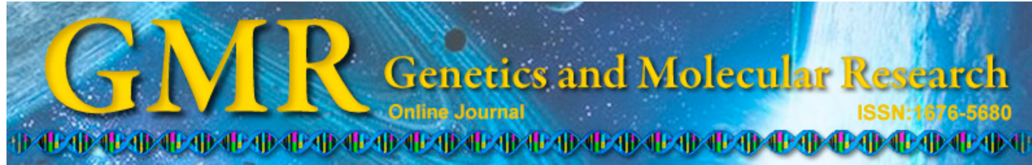

Allele frequencies of microsatellite loci for genetic characterization of a Sicilian bovine population

M. Cosenza, S. Reale, T. Lupo, F. Vitale and S. Caracappa

Experimental Zooprophylaxis Institute of Sicily, Palermo, Italy

Corresponding author: S. Reale
E-mail: stefano.reale@izssicilia.it

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ABSTRACT. Short tandem repeats are used as an effective method to trace DNA markers in genotyping. Using a standardized kit, we tested 11 microsatellite markers recommended by the International Society for Animal Genetics (ISAG) in a sample of 495 Sicilian cattle. The aim of this study was to investigate the allele frequencies in the Sicilian cattle population to provide a reference database and at the same time to assess the use of the ISAG microsatellite panel for pedigree analysis. DNA samples were collected from blood and amplified in an 11-plex polymerase chain reaction (PCR); PCR products were injected in a 3130 Genetic Analyzer. All loci showed high mean polymorphism information content (0.768), and the observed mean heterozygosity was less than the expected value (0.732 *vs* 0.794, respectively). The exact test for Hardy-Weinberg proportions, allele number, and inbreeding coefficient were calculated. Our results indicated that equilibrium was not always maintained. The observed mean homozygote value exceeded the expected value (132.81 *vs* 102.14), but no evidence for allele dropout was found. These results could be explained by a non-random mating; further studies using a larger number of animals could confirm or invalidate this hypothesis. The probability of identity and

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exclusion of a locus were also estimated and proved to be useful in paternity testing. The ISAG microsatellite panel is useful to screen the Sicilian bovine kinship. Currently, an allele frequency database is being constructed.

Key words: Microsatellite; Sicilian cattle; Frequency database; Allele frequencies

INTRODUCTION

In the last few years, the genetic structure of breeding animals has been described. Biodiversity conservation has been considered the main issue (Martín-Burriel et al., 2007) to prevent the loss of genetic variability. In September 2012, the cattle population in Sicily amounted to 347,268 animals (National Cattle Database of Teramo, Italy) with a strong dominance of females over males (275,016 *vs* 72,252). For this reason, breeding cattle was subjected to artificial selection (Bruford et al., 2003) that changed allele frequencies and created a serious loss of biodiversity. Today, the most representative cattle species in Sicily is a half-breed race (72% of the cattle population); Frisona and Limousine cattle comprise 7.1 and 4.5% of the population, respectively (National Cattle Database of Teramo, Italy). The use of short tandem repeats as DNA markers in genotyping represents an effective support for individual identification and parentage control in cattle (e.g., Řehout et al., 2006; Ozkan et al., 2009), horse breeds (Luis et al., 2002), sheep-goats (Lawson et al., 2007), and pigs (Putnová et al., 2003). In the last two decades, this approach has become the most reliable molecular marker for forensic analysis. Microsatellites are recognized as genetic markers because of their random distribution (Tautz and Renz, 1984), co-dominant mode of inheritance (Barbarà et al., 2007), discriminant power (e.g., Barendse et al., 1994), and overall ability for simultaneous analysis (Cañón et al., 2001). Microsatellite markers represent the gold standard for unequivocal determination of the genetic identity and/or parentage, even with limited sample quantities (Taberlet et al., 1996) or when DNA is degraded. They can also be used to trace the meat through the production chain (Vázquez et al., 2004), to study genetic diversity in cattle (Radko et al., 2005), and to select animals in breeding programs. Currently, more than 1000 microsatellite markers have been evaluated for cattle (e.g., Barendse et al., 1994; Kappes et al., 1997). Although many of these studies were conducted using European cattle (MacHugh et al., 1998), the Sicilian cattle population has not been studied. Kinship and/or identity analysis for traceability and consanguinity investigations use the allele frequencies of the local population (e.g., Radko et al., 2005; Cervini et al., 2006; Carolino et al., 2009) from a reference database. A reference database did not exist in Sicily until now, and microsatellite analysis has not been performed on Sicilian cattle. Additionally, allele frequency studies to evaluate microsatellite informativeness and their efficiency are lacking. In 2006, the International Society for Animal Genetics (ISAG) recommended a panel of nine microsatellite loci for cattle genetic analysis: TGLA227 (Georges and Massey, 1992), BM2113 (Sunden et al., 1993), ETH10 (Toldo et al., 1993), SPS115 (Baylor College of Medicine Human Genome Sequencing Center, 2006), TGLA126 (Georges and Massey, 1992), TGLA122 (Georges and Massey, 1992), INRA023 (Vaiman et al., 1994), ETH225 (Steffen et al., 1993), and BM1824 (Barendse et al., 1994). In 2008, the ISAG added three additional microsatellite loci: BM1818 (Bishop et al., 1994), ETH3

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(Toldo et al., 1993), and TGLA53 (Georges and Massey, 1992). The aim of this study was to test the 11 microsatellite markers that were suggested by the ISAG and to evaluate genetic variability to create a database of allele frequencies and improve parental definitions and genetic traceability of the Sicilian cattle population.

MATERIAL AND METHODS

DNA extraction and multiplex-polymerase chain reaction (PCR)

Blood samples of 495 registered related and unrelated cattle belonging to half-breed species were collected. Cattle were sampled from several different breeding areas that were representative of the Sicilian population. Genomic DNA was extracted and purified from whole blood using a commercial kit (PureLink Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol. DNA samples were stored at -20°C. DNA fragments were amplified in 11-plex PCRs using a certified commercial kit (StockMarks for Cattle Bovine Genotyping Kit, Applied Biosystems, Foster City, CA, USA) according to manufacturer instructions. PCRs were carried out using a thermocycler (9700 Applied Biosystems, San Diego, CA, USA). Multiplex-PCR products were analyzed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, San Diego, CA, USA) using the GeneScan-500 ROX Size Standard (Applied Biosystems, Foster City, CA, USA). Genotypic profiles were read and analyzed using the GeneMapper ID v4.0 software (Applied Biosystems).

Statistical analysis

Statistical analysis of data generated from the 11 markers that were analyzed was performed using GenAlEx (Peakall and Smouse, 2012), PowerMarker (Liu and Muse, 2005), and Micro-Checker (Van Oosterhout et al., 2004) softwares, which are useful tools for the analysis of genetic populations because they use algorithms that allow the management of a variety of data. The number of effective alleles (N_E), allele number (N_A), allele frequency, observed and expected heterozygosities (H_O and H_E, respectively), probability of identity of a locus (PI), probability of exclusion of a locus (PE), and the deviation probability from the Hardy-Weinberg equilibrium (HWE) were obtained using the GenAlEx v6.5 software. The expected homozygosity (Hom_E), observed homozygosity (Hom_O), homozygosity excess, evidence for null allele, evidence for large allele dropout, and evidence for scoring error due to stuttering were obtained using the Micro-Checker v2.2 software. The polymorphism information content (PIC), inbreeding coefficient, genotype number (N_G), major allele frequency (M_{AF}), and major genotype frequency (M_{GF}) were investigated using the PowerMarker v3.25 software.

RESULTS

In total, 495 related and unrelated animals were genotyped. Information about the 11 microsatellites that were investigated is presented in Tables 1-5. Using the method that was described by Barker (1994), the 11 microsatellite loci tested in this study were used to evaluate the genetic diversity in Sicilian cattle breeds ($N_A > 4$) (Table 1). All the evaluated microsatel-

lite loci were polymorphic with an N_A of 136, an average of 12.36 alleles per locus, and a range of 5 (BM1824) to 23 (TGLA122) alleles per locus. The N_E value differs from the N_A value and ranged from 3.27 (TGLA126) to 8.85 (TGLA53) alleles per locus with an average of 5.39 alleles per locus. The N_E/N_A ratio had a mean of 0.469 and ranged from 0.216 (TGLA122) to 0.817 (BM1824). The mean M_{AF} was 0.321 and ranged from 0.185 (TGLA227) to 0.494 (ETH3). Despite this, the polymorphism and informativeness of ETH10, ETH3, TGLA126, and SPS115 were lower than those of other loci because their M_{AF} values were close to 0.5. The PIC is a parameter that indicates the degree of marker informativeness. According to the parameters established by Botstein et al. (1980), all microsatellite loci in this study were highly informative (PIC > 0.5), with a mean PIC of 0.768 for all loci. The PIC varied from 0.877 (TGLA53) to 0.654 (TGLA126). The mean H_O and H_E were 0.732 and 0.794, respectively. TGLA53 showed the highest level of heterozygosity (0.887), whereas the SPS115 marker showed the lowest level of heterozygosity (0.628). The probability of finding another animal with the same genetic profile using these 11 markers (PI for increasing locus combinations) was 5.3×10^{-14} and is shown in Table 1 for each locus. The PE by locus for two putative parents had an average of 0.794, ranged from 0.92 (TGLA53) to 0.66 (TGLA126), and followed the pattern that was observed for PIC values. In the Sicilian cattle population, the combined PE exceeded 99% for the ISAG microsatellite panel when the genotypes of both parents are known, when the genotype of only one parent is known, and when the two putative parents are excluded (data not shown).

Table 1. Genetic characteristics of the microsatellite markers that were tested in the Sicilian cattle population (N = 495).

Locus	Observed size range (bp)	N_A	N_E	N_E/N_A	M_{AF}	H_O	H_E	PIC	PE	PI
TGLA227	74-102	15	7.878	0.525	0.185	0.772	0.873	0.86	0.9	0.029
BM2113	120-146	12	6.877	0.573	0.202	0.747	0.855	0.837	0.87	0.038
TGLA53	153-189	17	8.855	0.521	0.217	0.887	0.887	0.877	0.92	0.023
ETH10	208-224	9	3.933	0.437	0.398	0.695	0.746	0.709	0.72	0.1
SPS115	242-254	10	4.251	0.425	0.419	0.628	0.765	0.741	0.78	0.079
TGLA126	114-124	8	3.27	0.408	0.475	0.663	0.694	0.654	0.66	0.13
TGLA122	138-162	23	4.988	0.216	0.307	0.725	0.8	0.775	0.81	0.065
INRA023	198-220	15	6.597	0.439	0.228	0.786	0.848	0.83	0.87	0.041
ETH3	106-130	12	3.286	0.273	0.494	0.679	0.696	0.664	0.69	0.12
ETH225	134-146	10	5.312	0.531	0.318	0.723	0.812	0.788	0.82	0.059
BM1824	176-190	5	4.086	0.817	0.288	0.743	0.755	0.711	0.7	0.1
Mean		12.36	5.39	0.469	0.321	0.732	0.794	0.768	0.794	0.071

N_A = number of alleles; N_E = number of effective alleles; M_{AF} = major allele frequency; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content; PE = probability of exclusion; PI = probability of identity.

The frequencies of each allele at each microsatellite marker are shown in Table 2.

The Hom_E and Hom_O averaged 102.14 and 132.81, respectively (Table 3). All loci significantly deviated from HWE (P < 0.01) except ETH10 and TGLA126 (Table 4). The N_G value, the most representative genotypes for each locus and their M_{GF} are shown in Table 5. TGLA53 showed the highest number of genotypes (N = 89). The lowest N_G was 15 in BM1824, and the average was 47.18. Inbreeding coefficients were calculated on the basis of H_E versus H_O. The mean inbreeding coefficient was 0.081. SPS115 showed the highest value (0.179), whereas TGLA53 showed the lowest value (0.001).

Table 2. Allele frequencies (A_F) of the 11 microsatellite markers.

TGLA53		TGLA227		BM2113		SPS115		ETH10		TGLA126	
Allele size (bp)	A_F	Allele size (bp)	A_F	Allele size (bp)	A_F	Allele size (bp)	A_F	Allele size (bp)	A_F	Allele size (bp)	A_F
153	0.097	74	0.002	120	0.009	237	0.001	208	0.003	110	0.001
157	0.017	76	0.006	122	0.066	243	0.419	210	0.007	114	0.034
159	0.217	78	0.026	124	0.122	245	0.099	212	0.080	116	0.475
161	0.094	80	0.076	126	0.005	247	0.095	214	0.398	118	0.159
163	0.084	82	0.185	128	0.148	249	0.112	216	0.218	120	0.018
165	0.049	84	0.053	130	0.132	251	0.099	218	0.187	122	0.095
167	0.135	86	0.028	132	0.202	253	0.012	220	0.079	124	0.209
169	0.108	88	0.021	134	0.096	255	0.131	222	0.027	126	0.008
171	0.038	90	0.179	136	0.190	257	0.030	224	0.001		
173	0.024	92	0.115	138	0.011	265	0.001				
175	0.069	94	0.125	140	0.017						
177	0.014	96	0.026	146	0.001						
181	0.011	98	0.143								
183	0.023	100	0.010								
185	0.007	102	0.004								
187	0.004										
189	0.007										

TGLA122		INRA023		ETH3		ETH225		BM1824	
Allele size (bp)	A_F	Allele size (bp)	A_F	Allele size (bp)	A_F	Allele size (bp)	A_F	Allele size (bp)	A_F
136	0.001	195	0.005	96	0.001	134	0.110	180	0.179
140	0.020	197	0.063	106	0.024	136	0.144	182	0.262
142	0.287	199	0.060	112	0.018	138	0.006	184	0.245
144	0.005	201	0.021	114	0.494	140	0.118	190	0.288
148	0.070	203	0.011	116	0.133	142	0.059	192	0.025
150	0.034	205	0.228	118	0.021	144	0.188		
152	0.307	207	0.175	120	0.011	146	0.318		
154	0.107	209	0.054	122	0.162	148	0.010		
156	0.018	211	0.161	124	0.117	150	0.030		
158	0.016	213	0.177	126	0.013	152	0.016		
160	0.001	215	0.027	128	0.003				
162	0.070	217	0.014	130	0.001				
164	0.010	221	0.003						
166	0.003	223	0.001						
170	0.002	235	0.001						
174	0.004								
176	0.007								
178	0.001								
180	0.006								
182	0.013								
184	0.005								
186	0.010								
192	0.002								

Table 3. Micro-Checker analysis.

Locus	Hom_E	Hom_O	Hom_{EXC}	Null-Al	Dropout	Stutter
TGLA227	62.83	113	*	*	-	-
BM2113	71.98	125	*	*	-	-
TGLA53	55.89	56	-	-	-	-
ETH10	125.87	151	*	*	-	-
SPS115	116.43	184	*	*	-	-
TGLA126	151.37	167	-	-	-	-
TGLA122	99.24	136	*	*	-	-
INRA023	75.03	106	*	*	-	-
ETH3	150.61	159	-	-	-	-
ETH225	93.18	137	*	*	-	*
BM1824	121.15	127	-	-	-	-
Mean	102.14	132.81				

Hom_E = expected homozygosity; Hom_O = observed homozygosity; Hom_{EXC} = excess homozygosity; Null-Al = evidence for null allele; Dropout = evidence for large allele dropout; Stutter = evidence for scoring error due to stuttering; (*) presence; (-) absent.

Table 4. Hardy-Weinberg equilibrium of the Sicilian cattle population based on the 11 microsatellite markers.

Locus	Probability	P value
TGLA227	0.000	***
BM2113	0.000	***
TGLA53	0.001	***
ETH10	0.128	ns
SPS115	0.000	***
TGLA126	0.177	ns
TGLA122	0.000	***
INRA023	0.000	***
ETH3	0.000	***
ETH225	0.000	***
BM1824	0.005	**

ns = not significant. **P < 0.01; ***P < 0.001.

Table 5. Genotypic information about the 11 investigated short tandem repeat loci.

Locus	N_G	Allele 1	Allele 2	Count	M_{GF}	F
TGLA227	70	10	6	28	0.056	0.117
BM2113	51	11	9	36	0.072	0.126
TGLA53	89	12	7	28	0.056	0.001
ETH10	25	9	9	88	0.177	0.069
SPS115	35	5	5	123	0.248	0.179
TGLA126	25	7	7	120	0.242	0.046
TGLA122	74	10	5	73	0.147	0.093
INRA023	59	10	7	41	0.082	0.074
ETH3	34	13	13	122	0.246	0.025
ETH225	42	7	8	75	0.151	0.11
BM1824	15	11	7	85	0.171	0.016
Mean	47.18				0.149	0.081

N_G = number of genotypes; Count = number of times in which genotype Allele 1/Allele 2 appears in the population; M_{GF} = major genotype frequency (refers to Allele 1/Allele 2); F = inbreeding coefficient.

DISCUSSION

This is the first study that used a standardized kit to evaluate microsatellite DNA markers to determine the genetic variability of the Sicilian cattle population. This study aimed

to create an allele frequency database and to assess the use of the ISAG microsatellite panel to verify the parentage. This study proved the ability of the ISAG microsatellite panel to test the parental definition and genetic traceability of the Sicilian cattle population. In our study, the population showed a significant deficiency in the H_O value compared to the H_E value according to the HWE. This fact can often indicate positive assortative mating with deviations from panmixia. Decreased panmictic indices increase the number of homozygous individuals in the population. Loss of heterozygosity also can include the Wahlund effect because populations are fragmented into subpopulations, allele dropout, and null alleles caused by mutations in primer binding site (Pemberton et al., 1995). In the last case, an undetected allele may cause parents to be incorrectly assigned (Holm et al., 2001) and incorrect genotypes. Because of this, we also recognize the heterozygote deficiency of some loci (ETH10, SPS115, TGLA126, and ETH3), in which genotypes showed predominant allele 1/allele 2 homozygosis. This finding was related to the lower informativeness of the same loci because their M_{AF} values were close to 0.5. Finally, we observed a homozygote excess compared to the expected value. The N_E, which is a function of the expected heterozygosis, showed a strong uneven distribution compared to respective N_A values. According to the study by Carolino et al. (2009), the N_E/N_A ratio describes the allelic distribution depending on the number of alleles at a given locus. Therefore, a high ratio indicates a prevalence of many alleles at a given locus. A low N_E/N_A ratio shows the prevalence of only a limited number of alleles despite a high number of alleles, for example, marker TGLA122. Only BM1824 revealed a nonlinear relationship with the H_E. Thanks to this result, we concluded that allele sets have very different frequencies. We found statistically significant deviations from HWE for all loci except ETH10 and TGLA126. This could be explained by several consequences of the Wahlund effect, such as a loss of heterozygosity and inbreeding. The inbreeding coefficient value is probably explained by the genetic isolation of breeding farms; therefore, breeding cattle has been subjected to nonrandom mating, which is also because the result of the strong disproportion between males and females.

The cumulative PI demonstrated the high discrimination power of these 11 markers in paternity testing of the Sicilian cattle population, with PE > 99.99% (1 - PI). Many authors (e.g., Heyen et al., 1997; Luikart et al., 1999) described paternity incompatibility if putative parents and offspring differed at least in two loci to avoid misidentification due to allelic dropout or null alleles. In this report, we rejected a direct relationship if the genotypic profiles of parents and offspring differed at a single locus if they were heterozygotes at the given locus. However, when genotypes differed only in one homozygous locus, we required one more mismatched locus to discard paternity. The disproportion between male and female animals (1:4) leads to an increased level of inbreeding in Sicily and to the spread of very similar genotypes in the population. To validate our results, seven loci that were analyzed using MicroCheker showed evidence for null alleles, which was suggested by the excess homozygotes for most alleles. Only ETH225 showed evidence for scoring error due to stuttering, and none of these loci had large allele dropout. It is not clear whether the homozygote excess is due to null alleles or if it really reflects the genotypes of the Sicilian cattle population genotypes.

Our purpose was to develop the first Sicilian cattle allele frequency database for genetic traceability. The ISAG panel showed that some alleles are widely distributed while others are present discontinuously within the population. The 11 ISAG microsatellite markers are reliable in parentage verification and identity tests of Sicilian cattle population. Nevertheless, their reliability depends on the consanguinity of the breeding. Additional information supplied by extra-microsatellite markers could provide more useful information to better define the

relationships. Until now, little information was available about Sicilian cattle population microsatellites, and more investigations using a large number of animals are necessary to widen the allele frequency database.

Conflicts of interest

The authors declare no conflict of interest.

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