

Short communication

Biodiversity in Mediterranean buffalo using two microsatellite multiplexes

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Received 25 July 2006; received in revised form 29 September 2007; accepted 1 October 2007

Abstract

The present study was carried out to investigate genetic diversity in Nile-Delta and Southern-Egypt buffalo populations in comparison with the Italian buffalo utilizing two microsatellite multiplexes. A total of 104 animals classified into three groups were used, 28, 38 and 38 representing the Nile-Delta, Southern-Egypt and Italian buffalo, respectively. The 15 studied microsatellites were CSSM38, CSSM70, CYP21, CSSM42, CSSM60, MAF65, BM0922, CSSM19, INRA006, ETH02, BM1706, BMC1013, CSSM47, INRA026 and CA004. All studied microsatellites showed allelic polymorphism. Number of polymorphic alleles ranged between 4 alleles (CSSM38, CSSM70 and CYP21) and 11 alleles (CA004). Pairwise Chi-square test for Nile Delta and Southern Egypt showed significant differences in allelic distribution at five loci, CSSM70, CSSM38, BM0922, ETH02 and BM1706. Italian buffalo showed the lowest percentage of observed heterozygotes (65%), while the Southern Egypt showed the highest (71%). Both the Italian and the Delta populations deviated significantly ($P < 0.05$) from HW equilibrium. Italian buffalo is relatively the most inbred population while the Southern-Egypt buffalo is the only outbred population. High level of genetic differentiation (F_{ST} estimates) between the Italian group and each of the Delta and Southern-Egypt group (0.083 and 0.076, respectively) was observed while Southern-Egypt group showed a lower level of genetic differentiation with the Delta group (0.014). Italian buffalo had the greatest genetic distance values with the two Egyptian groups (0.25 and 0.23) while much lower values between the Southern-Egypt and the Delta groups (0.06) was observed. Genetic variation between the Italian buffalo and the Egyptian buffalo was detected in 14 (out of 15) microsatellite loci. While a lower level (than that between Egyptian and Italian) of genetic variation between Southern-Egypt and Delta buffalo populations was expressed by 5 loci. It was concluded that the Southern-Egypt buffalo could be considered as distinct population from the Delta buffalo. In addition, Southern-Egypt group, being the only group with non-significant global deviation from HW equilibrium, the most heterozygous, and the only outbred population as well, is expected to respond more favorably to selection.
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Keywords: *Bos bubalus bubalis*; Biodiversity; Egyptian buffalo; Italian buffalo; Microsatellites

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1. Introduction

Buffalo sub-populations (e.g. breeds) in Egypt are mainly based on geographical origin within the country

with little or no documented phenotypic or genetic differences that may help in characterizing these sub-populations and relating them to other Mediterranean breeds. Examples of the breed names mentioned in the Egyptian and international literature are the Baladi (Native), Beheri, Monoufi, Masri and Saiedi (Southern Egypt) (FAO Domestic Animal Diversity Information System (DAD-IS)). Minor phenotypic differences are reported concerning size, color and performance that have not been updated or verified since the establishment of DAD-IS. These differences are not well defined to be relied on as taxonomic classification for those populations.

Limited number of researches has been conducted globally exploring the genetic diversity on molecular genetic basis in buffalo in comparison with other farm animal genetic resources (FAnGR). Only very few studies were conducted including the Egyptian buffalo, e.g. Moiola et al. (2001), in which a limited sample size and particular regions of Egypt were selected as sample sources.

The present study aimed at investigating genetic diversity in Nile-Delta and Southern-Egypt buffalo populations in comparison with the Italian buffalo and investigating the distinction between Delta and Southern-Egypt buffalo populations.

2. Materials and methods

2.1. Sampling of animals

Hundred and four animals classified into three groups were sampled as: i. Nile-Delta buffalo represented by 24 females and 4 males kept in three adjacent buffalo experimental farms of the Animal Production Research Institute (APRI) located in Kafr Elsheikh governorate in Mid Nile Delta which represent an admix of non-characterized buffalo germplasm in the Delta region. Selected animals were genealogically traced to be unrelated as far as three generations back. ii. Southern-Egypt buffalo represented by 38 females kept in 23 small scale private animal holdings scattered in Sohag governorate. Sohag was selected for being a remote governorate in Southern Egypt. Animals were selected for belonging to various holdings and different generations (age class) to minimize co-ancestry. iii. Italian buffalo, represented by 38 animals belonging to various farms in central and southern Italy.

2.2. Specimen collection and DNA extraction and quantification

For the Egyptian buffalo populations, blood samples were collected from all the females while hair pulps were collected from the males. DNA samples of the Italian animals were supplied by the Laboratory of Genetic Service (LGS), Cremona, Italy.

DNA of the Egyptian animals was extracted using either the “reversible binding to silica membrane” or “salting out” (Miller

et al., 1988) techniques, while the DNA of the Italian animals was extracted at LGS using CHELEX/Proteinase-K procedure for DNA release using Chelex® 100 (BioRad) resin (Cano and Poinar, 1993). Extracted DNA from Egyptian buffalo was quantified using BioPhotometer, Eppendorf AG, Germany.

2.3. Utilized microsatellite markers

All 15 markers used were microsatellite type recommended by Laboratory for Genetics and Services, LGS, and shown to be polymorphic in cattle and buffalo (Moore et al., 1995; Barker et al., 1997; FAO, 2004). Studied microsatellites were amplified using two multiplexes. The first multiplex consisted of nine microsatellites (being MAF65, INRA006, CSSM47, CSSM19, CA004, CSSM42, CYP21, ETH02, and BMC1013) while the second multiplex consisted of six microsatellites (CSSM70, CSSM60, INRA026, BM0922, BM1706, and CSSM38). The labeling dye for each forward segment of primers is presented in Table 1.

2.4. Polymerase chain reaction (PCR) and allelic fragmentation

Studied microsatellites were amplified using PTC-200 thermal cycler (M. J. Research, Watertown, USA). PCR cocktail, in each of the multiplexes, was composed of 300 ng template DNA, 1× PCR buffer, 3 mM MgCl₂, 2 U of Taq Gold (Applied Biosystems), 400 μM of dNTP's mix (Applied Biosystems). The first multiplex contained 400, 200, 400, 100, 400, 100, 200, 200, and 200 pM from each of the forward and reverse segments of MAF65, INRA006, CSSM47, CSSM19, CA004, CSSM42, CYP21,

Table 1

Observed number of alleles in each of the three sampled buffalo groups/populations and detected allele size range

Locus	Number of observed alleles in				Allele size range (bp)	Labeling dye
	Delta buffalo	Southern-Egypt buffalo	Italian buffalo	All		
CSSM38	4	3	3	4	159–181	VIC
CSSM70	3	4	3	4	125–135	NED
CYP21	4	4	4	4	180–188	NED
CSSM42	3	5	5	6	176–192	VIC
CSSM60	6	4	3	6	90–126	FAM
MAF65	5	4	5	6	111–127	VIC
BM0922	5	7	6	7	34–76	NED
CSSM19	6	6	4	7	131–159	FAM
INRA006	5	6	7	7	112–128	FAM
ETH02	5	5	6	8	195–221	FAM
BM1706	9	8	5	9	227–253	FAM
BMC1013	8	7	5	9	221–245	VIC
CSSM47	9	5	5	9	127–169	NED
INRA026	8	8	6	10	72–100	VIC
CA004	6	8	6	11	131–165	VIC
TDA ¹	86	84	73	107		

TDA = Total detected alleles.

ETH02, and BMC1013 primers, respectively. The second multiplex contained 150, 500, 500, 75, 400, and 500 pM of each segment of CSSM70, CSSM60, INRA026, BM0922, BM1706, and CSSM38, respectively. Final reaction volumes were made to 10 and 11 μ L for the first and the second multiplexes, respectively. Thermal profile was a pre-run at 95 °C for 10 min., followed by 30 tri-phasic cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for a minute and extension at 72 °C for a minute, and final extension for 10 min at 72 °C. PCR product was then denatured in a denaturation mix composed of 0.9 μ l PCR product+2.1 μ l loading master mix, while the loading master mix was composed of 250 μ l deionized Hi-Di-Formamide (Applied Biosystems, P/N. 4311320), 50 μ l GENESCAN-350 size standard (Applied Biosystems, P/N. 401735), and 50 μ l loading stain (Blue dextrane, 50 mg/ml in EDTA, 25 mM, PHARMACIA P/N. 17-0360-01). The denaturation mix was incubated at 95 °C for 4 min and immediately chilled in ice to prevent re-annealing. A volume of 1.5 μ l of such mix was loaded to the Applied Biosystems 377 DNA sequencer. GeneScan v. 3.1 software was utilized by the equipment for fragmentation of various alleles by comparison against the GENESCAN-350 standard.

2.5. Quantitative measures

Quantitative measures, at the molecular level, were classified into four main categories: a. microsatellite polymorphism in order to assess polymorphism profile in the studied populations. Detected alleles and their molecular length (in base pairs) in every studied group and all over the groups were determined using GenePop software, Version 3.3, March 2001 updated from Raymond and Rousset (1995a). b. Genetic variability, in which five measures were estimated. i. Allele frequency. ii. Chi-square test for the heterogeneity of genetic frequencies. iii. Observed H_{obs} and expected H_{exp} heterozygosity for each studied group all over studied loci and mean heterozygosity (Nei, 1978). H_{exp} was calculated as defined by Nei (1973). iv. “ F_{IS} ”, as a measure of the within group heterozygosity deficit or inbreeding, representing Wright’s fixation indices (Wright, 1943, 1978) was estimated using permutation tests (Goudet, 2002) with determined level of significance of 0.01 and 2000 permutation tests of alleles within populations and was adjusted with the sequential Bonferroni procedure (Holm, 1979; Hochberg, 1988) applied over loci. v. Deviation from HW equilibrium of population per locus and globally over all loci. The test was performed using the unbiased estimates of HW exact P values according to Raymond and Rousset (1995b) using Markov Chain Monte Carlo method applied to compute unbiased estimate of the exact test probability, P, (Guo and Thompson, 1992). Significant levels for each test were determined by applying the probability estimates calculating the sequential Bonferroni procedure for multiple tests (Hochberg, 1988; Lessios, 1992). Test for HW equilibrium was performed using the GenePop software, Version 3.3, while the first four genetic variability measures were estimated using FSTAT software, version 2.9.3.2 (Feb. 2002), updated from Goudet (1995). c. Genetic

differentiation measures which included: i. “ F_{ST} ” as a measure of the between populations heterozygosity deficit or population differentiation assessing the variation in the group relative to that in the total groups, calculated for each pair of groups. Similar to the “ F_{IS} ”, “ F_{ST} ” was also computed using permutation tests (Goudet, 2002) with a level of significance of 0.01 and 2000 permutation test of genotypes within the total population (F_{ST}) and was adjusted with the sequential Bonferroni procedure applied over loci. ii. Genetic distance; (D), a measure of the number of detectable genetic substitutions that have accumulated in two populations since they diverged (and therefore a measure of genetic difference). Nei’s standard genetic distance D_s (Nei, 1987) was estimated. Both Genetic differentiation measures were estimated using FSTAT. d. Phylogenetic analysis, representing the inter-individual-genetic distance based on dendrogram relationship in population pairs aiming at studying proposed evolutionary relationships between pairs of studied buffalo populations. Standard genetic distance estimates, D_A of Nei and Takezaki (1994) were used to construct dendrograms of phylogenetic relationship trees with the neighbor-joining (NJ) on clustering algorithm (Saitou and Nei, 1987) using PHYLIP, Phylogeny Inference Package, version 3.6 (Jul., 2004), updated from (Felsenstein, 1985), NEIGHBOR module. Tree-View 32 was used to draw the dendrogram presentations.

3. Results

3.1. Microsatellite polymorphism

All studied microsatellites showed polymorphism in all studied buffalo groups. Number of polymorphic alleles ranged between four (CSSM38, CSSM70 and CYP21) and eleven (CA004) as shown in Table 1. The highest number of detected alleles over all loci was shown by the Nile-Delta group (86), while the lowest polymorphic alleles were shown by the Italian buffalo (73 alleles).

3.2. Allele frequency

Allele frequency of studied loci over the buffalo groups showed the presence of exclusive alleles with low frequencies (between 0.01 and 0.17 for a single population).

3.3. Homogeneity test of allelic distribution across all groups and pairwise comparison

Results of the Chi-square test are presented in Table 2 as a test of homogeneity between populations. Generally, all the studied groups are genetically distinct from each other at 14 loci. CYP21 is the only locus that did not show significant heterogeneous distribution of alleles among studied groups. In the pairwise comparison, Delta

Table 2

Chi-square test of significance of the population homogeneity for the 15 loci studied in the three buffalo groups, and in a pairwise comparisons between the Nile-Delta and the Southern-Egypt groups

Locus	No. Alleles	All groups		Delta and Southern-Egypt groups	
		D.F.	χ^2	D.F.	χ^2
CSSM38	4	12	29.73**	3	12.52**
CSSM70	4	12	50.21**	3	8.28*
CYP21	4	16	21.95 ^{ns}	3	5.71 ^{ns}
CSSM42	6	20	124.87**	4	2.19 ^{ns}
CSSM60	6	32	52.65*	5	4.14 ^{ns}
MAF65	6	20	51.63**	4	8.30 ^{ns}
BM0922	7	24	201.43**	6	16.29*
CSSM19	7	28	104.34**	6	7.12 ^{ns}
INRA006	7	24	75.45**	5	4.68 ^{ns}
ETH02	8	28	148.60**	5	22.57***
BM1706	9	40	125.24**	8	20.53**
BMC1013	9	40	102.87**	8	13.07 ^{ns}
CSSM47	9	44	310.16**	8	13.65 ^{ns}
INRA026	10	40	122.19**	8	7.59 ^{ns}
CA004	11	44	139.88**	9	13.41 ^{ns}

* $P < 0.05$, ** $P < 0.001$ *** $P < 0.0001$ and ns = non-significant.

vs. Southern Egypt, five loci showed significant heterogeneity (Table 2).

3.4. Observed and expected heterozygosity

Generally, the average expected heterozygosity for studied groups was higher than the observed except the Southern-Egypt group, where, the average observed was slightly higher than the expected (Table 3), indicating heterozygosity deficit, in both Italian and Delta buffalo groups. Italian buffalo showed the lowest percentage of observed heterozygotes (0.65).

3.5. Deviation from Hardy–Weinberg equilibrium

Deviation from Hardy–Weinberg equilibrium (Table 3) was statistically significant ($P < 0.05$) in 17 locus-breed combinations. Four loci showed significant deviation in all of the three studied breeds (CYP21, CSSM60, CSSM70

Table 3

Mean observed, H_{obs} , and mean expected, H_{exp} heterozygosity, within population heterozygosity deficiency, F_{IS} , and number of loci deviating from HW equilibrium

Buffalo population	H_{obs}	H_{exp}	F_{IS}	No. of loci deviating from HW equilibrium
Delta	0.661	0.680	0.052	6
Southern Egypt	0.709	0.695	-0.005	4
Italian	0.65	0.677	0.059	7

and CSSM38). The three loci CSSM19, CA004, and ETH02 showed significant deviation only in the Italian population while CSSM42, INRA026 showed the significant deviation in the Delta population. The global test of deviation overall loci indicated that both Nile-Delta and Italian buffalo populations deviated from HW equilibrium due to heterozygosity deficiency.

3.6. Within population heterozygosity deficit (inbreeding) measure

Averaging F_{IS} (within population heterozygosity deficit) over all loci as an estimator of inbreeding (Table 3) indicates that the Italian buffalo is relatively the most inbred population while the Southern Egypt is the only outbred population. Obtained results are compatible with heterozygosity results.

3.7. Population differentiation measure, F_{ST}

Table 4 shows the high level of genetic differentiation between the Italian group and each of the two Egyptian groups, while Southern-Egypt group showed a lower level of genetic differentiation with the other Egyptian group. All pairwise population differentiation estimates, after the Bonferroni correction, showed highly significant differences ($P < 0.01$).

3.8. Genetic distance

Italian buffalo had the greatest distance values with the two Egyptian groups and expectedly, the lowest distance estimate was shown between the two Egyptian groups (Table 4).

3.9. Relationship dendrogram

Fig. 1 (A, B and C) represent the inter-individual-genetic distance based dendrogram relationship in population pairs. The radial trees of individuals' genetic distances show a separated pattern between the individuals of the Italian and both the Egyptian groups

Table 4

Estimated pairwise F_{ST} (above diagonal) and genetic distance (below diagonal) between pairs of studied buffalo groups

Group	Delta	Italian	Southern Egypt
Delta		0.0827**	0.0142**
Italian	0.2525		0.0762**
Southern Egypt	0.0574	0.2295	

** $P < 0.01$; P value obtained after 2000 permutation, after standard Bonferroni correction.

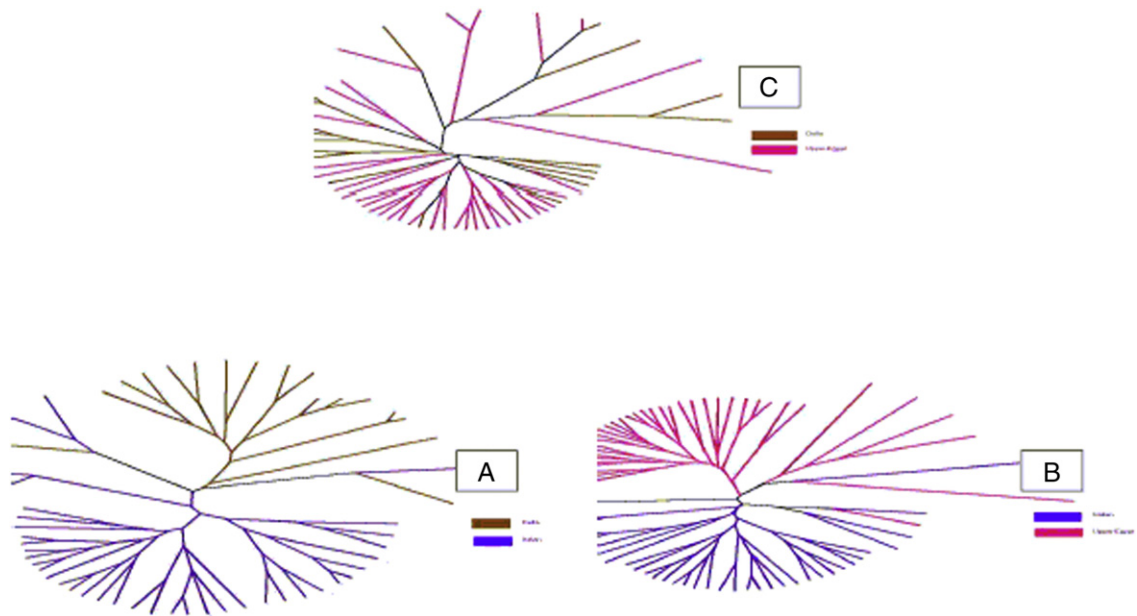


Fig. 1. Radial (unrooted) phylogenetic tree representing inter-individuals genetic distance within the Delta and the Italian (A), the Southern-Egypt and the Italian (B) and the Delta and the Southern-Egypt (C) buffalo groups using D_A genetic distance on 15 microsatellite loci and NJ method of clustering.

(A and B) while a mixture of individuals from the two Egyptian groups is shown. Individual-pairs distance was only estimated for the individuals with no missing data in the allelic forms for all the studied 15 loci and only those individuals appeared in the unrooted trees.

4. Discussion

In comparison of microsatellite polymorphism results with other studies on water buffalo (*Bubalus bubalis*) for some similar loci, Barker et al. (1997) working on Asian water buffalo, reported number of alleles of 5, 9, 18 and 9 for the CSSM19, CSSM38, CSSM47 and CSSM60, respectively. Their results showed that Asian water buffalo carries higher corresponding number of alleles in CSSM38 (9 vs. 4), CSSM60 (9 vs. 6) and CSSM47 (18 vs. 9) loci, and lower number of alleles in locus CSSM19 (5 vs. 7). Moiola et al. (2001) detected a total number of alleles (in the three populations of Italian, Greek and Egyptian buffalo) of 8, 6, 9, 10 and 16 alleles for loci CSSM70, CSSM38, CSSM60, BMC1013, and CSSM47, respectively, which is higher than the corresponding markers in the present study (Table 1). Kumar et al. (2006), studying eight Indian buffalo breeds, reported 9 alleles for each of CA004 and CSSM47 loci, which is a similar number for the latter in the present study, but lower than those detected for the CA004 (being 11 in the present study).

Lower degree of polymorphism shown by the Italian population may possibly be explained by the selection practiced in the Italian buffalo population that could have eroded polymorphism throughout successive generations, assuming the presence of a degree of linkage disequilibrium between some of the studied loci and the QTL or functional segments affecting economic traits, mainly milk.

The lowest percentage of observed heterozygotes shown by the Italian buffalo (0.65), may be partially explained by reasons given above for lower degree of polymorphism. Barker et al. (1997) reported observed heterozygosity range between 0.531 and 0.613 and expected heterozygosity range between 0.564 and 0.607. Kumar et al. (2006) reported ranges of 0.63–0.71 and 0.71–0.78 for the observed and the expected heterozygosity, respectively, indicating higher expected than observed heterozygosity in all the studied breeds. Generally, heterozygosity estimates of the present study are agreeable with other estimates reported for the Asian water buffalo.

The non-significant global deviation from Hardy–Weinberg equilibrium shown by the Southern-Egypt population might be due to a sort of random mating (no specific genetic improvement plan is reported to be applied within this group). The higher heterozygosity shown in that population is an indication of higher variability, thus allowing the possibility of genetic improvement.

Conversely, global significant deviation from HW equilibrium in groups selected from experimental herds of Nile Delta, as well as the Italian group, might be due selection applied for the genetic improvement of economic traits.

Results of the F_{IS} average over all loci indicate that the Italian buffalo is relatively the most inbred population while the Southern Egypt is the only outbred population. This is compatible with heterozygosity results since the Southern-Egypt group was the most heterozygous group while the Italian was the least.

The dendrographic relationships of individuals' genetic distances, showing a separated pattern between the individuals of the Italian and both the Egyptian groups (Fig. 1, A and B) and a mixture of individuals from the two Egyptian groups (Fig. 1, C), emphasize the distinctiveness of the Italian buffalo from the Egyptian and the common ancestry for both the Egyptian buffalo populations.

5. Conclusion

On one hand, there was a reasonable genetic variation, at the molecular level, between Italian and the Egyptian buffalo. On the other hand, there was a lower but significant level of genetic variation between Southern-Egypt and Nile-Delta buffalo, expressed in 5 loci. Therefore, Southern-Egypt buffalo could be considered as a distinct population from the Nile-Delta buffalo. More studies need to be carried out to study probable variations among further putative sub-populations (breeds/types) of Nile-Delta buffalo.

The Southern-Egypt group, being the only group with non-significant deviation from HW equilibrium and the most heterozygous group, as well, can more significantly respond to genetic improvement based on selection.

Acknowledgements

Authors gratefully acknowledge the staff of the Laboratory of Genetic Service, LGS, in both Cremona and Potenza, Italy, especially Mrs. Giovanna Berrotta, Mrs. Adele Lanza, Mrs. Cinzia Senese and Mr. Mario Pertoletti for their technical support and personal help in PCR optimization, the AB 377 sequencer maintenance and operation, allele size determination and standardization between the AB 377 and AB 3100 sequencing machines.

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