การตรวจความเป็นพ่อ-แม่-ลูกในกระบือปลักไทยโดย microsatellite markers

Parentage Testing in Thai - Swamp Buffaloes by Microsatellite Markers

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บทคัดย่อ

การศึกษาในครั้งนี้เพื่อตรวจความเป็นพ่อ-แม่-ลูกกระบือปลักไทย โดยใช้เครื่องหมายไมโครแซทเทิลไลท์และทดสอบความแม่นยำของชุดตรวจ ตัวอย่างที่ทำการศึกษาจำนวน 195 ตัวอย่าง ประกอบด้วยพ่อกระบือ 23 ตัว แม่กระบือ 67 ตัว ลูกกระบือ 87 ตัว และกระบือไม่ทราบประวัติ 18 ตัว ตัวอย่างดีเอ็นเอได้จากการสกัดเลือดและน้ำเชื้อแช่แข็งกระบือ ชุดตรวจสอบประกอบด้วยเครื่องหมายไมโครแซทเทิลไลท์จำนวน 10 เครื่องหมาย สำหรับเพิ่มขยายชิ้นส่วนดีเอ็นเอจากตัวอย่างด้วยปฏิกิริยาลูกโซ่โพลีเมอเรส 2 ชุด และหาขนาดชิ้นส่วนดีเอ็นเอด้วยเครื่องหาลำดับเบส ผลการวิเคราะห์ได้จำนวนอัลลิล 5 ถึง 29 อัลลิล ซึ่งมีค่าเฉลี่ยเท่ากับ 16.3 อัลลิล ขนาดของอัลลิลที่ได้อยู่ระหว่าง 110 ถึง 264 คู่เบส มีค่า Ho และ He เฉลี่ยเท่ากับ 0.762 และ 0.834 มี 7 เครื่องหมายที่มีการกระจายของชิ้นส่วนดีเอ็นเออยู่ในภาวะสมดุลของ HWE มี 9 เครื่องหมายที่มีค่า PIC มีค่า > 0.7 เฉลี่ยเท่ากับ 0.811 เมื่อทำการวิเคราะห์ค่าความถูกต้องของหลายเครื่องหมายรวมกัน ตั้งแต่ 8 ถึง 10 เครื่องหมาย พบค่าความถูกต้องในการตรวจสอบความเป็นพ่อ-แม่-ลูกกระบือปลักไทยในการศึกษาครั้งนี้อยู่ระหว่าง 0.9998 ถึง 0.9995 ซึ่งให้ความเชื่อมั่นสูงระหว่าง 99.98 ถึง 99.95 %

ABSTRACT

The present study was undertaken to construct a multiplex microsatellite panel for parentage testing in swamp buffalo. The study was based on total of 195 swamp buffalos (23 sires, 67 dams, 87 off springs and 18 unrelated buffaloes). Genomic DNA was extracted from blood and semen samples. Panel of 10 microsatellite markers was amplified in two panels of multiplex polymerase chain reaction and analyzed by capillary electrophoresis on automated DNA sequence. The number of alleles ranged from 5 to 29 with a mean of 16.3 in 10 loci. The allele size ranged from 110 to 264 bp. The mean Ho and He was 0.762 and 0.834, respectively. The results of the X^2 test of goodness of fit revealed that the population was in HWE proportion for 7 microsatellite loci. Nine out of ten microsatellite loci revealed relatively high PIC (> 0.7) with mean 0.8109. The CEP using 10 microsatellite loci was 0.9994965. The accurate for parentage testing (CEP) using 8 to 10 microsatellite loci with was 0.9998 to 0.9995 (99.98% to 99.95%).

Key words: microsatellite markers, parentage, swamp buffalo
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INTRODUCTION

Asian water buffalos (*Bubalus bubalis*) were classified into two clades (swamp and river buffalos) based on 21 microsatellite loci variation and a comparison with 25 protein coding loci (Barker et al., 1997a). Significant genetic differentiation among swamp buffalos based on 53 protein coding loci was also reported by Barker et al., (1997b). Differences among swamp buffalo population may be due to geography and domestication. Thai-swamp buffalo population of approximately 1.35 million in 2006, while the population of approximately 4.61 million in 1989. The decreased buffalo population was due to socio-economic changes in Thailand since nearly 20 years ago. At present, some agricultural crops eg. sugar cane, cassava, palm oil tree, rubber tree are more attractive incomes for farmers than livestock sector in the rural area. The Department of Livestock Development had an implemented pilot project of high performance and genetic buffalo sire production in the village since 2007. The project was implemented by artificial insemination with frozen semen of champion buffalos from several nation contests into buffalo cows after synchronization.

Due to a problem with sire misidentifying of buffalo offsprings were born in the village, while other buffalo bulls were available in the village. Thus, parentage testing by microsatellite markers might be a valuable tool for solving this problem. Parentage analysis comes in several forms depending on the question being asked and the data available. Paternity analysis is the most familiar form of parentage analysis. The objective is to assign a male parent to each offspring. Maternity analysis is identical to paternity analysis except that the objective is to assign female parents to offspring. Parent pair analysis is the optimal method of parentage testing when neither parent is known and the objective is to assign both parents.

Recently, Jakhesara et al. (2012) reported that ten microsatellite markers (CSSM61, ILSTS29, ILSTS17, ILSTS28, CSSM57, CSSM22, ILSTS61, CSSM8, ETH152, and ILSTS11) for parentage testing in 212 a river buffalo; Mehsana (100 dams, 100 daughters and 12 sires). The expected heterozygosity ranged from 0.642 to 0.833 (mean 0.762). Seven out of ten microsatellite loci revealed relatively high PIC (> 0.7). The total exclusion probability (EP) using 10 microsatellite loci with one known parent was 0.993.

The object of this study was to construct a multiplex microsatellite panel for parentage testing in Thai-swamp buffalo.

MATERIALS AND METHODS

1. Animals

A total of 195 swamp buffalos (23 sires, 67 dams, 87 offsprings and 18 unrelated buffaloes) from 6 provinces (Chonburi, Saraburi, Lopburi, Uthaithani, Buriram, Mahasarakam) of Thailand were used in this study.

2. DNA samples

Blood samples of each animal were collected from the jugular vein into 6 ml of EDTA tube. The blood samples were kept in ice box. Two hundred µl of each blood sample was extracted genomic DNA by commercial kit (AxyPrep Blood Genomic DNA Miniprep kit: Axygen Bio science; USA). Frozen semen samples of swamp buffalo bulls were extracted DNA by phenol: chloroform method (Sambrook et al., 1989). Twenty ng gram of the DNA samples were prepared and kept at -20 ^{o} C until used.

3. Microsatellite markers

Eight microsatellite markers as recommended by ISAG'EC 2010 for water buffalo CT (INRA006, CSSM47, RM004, BM1706, MAF65, CSSM38, CSSM42 and CSSM19) as well as 2 additional markers (D5S2 and INRA189) was used to amplified the DNA samples by multiplex polymerase chain reaction in 2 panels as shown in table 1.

4. Multiplex polymerase chain reaction

A panel of multiplex PCR mixture contained 2 µl of 10x PCR buffer, 2 µl of 2 mM dNTP, 1.2 µl of 50 mM $MgCl_2$, 0.4 µl of primer mixed of each 5 µM primers, 0.4 µl of 5U/µl Platinum *Taq* polymerase, 1 µl of 20 ng/µl DNA template and DDW adjusted to 20 µl of final PCR mixture. The DNA samples were amplified by a thermal cycler (MJ Mini Personal Thermal cycler). The PCR condition was initial denaturation at 94 ^{o}C for 5 min, followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec, extension at 72 ^{o}C for 1 min, following final extension for 1 min at 72 ^{o}C.

5. DNA fragment size

The PCR product of each sample was purified and analyzed DNA fragment size by capillary electrophoresis on automated DNA sequencer (3130 Genetic Analyzer: Applied Biosystem) using the GeneScan™-500 ROX™ Size Standard (Applied Biosystem). Results were read and interpreted using GeneScan®and Genemapper software, respectively.

6. Statistical analysis

Number of alleles, observed and expected heterozygosity, chi-square values of Hardy-Weinberg Equilibrium (HWE), polymorphism information content (PIC) values and exclusion probability (EP) of 10 microsatellite markers loci were analyzed from 195 Thai-swamp buffalo samples by the Cervus 3.0, Genetic Parentage Analysis Software (Kalinowski et al. 2007).

7. Parentage testing

We analyzed 28 known records among sires and offsprings of the swamp buffalos for paternity testing. For maternity testing, we analyzed 21 known records among dams and offsprings of the swamp buffalos. For parent pair analysis, we analyzed 11 known records among dams and offsprings of the swamp buffalos.

RESULTS AND DISCUSSION

A panel of 8 microsatellite markers as recommended by ISAG'EC 2010 for water buffalo CT (INRA006, CSSM47, RM004, BM1706, MAF65, CSSM38, CSSM42 and CSSM19) as well as 2 additional markers (D5S2 and INRA189) was effective amplified in two panels of multiplex PCR and analyzed the DNA fragment sizes by capillary electrophoresis on automated DNA sequencer. In table 2, the number of alleles ranged from 5 to 29 with a mean of 16.3 in 10 loci. The allele size ranged from 110 to 264 bp. The mean observed and expected heterozygosity was 0.762 and 0.834, respectively. The results of the X^2 test of goodness of fit revealed that the population was in Hardy-Weinberg Equilibrium proportion for 7 microsatellite loci (INRA006, CSSM47, RM004, BM1706, CSSM38, CSSM19 and D5S2). The remaining 3 loci (MAF65, CSSM42 and INRA189) showed significant ($P<0.05$ in MAF65; $P<0.01$ in CSSM42 and INRA189) departure from HWE which might be due to both the systemic (selection, mutation and migration), dispersive (genetic drift and inbreeding) forces operating in the population. Nine out of ten microsatellite loci revealed relatively high PIC (> 0.7) with mean 0.8109. Based on the PIC values, it was found that all markers used in this study showed values of more than 0.5, indicating that microsatellite markers can effectively be used for molecular characterization and genetic diversity studies in swamp buffalos.

Table 1 Microsatellite loci and primer sets for 2 panels of multiplex polymerase chain reaction to amplify genomic DNAs of Thai-swamp buffaloes in this study.

Locus	Chromosome	Primer Sequence	Fluorescent Dye	Panel	Annealing Temp.(°C)
INRA006	3	5'-AGGAATATCTGTATCAACCTCAGTC-3'	FAM	1	58
		5'-CTGAGCTGGGGTGGGAGCTATAAATA-3'			
CSSM47	8	5'-TCTCTGTCTCTATCACTATATGGC -3'	FAM	1	58
		5'-CTGGGCACCTGAAACTATCATCAT-3'			
RM004	15	5'-CAGCAAAATATCAGCAAACCT-3'	PET	1	58
		5'-CCACCTGGGAAGGCCTTTA-3'			
BM1706	16	5'-ACAGGACGGTTTCTCCTTATG-3'	PET	1	58
		5'-CTTGCAGTTTCCCATACAAGG-3'			
MAF65	15	5'-AAAGGCCAGAGTATGCAATTAGGAG-3'	NED	1	58
		5'-CCACTCCTCCTGAGAATATAACATG-3'			
CSSM38	10	5'-TTCATATAAGCAGTTTATAAACGC-3'	NED	1	58
		5'-ATAGGATCTGGTAACTTACAGATG-3'			
CSSM42	2	5'-GGGAAGGTCCTAACTATGGTTGAG-3'	FAM	2	58
		5'-ACCCTCACTTCTAACTGCATTGGA-3'			
CSSM19	1	5'-TTGTCAGCAACTTCTTGTATCTTT-3'	VIC	2	58
		5'-TGTTTTAAGCCACCCAATTATTTG-3'			
D5S2	5	5'-TACTCGTTAGGGGCAGGCTGCCTG-3'	VIC	2	58
		5'-GAGACCTCAGGGTTGGTGATCAG-3'			
INRA189	Y	5'-TTTTGTTTCCCGTGCTGAG-3'	NED	2	58
		5'-GGACCTCGTCTCCTTGTAGCC-3'			

Remarks:

Microsatellite markers were selected and used in this study from recommendation of ISAG'EC 2010 for water buffalo CT. *In* Proceedings of the 32nd International Conference on Animal Genetics, 32nd Conference of the International Society of Animal Genetics. 26-30 July 2010. Edinburgh, Scotland (UK). The primer sets were derived from GenBank at www.ncbi.nlm.gov

Table 2 Number of alleles, observed and expected heterozygosity, chi-square values of Hardy-Weinberg Equilibrium (HWE), polymorphism information content (PIC) values and exclusion probability (EP) of 10 microsatellite markers loci were analyzed in 195 Thai-swamp buffalos.

Locus	n_o	Allele size range (bp)	H_o	H_e	HWE (X^2 values)	PIC	EP
INRA006	12	110-121	0.749	0.897	7.557^{NS}	0.885	0.647
CSSM47	15	120-153	0.821	0.846	14.172^{NS}	0.826	0.525
RM004	17	123-159	0.788	0.812	7.258^{NS}	0.787	0.463
BM1706	21	237-264	0.831	0.882	5.831^{NS}	0.868	0.612
MAF65	16	110-133	0.703	0.820	18.604**	0.796	0.479
CSSM38	13	161-183	0.687	0.702	4.612^{NS}	0.651	0.292
CSSM42	17	179-208	0.339	0.884	174.223***	0.872	0.623
CSSM19	29	126-163	0.846	0.896	0.277^{NS}	0.886	0.660
D5S2	18	194-220	0.862	0.844	5.277^{NS}	0.826	0.533
INRA189	5	244-258	0.989	0.755	194.993***	0.712	0.348
Mean	16.3		0.762	0.834		0.811	0.518

n_o: Observed alleles; H_o: Observed heterozygosity; H_e: Expected heterozygosity

HWE: Hardy-Weinberg Equilibrium; PIC: Polymorphism information content; EP: Exclusion probability

** P<0.05, *** P<0.01

Table 3 Combined exclusion probability (CEP) of 10 microsatellite loci used in the study.

	Exclusion probability (EP) in each locus									
Locus	10	9	8	7	6	5	4	3	2	1
INRA006	0.6471	0.6471	0.6471	0.6471	0.6471	0.6471	0.6471	0.6471	0.6471	0.6471
CSSM47	0.5254	0.5254	0.5254	0.5254	0.5254	0.5254	0.5254	0.5254	0.5254	
RM004	0.4629	0.4629	0.4629	0.4629	0.4629	0.4629	0.4629	0.4629		
BM1706	0.6119	0.6119	0.6119	0.6119	0.6119	0.6119	0.6119			
MAF65	0.4789	0.4789	0.4789	0.4789	0.4789	0.4789				
CSSM38	0.2921	0.2921	0.2921	0.2921	0.2921					
CSSM42	0.6232	0.6232	0.6232	0.6232						
CSSM19	0.6596	0.6596	0.6596							
D5S2	0.5325	0.5325								
INRA189	0.3479									
CEP	0.9995	0.9992	0.9998	0.9951	0.9871	0.9818	0.9650	0.9100	0.8325	0.6471

In table 2, the combined exclusion probability (CEP) using 8 to10 microsatellite loci was 0.9998 to 0.9995 in Thai-swamp buffalos, similarly to the report of using 11 microsatellite markers in river buffalos

with CEP of 0.999 (Kathiravan et al., 2012). It was noticed that the CEP for parentage testing in Thai-swamp buffalo using 8 microsatellite markers as recommended by ISAG'EC 2010 for water buffalo CT (INRA006, CSSM47, RM004, BM1706, MAF65, CSSM38, CSSM42 and CSSM19 was higher (0.9998) than the report of Jakhesara et al. (2012) in Mehsana buffalos (CEP=0.993).

In this study, we decided to use 8 to10 markers for parentage verification in Thai-swamp buffalos. The frequencies of 10, 9 and 8 matching loci was 18.2, 54.5 and 27.3 %, respectively for paternity testing in 28 known records of swamp buffalos. For maternity testing with 21 known records, the frequencies of 10, 9 and 8 matching loci was 42.9, 38.1 and 19.0 %, respectively, whereas the frequency of 10, 9 and 8 matching loci was 100, 0 and 0 % in parentage testing with 11 known records.

The present study proposes two multiplex sets with six and four markers respectively for routine parentage testing in swamp buffalo and would be an additional set of markers for doubtful cases of paternity and maternity testing.

CONCLUSION

A panel of 8 microsatellite markers as recommended by ISAG'EC 2010 for water buffalo CT as well as 2 additional markers was able to perform multiplex PCR. The results suggest that multiplex microsatellite panel is a fast, robust, reliable and economic tool to verify the parentage as well as to assign the putative sire to offsprings under progeny testing with very high accuracy and hence can be used in routine parentage testing in Thai-swamp buffalos. Implementation of artificial insemination programs and DNA testing to identify sires are the keys for increasing genetic progress in the Thai-swamp buffalo population.

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