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VALIDATION OF PEDIGREE RELATIONSHIPS USING A MULTIPLEX MICROSATELLITE MARKER ASSAY IN IRANIAN HOLSTEIN CATTLE*

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Abstract

In the present study, the pedigree and genotype data of 94 individuals were examined for accuracy of parentage allocations and identity test using 12 fluorescent-labelled microsatellite markers in a highly sensitive and accurate ABI system. The panel of 12 markers showed mean PIC value of 0.71 and Shannon index of 1.65 and 7.58 alleles per locus, which suggests that these markers are highly polymorphic and could be useful for parentage control. Based on scoring allele sizes, a total of 91 alleles were observed within the studied population. The highest and lowest number of alleles was observed for TGLA227 locus and TGLA126 and BM1818 loci, respectively. The pedigree was considered incorrect in seven (35%) out of all the evaluated progeny, as their genotype did not match their parents. Combined EP value obtained for all loci in both parentage and identification analysis was 0.99, which indicates the high efficiency of the studied marker set and the accuracy of genotyping in ABI systems. Finally, the present findings confirmed the importance of surveying the pedigree structure and efficiency of 12 fluorescent-labelled microsatellite markers in a single multiplex PCR for parentage testing in the sampled Holstein cattle population.

Key words: parentage testing, microsatellite, Holstein cattle, pedigree, multiplex PCR, exclusion probability

Per capita milk consumption in Iran has been reported to be 73 kg while the average world figure is between 300 and 350 kg. Holstein cattle are the dominant industrial breed for providing main dairy products in Iran. The population of this breed was established with the importation of registered heifers from Europe, the

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USA and Canada during the 1970s and in the early 1980s. Each year approximately 80 young bulls are entered into the progeny-testing programme by the Iranian animal breeding centre, of which 12–20 bulls would be selected as proven sires (Dadpasand et al., 2008).

The increase in genetic progress depends highly on accurate evaluation based on the entire and correct pedigree (Zhang et al., 2010). Inaccurate pedigree information is a common problem in the livestock industry, and paternity pedigree errors always have a substantial negative impact on the national genetic evaluation and estimates of inbreeding (Kios et al., 2011). Van Vleck (1970 a, b) demonstrated that incorrect identification of sires, in cattle data, could cause biased estimates of heritability, evaluations of sires and estimates of genetic progress due to selection.

Selection decisions based on the best linear unbiased prediction (BLUP) are more accurate because BLUP takes account of all available relationships and pedigree information. However, when pedigrees contain errors, estimation of heritability is mostly doubtful. The use of Genomic Selection (GS) also depends on the accuracy of the GS models to predict the breeding values (BV). Improvements in BLUP BV can be obtained simply by correcting errors in the pedigree or using more complex approaches, such as applying a realized relationship matrix (RRM) in the BLUP prediction as an alternative to the relationship matrix (A) based on expected values derived from the pedigree (Munoz et al., 2011).

Due to the above facts, there is a need for tools or indicators to check the correct paternity relationships. Nowadays DNA analysis and microsatellite markers have become a powerful tool for verifying the parentage and identification of individual animals (Rehout et al., 2006).

Microsatellite markers, because of several advantages such as their high polymorphism content, widespread distribution in the genome and easily interpretable results are markers of choice (Baron et al., 2002). Regardless of the importance of microsatellite markers for parentage test, it also may be prone to errors in every step of the genotyping process, from initial sampling to allele scoring and data entry, which could affect parentage analysis (Bonin et al., 2004). Applying advanced methods such as capillary electrophoresis and their many advantages, including separation efficiency, short analysis time, low sample and solvent consumption, low cost of running and lower effect of matrices compared with the other separation techniques could determine accurate allele size (Mitchelson and Cheng, 2001).

The aim of the present study was to validate pedigree relationships using a multiplex microsatellite marker assay as a critical step for Holstein cattle genetic evaluation and also to investigate the efficiency of 12 ISAG/FAO recommended microsatellite loci used for parentage tests of Iranian Holstein cattle.

Material and methods

Animals

Overall, the pedigree of 94 animals was tested for both parentage control and identity. Forty 40 DNA samples of proven Holstein bulls were provided as detailed

information from the Animal Science Research Institute of Iran (ASRI) DNA bank. Regarding the pre-assumption of pedigree, nine sires along with 21 offspring and 13 dams were selected for paternity allocations. In addition, 20 unknown animals were provided from the Animal Breeding Station of Iran with no prior information about their relationships to identify pedigree relationships and pattern of heredity.

DNA extraction

Blood samples in offspring and dams were collected from the jugular vein, supplemented with 0.5 M EDTA (pH=8) and transferred to the laboratory freezer (−20°C). Genomic DNA was extracted by modified salting out method (Miller et al., 1988) and purity of all extracted DNA was assessed by calculating the 260/280OD ratio determined with the Nanodrop (Model ND1000).

Primer sets and method of genotyping

In the present study, 12 microsatellite loci from an ISAG/FAO joint recommended list of markers in bovine genotyping were co-amplified using 12 primer pairs. The forward primer of each locus was end labelled with a fluorescent dye. Table 1 shows the loci characteristics and the primers used in amplifying each locus.

The amplification of microsatellite sequences was performed by multiplex PCR reaction using commercially available bovine genotype panel 1.2 (FINNZYMES DIAGNOSTICS, F-904, FINLAND) in a 25 µl of the reaction volume containing 2 µl (50-100 ng) of genomic DNA and 18 µl of master mix according to manufacturer's instructions. Polymerase chain reaction was carried out in a Master Cycle gradient PCR system (Eppendorf) with the following PCR programme: initial denaturation for 1 min at 98°C, followed by 30 cycles of denaturation at 98°C for 20 s, annealing temperature at 60°C for 75 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were run on ABI PRISM Genetic Analyser 3130 (Applied Biosystems Inc., Foster City, CA) capillary electrophoresis in the presence of GeneScan-500 LIZ internal size standard (Applied Biosystems Inc., Foster City, CA), which is designed for sizing DNA fragments in the 35–500 bp range and provides 16 single-stranded labelled fragments.

During the process and due to normalization of data we also used bovine genomic DNA at the 0.5 mg/µl concentration as a control for verification of acceptable PCR and electrophoresis conditions. The raw data were collected using Data Collection software version 4.0, which was installed on the ABI system. Fragment analysis of PCR products was then performed by GeneMapper software version 4.0 (Applied Biosystems Inc., Foster City, CA).

Statistical analysis

After the adjustment and normalization process using binning alleles in Excel, measures of genetic variation including observed and effective number of alleles and their frequencies, observed and expected heterozygosity, and Shannon index were calculated using GeneAlex 6.4 software. Polymorphism information content (PIC) and exclusion probability (EP) value of each locus were obtained by CERVUS 3.0 software (Kalinowski et al., 2007). Parentage and identity test according to most-likely candidate parent was also done with CERVUS 3.0.

Table 1. Chromosome number, motif repeat, primer sequence, allele size range and fluorescent-labelled dye

Locus	Chromo-some	Repeat motif	Primer sequence	Allele size range (bp)	Dye
TGLA227	18	$(TG)_n$	F: CGA ATT CCAAATCTGTTA ATT TGC T R: ACAGAC AGA AACTCAATG AAA GCA	63–115	FAM
BM2113	2	$(CA)_n$	F: GCTGCCTTCTACCAA ATA CCC R: CTTCCT GAG AGA AGCAACACC	116–146	FAM
TGLA53	16	$(TA)_n$	F: GCTTTCAGAAATAGTTTGCATTCA R: ATCTTCACATGATATTACAGCAGA	147–197	FAM
ETH10	5	$(AC)_n$	F: GTT CAG GACTGG CCC TGCTAA CA R: CCTCCAGCCCACTTT CTC TTCTC	198–234	FAM
SPS115	15	$(CA)_n$	F: AAA GTGACACAA CAG CTT CTC R: AAC GAG TGTCCTAGTTTGGCTGTG	240–270	FAM
TGLA126	20	$(TG)_n$	F: CTA ATT TAG AAT GAG AGA GGCTTC T R: TTGGTCTCT ATT CTC TGA ATA TTC C	104–132	VIC
TGLA122	21	$(AC)_n (AT)_n$	F: CCC TCCTCCAGGTAAATCAGC R: AATCACATGGCAAATAAGTAC ATA C	133–193	VIC
INRA023	3	$(AC)_n$	F: GAG TAG AGCTACAAG ATA AACTTC R: TAA CTA CAG GGTGTT AGA TGA ACT C	194–236	VIC
BM1818	23	$(TG)_n$	F: AGCTGGGAATATAACCAAAGG R: AGTGCTTTCAAGGTCCATGC	148–276	VIC
ETH3	19	$(GT)_n$	F: GAACCTGCCTCTCCTGCATTGG R: ACTCTGCCTGTGGCCAAGTAGG	89–131	NED
ETH225	9	$(CA)_n$	F: GAT CACCTTGCC ACT ATT TCC T R: ACATGA CAG CCAGCTGCT ACT	132–166	NED
BM1824	1	$(GT)_n$	F: GAG CAAGGTGTTTTTCCAATC R: CAT TCTCCA ACT GCTTCCTTG	170–218	NED

F: Forward R: Reverse bp: Base pair

Table 2. The number of observed (N) and effective alleles (N_e), overall number of genotypes (N_g), MFA, the observed (H_o) and expected (H_e) heterozygosities, polymorphism information content (PIC) and Shannon index (I) for genotyped microsatellite markers

Marker	Na	Ng	Ne	MFA (bp)	Frequency of MFA	H_e	H_o	PIC	I
TGLA227	13	93	7.157	99	0.263	0.860	0.892	0.847	2.224
BM2113	6	92	4.443	133	0.321	0.775	0.783	0.741	1.611
ETH10	8	92	4.093	219	0.435	0.756	0.630	0.732	1.726
SPS115	6	90	3.369	252	0.472	0.703	0.522	0.667	1.459
TGLA126	5	93	2.778	113	0.495	0.640	0.624	0.581	1.224
TGLA122	10	89	4.263	139	0.438	0.765	0.764	0.748	1.861
INRA023	6	90	4.401	206	0.322	0.773	0.822	0.738	1.589
BM1818	5	89	2.850	266	0.427	0.649	0.719	0.583	1.209
ETH3	6	94	3.187	115	0.489	0.686	0.766	0.647	1.381
ETH225	8	92	4.339	144	0.348	0.770	0.783	0.738	1.713
BM1824	8	92	5.343	180	0.255	0.813	0.848	0.787	1.812
TGLA53	10	79	6.176	158	0.259	0.838	0.506	0.820	2.018
Mean	7.58	90.41	4.367			0.752	0.722	0.719	1.652

Na: Number of alleles, Ng: Overall number of genotypes, Ne: Effective number of alleles, MFA: Most frequent allele, He: Expected heterozygosity, Ho: Observed heterosygosity, PIC: Polymorphism information content, I: Shannon index.

Table 3. Comparison between loci allele size ranges in the present study, ISAG and literature data (bp)

Breed Locus	Holstein (Present study)	Fleck-vieh (Putnova et al., 2011)	Charolais (Putnova et al., 2011)	Beef Simmental (Putnova et al., 2011)	Holstein (Riojas--Valdes et al., 2009)	Brown Swiss Riojas--Valdes et al., 2009	Beefmaster (Riojas--Valdes et al., 2009)	Brahman (Riojas--Valdes et al., 2009)	Brangus (Riojas--Valdes et al., 2009)	Gyr (Curiet et al., 2002)	Serbian beef cattle (Jevrosi-ma et al., 2009)	ISAG
TGLA227	69–105	79–97	77–97	79–97	66–102	76–97	70–99	76–83	76–97		70–114	63–115
BM2113	123–137	125–139	125–139	127–139	126–144	128–146	127–150	129–148	108–149		122–142	116–146
ETH10	207–225	213–223	217–223	213–221	105–229	213–230	206–226	213–225	173–225	212–216	206–220	147–197
SPS115	252–264	248–260	248–260	248–260	245–262	223–260	240–262	140–156	227–262	248–256	242–254	198–234
TGLA126	111–119	111–125	115–123	115–123						115–121	114–124	240–270
TGLA122	139–179	141–183	141–179	141–181	128–162	136–175	136–160	136–167	136–160	135–163	138–162	104–132
INRA023	202–214	198–222	198–218	198–218							198–220	133–193
BM1818	262–270	258–270	258–270	258–268								194–236
ETH3	115–129	117–127	117–125	117–127	92–126	90–124	99–124	100–114	91–124	113–119	112–124	148–276
ETH225	134–148	140–152	138–152	140–158	114–154	142–160	142–161	142–161	108–161	139–161	134–150	89–131
BM1824	178–214	178–190	178–190	178–190	175–190	176–188	178–189	177–192	150–188	82–188	178–190	132–166
TGLA53	152–18										150–184	170–218

All allelic sizes are in base pair (bp).

Results

Diversity of microsatellites

According to calculated diversity indices, all microsatellite loci were polymorphic and a total of 91 alleles were identified in the present study. The number of alleles per locus ranged from 5 (TGLA126 and BM1818) to 13 (TGLA227) with the overall mean number of 7.58 alleles per locus. The results of the microsatellite marker potential, expressed by expected heterozygosity, Shannon index and polymorphism information content (PIC) are shown in Table 2.

Multiplex PCR

In the present study, a single multiplex PCR using primer pairs of 12 microsatellite markers was first evaluated for each individual locus by the sharpness of band and easy optimization and then the PCR products of the loci were mixed and based on fragment size they were grouped for multiplex system. Using multiplex PCR allows the target sequence to be amplified simultaneously by using several pairs of primers in the reaction which substantially saved time and cost in this study. To prevent overlapping among loci with the same colour labelling, a suitable distance should be considered between loci, which was done for 12 microsatellites in the present study (Table 1).

A comparison between the allelic size ranges of the studied loci and ISAG allelic size range showed the following differences: TGLA227 and BM2113 were in the same allelic size range with ISAG. ETH10, SPS115, TGLA122, INRA023, BM1818, ETH225 and BM1824 showed higher ranges. TGLA126 and ETH3 were in the lower range, TGLA53 was in a different and slightly lower range than ISAG reported range. Table 3 shows this comparison and some other size ranges in different cattle breeds.

Fragment analysis

Fluorescently labelled PCR fragments were detected in Genetic Analyser 3130 and then analysed in Genemapper software. This software uses the size standard (GeneScan 500 LIZ) to create a standard curve for each lane and then determines the length of each dye-labelled fragment by comparing it with the standard curve for that specific lane. An example of individual genotyping in loci labelled with NED and VIC are shown in Figure 1. Each single peak in the Figure shows a homozygote genotype and double peaks indicate the heterozygote genotype of the sample.

Parentage and identity tests

In parentage testing, the usefulness of any co-dominant marker is defined as the probability of it making exclusion and called exclusion probability (EP). The EP values of this population were calculated in CERVUS 3.0. For each offspring, CERVUS calculates the likelihood of parentage of every candidate parent or parent pair. In this software EP values were yielded as an average non-EP for first parent, second parent, parent pair, identity and sib identity. The EP values were obtained by subtracting non-EP values from one. In this way the EP values for parentage test with

both parents ranged from 0.87 (locus TGLA227) to 0.43 (locus BM1818), which was consistent with polymorphic values of these loci. In the identity test all loci had EP values higher than 0.7, ranging from 0.95 (TGLA227 and TGLA126) to 0.73 (BM1818). These high EP values along with excellent electrophoresis results and easy judgement of genotypes implicate complete efficiency of this combination of loci in individual identification and paternity test of this population. Table 4 shows the average and combined EP values for each locus in parentage with parent pair and identity test. Because we only performed identity and parentage tests with both parents in this study, only the EP values of these tests are cited here and other measures are not mentioned.

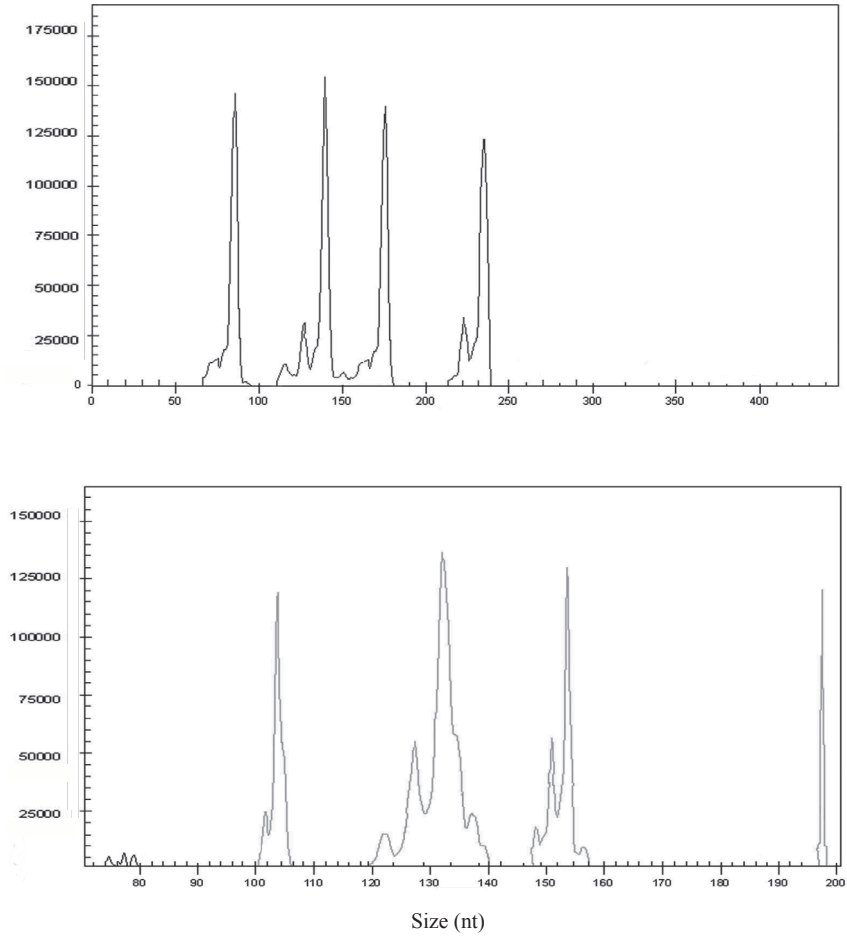

Figure 1. Size fragmentation of multiplex 2 (labelled with VIC) and multiplex 3 (labelled with NED) loci

Table 4. Exclusion probability (EP) values of parentage and identity test in 12 microsatellite loci

Locus	EP – parent pair	EP – identity test
TGLA227	0.873	0.959
BM2113	0.661	0.871
ETH10	0.767	0.918
SPS115	0.618	0.832
TGLA126	0.446	0.737
TGLA122	0.748	0.902
INRA023	0.684	0.889
BM1818	0.439	0.738
ETH3	0.649	0.855
ETH225	0.631	0.854
BM1824	0.748	0.914
TGLA53	0.798	0.932
Cumulative EP	0.9999	0.999

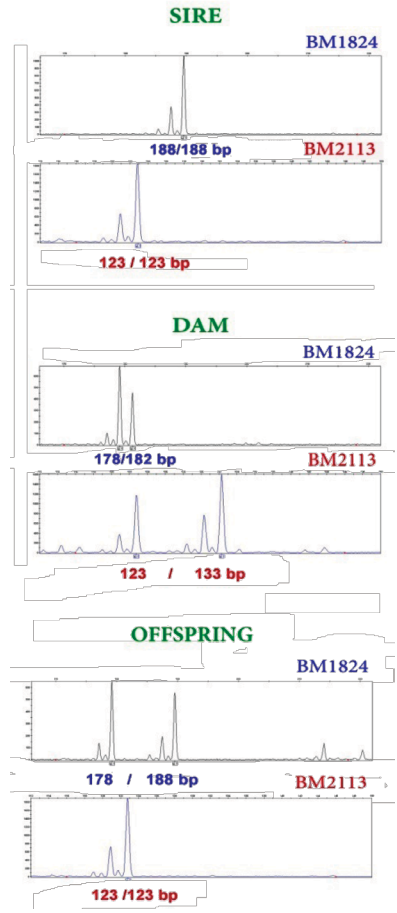

Figure 2. Mendelian heredity pattern in BM1824 and BM2113 loci

Results of parentage verification unfortunately indicated that only six offspring had accurately registered parental relationships. Seven offspring were incompatible with both parents, two offspring were incompatible with sire and five offspring had incorrectly assigned dams.

The identity test of 20 unknown samples in this population revealed different relationships including one and two parents for offspring and also repeated samples were observed. An example of Mendelian heredity and identification of parent pair's relationship between these unknown samples is shown in Figure 2 for two loci.

Discussion

Efficiency of paternity or parentage testing certainly depends on the level of information provided by the markers, as high genetic variability of markers implied their high effectiveness for parentage testing. In the present study, the highest and lowest value of observed heterozygosity was for the TGLA227 and TGLA53 loci (0.89 and 0.50, respectively). The expected heterozygosity ranged from 0.86 (TGLA227) to 0.64 (TGLA126 and BM1818), averaging 0.75. The high value of expected heterozygosity in locus TGLA227 was similar to the findings of Putnova et al. (2011). The mean PIC value was 0.71 and, as expected from their allelic number, ranged from 0.58 (TGLA126, BM1818) to 0.84 (TGLA227), confirming the high level of polymorphism in each analysed microsatellite marker. Rehout et al. (2006) and Ozkan et al. (2009), who analysed 10 and 12 loci, have reported the maximum PIC in locus TGLA227 (0.81 and 0.84, respectively), which was very close to the value obtained in our study. In general, the higher the heterozygosity, the higher the genetic variation of a population and its genetic polymorphism, and more suitable the marker is for individual identification.

In the present study all loci but three (TGLA126, BM1818 and ETH3) had expected heterozygosity higher than 0.7. The highest and lowest value of the Shannon index, which is a popular diversity index in the literature, was 2.22 for TGLA227 and 1.20 for BM1818. In this study the combined EP values of 12 microsatellite loci as a single multiplex in both parent pair and identity analysis was 0.999, which shows the usefulness of these microsatellites for parentage and identity tests in Iranian Holstein cattle.

Despite using the ABI system and its powerful performance in genotyping, some samples showed low quality in genotyping, especially in locus TGLA53. In these samples locus TGLA53 was excluded from further analysis due to the low quality of alleles and genotype detection. Unfavourable genotypes and elimination of locus TGLA53 from further analysis was also reported by other researchers (Visscher et al., 2002; Putnova et al., 2011).

Paternity misidentification was studied in many populations and by different researchers. For example Carolino et al. (2009) also used a panel of 10 microsatellite markers for parentage control in a group of 140 calves from several breeds. Overall 76.4% of the calves in this group were compatible with the recorded parents, with

most incompatibilities due to misidentification of the dam. Christensen et al. (1982) cited seven reasons for errors in paternity recording: 1) mistakes in labelling sperm by Artificial Insemination (AI) institutes; 2) AI technicians' mistakes in identifying correct semen sample; 3) The insemination of cows already pregnant with previous insemination; 4) Errors in entering bull's herd book number or its name into the insemination record; 5) The use of natural-service bulls which leads to pregnancies of previously inseminated cows, which were assumed pregnant from AI bulls; 6) Misidentification of sire when a new cow entered a milking herd in schemes where pedigree information is obtained through the milk-recording programme; and 7) Interchange of calves on a farm.

The pedigree error rates in a population of Holstein cattle were reported in many countries, including 5-15% in Denmark (Christensen et al., 1982), 4–23% in Germany (Geldermann et al., 1986), 10% in UK dairy herds (Visscher et al., 2002), 11.7% in Israeli Holstein (Weller et al., 2004), 10.73% in the Czech Republic (Rehout et al., 2006) and 4.7% in Turkey (Ozkan et al., 2009). According to the results of the present study on Iranian Holstein cattle, pedigree was considered incorrect in seven (35%) out of all the evaluated progeny, as their genotype did not match their parents. 25% of the offspring showed dam misidentification and 10% had mis-paternity. The possible reasons for this high error could be mistakes in labelling sperm by artificial insemination companies or carelessness of their technicians in identifying correct semen sample and re-insemination of pregnant cows because of weak farm management.

It is logical to suggest that the differences in the polymorphism level of candidate loci or a range of allele sizes in the Holstein breed in Iran and other breeds are due to different genetic makeup, breed adaptation, natural selection, history of synthesis and artificial selection, migration, mutation, as well as the way families are selected for genotyping, and technical staff.

In conclusion, because of the high proportion (35%) of incorrect pedigree the parentage control is very important for this breed and country to design an efficient selection programme. In addition, the implementation of the presented test in Iran could have a remarkable role in preventing the export of genetic resources and also in exit of exchange.

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Walidacja rodowodów irańskiego bydła holsztyńskiego przy użyciu analizy multipleks markerów mikrosatelitarnych

STRESZCZENIE

Dane rodowodowe i genotypowe 94 osobników badano pod kątem dokładności ustalania rodzicielstwa i identyfikacji, analizując 12 fluorescencyjnie znakowanych markerów mikrosatelitarnych przy użyciu niezwykle czułego i dokładnego systemu ABI. Dla panelu 12 markerów uzyskano współczynnik polimorfizmu (PIC) wynoszący 0,71 oraz indeks Shannona wynoszący 1,65 i 7,58 alleli na locus, co wskazuje na wysoki stopień polimorfizmu tych markerów i możliwość ich wykorzystania w kontroli rodowodów. W oparciu o analizę wielkości alleli, w badanej populacji stwierdzono ogółem 91 alleli. Najwyższą i najniższą liczbę alleli zaobserwowano odpowiednio w locus TGLA227 oraz w loci TGLA126 i BM1818. Błędy w rodowodzie wykryto u siedmiu osobników (35%) z całości badanego potomstwa, których genotyp nie pokrywał się z genotypem rodziców. Łączne prawdopodobieństwo wykluczenia (EP) uzyskane dla wszystkich loci w analizie zarówno pochodzenia, jak i identyfikacji wyniosło 0,99, wskazując na wysoką skuteczność badanego zestawu markerów oraz dokładność genotypowania przy zastosowaniu analizatorów ABI. Uzyskane wyniki potwierdziły także znaczenie kontroli struktury rodowodowej i skuteczności 12 fluorescencyjnie znakowanych markerów przy użyciu metody multipleks PCR w badaniach pochodzenia populacji bydła holsztyńskiego.