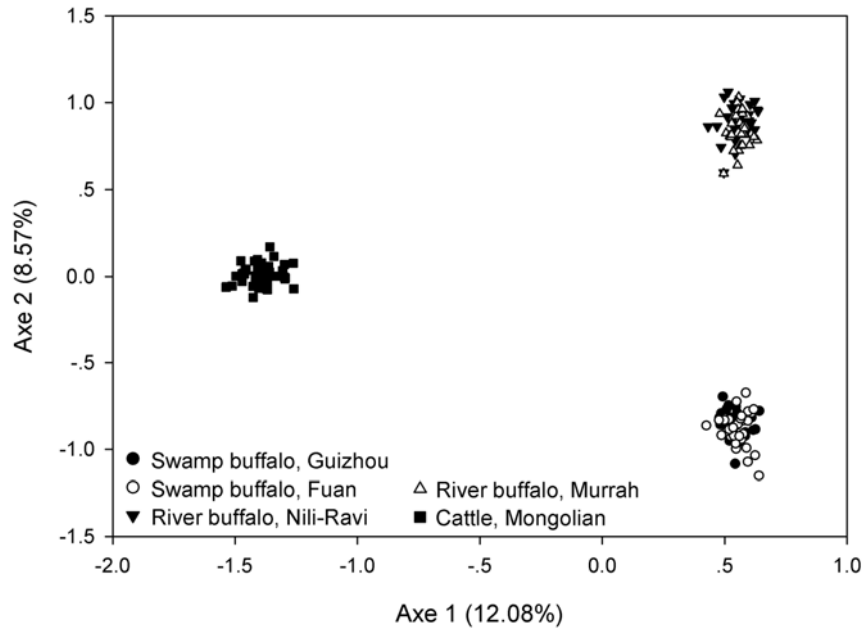

Figure 2. Correspondence analysis of individual multi-locus genotypes of 30 microsatellite markers in the five populations surveyed.

Phylogenetic analysis and genetic divergence

In the obtained evolutionary tree (Figure 1), as expected, the two swamp buffalo populations and the two river buffalo populations clustered respectively and formed distinct clades with bootstrap confidence values of one hundred percent. The branch connecting buffalo with cattle was long, indicating their marked differentiation. Moreover, the clear separations among the three groups of animals were also demonstrated by the multivariate analysis. Figure 2 illustrates the population relationships based on the correspondence analysis using individual multi-locus genotypes of 30 microsatellite markers. The first two factors contributed 12.08% and 8.57% of the total inertia respectively. Notably, the first factor (Axe 1) corresponded to the buffalo-cattle divergence while the second factor (Axe 2) represented the split of swamp and river buffalo (Figure 2). The individuals from different populations within the two buffalo types, however, clustered together and no sub-groups could be recognized, indicating relatively close genetic relationship between them.

The $(\delta\mu)^2$ genetic distances were 11.11 between swamp and river buffalo, 68.27 between swamp buffalo and cattle and 62.58 between river buffalo and cattle. Using cattle as an outgroup, it is possible to estimate the divergence time between the two buffalo types. Paleontological evidence (Savage and Russell, 1983) indicated at least 10 million years of divergence between cattle (*Bos*) and Asian buffalo (*Bubalus*). With this benchmark and the average $(\delta\mu)^2$ distance between cattle and the two buffalo types (= 1/2(68.27+62.58)), we extrapolated a rough estimate of divergence as 1.7 million years between swamp and river buffalo. This value was consistent with those derived from mitochondrial DNA sequences (Tanaka et al., 1996) and 20 microsatellite loci data (Ritz et al., 2000). The deep

divergence strongly supported the opinion that swamp and river buffalo originated from two separated domestication events (Kumar et al., 2007).

In summary, we investigated genetic variation and divergence among three groups of animals representing swamp buffalo, river buffalo and cattle, using 30 microsatellite markers. Cross-species comparisons reflected substantial genetic differences in the allele profiles and varied levels of genetic variability between buffalo and cattle. These findings shed new light on the genetic diversity within and among cattle and the two buffalo types.

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