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EVALUATION OF MICROSATELLITE MARKERS FOR USE DURING GENOTYPING AND PARENTAGE EXCLUSION FOR DROMEDARY CAMELS IN QATAR

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ABSTRACT

This study was aimed to evaluate the efficiency of 20 previously published microsatellite markers for the determination of parentage within the racing camel population in Qatar, using multiplex polymerase chain reaction (PCR), capillary electrophoresis, and genotyping. These markers amplified 127 alleles, and 15 out of 20 loci were polymorphic among the dromedary camels in Qatar, with an average of 8.13 alleles per locus. The mean expected heterozygosity (H_e) among the studied population was 0.562 (range 0.114–0.867). The polymorphic information content (PIC) ranged from 0.107 to 0.852, with an average value of 0.516. These results indicated a low probability of identity ($2.10E^{-11}$), with a high parentage exclusion probability if either one (0.99959) or both parents (0.99999) were putative. In those study cases with parentage assignment, the 15 microsatellite loci successfully assigned 135 young calves to the correct parents, with 95% confidence. Our results demonstrated that a set of nine microsatellite DNA markers could provide highly precise individual identification and paternity assignment within the studied camel population.

Key words: Dromedary camels, genotyping, microsatellite markers, parentage assignment, pedigree

Microsatellites or short tandem repeats (STRs) have been used as widely accepted genetic markers to study DNA profiling and phylogenetic relationships between closely related genomes because they represent highly polymorphic, abundant sequences in DNA, which are inherited in a Mendelian co-dominant manner and are readily adaptable to polymerase chain reaction (PCR) methods (MacHugh *et al,* 1997; Schlötterer, 2004).

In 2014, a camel comparison test was introduced, for the first time, by the International Society of Animal Genetics (ISAG), which was able establish unique binning through the use of a core panel of 7 loci, LCA8, LCA37, LCA56, LCA65, LCA66, YWLL29, and YWLL44, and 10 additional STRs, CVRL01, CVRL04, CVRL05, LCA99, LGU49, VOLP3, VOLP32, VOLP59, YWLL08, and YWLL36. In 2016, LCA19 was added to the core panel and CVRL01, CVRL04, and CVRL05 were excluded from the backup panel.

Many researchers have used STR loci to investigate genetic polymorphisms within and between dromedary camel populations (Mahmoud *et al,* 2012; Nolte *et al,* 2005; Spencer *et al,* 2010). Few efforts have been made to use STRs during camel genotyping in Qatar (Hashim *et al,* 2014). Therefore, the present study was performed to refine the recommended ISAG panels of microsatellite markers for use during the genotyping and parentage testing of dromedary camels in Qatar.

Materials and Methods

Sampling and DNA extraction

A total of 297 blood samples (135 dams, 27 sires, and 135 calves) were collected from dromedary camels reared in Qatar. Blood samples were collected from the jugular vein and placed into tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were collected utilising the pedigree records maintained by camel owners (2016-2017). Genomic DNA was extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche diagnostics, #03730964001/Germany).

PCR amplification and fragment analysis

Twenty camelid microsatellite primer pairs, which were recommended by the ISAG for use

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during dromedary parentage testing, were selected for DNA amplification using PCR. Forward primers were fluorescently labelled with FAM, VIC, NED, and PET dyes (Table 1). The microsatellite markers were amplified individually and then optimised using the following three multiplex reactions: multiplex I included YWLL36, YWLL44, YWLL29, CVRL04, LCA77, VOLP59, LGU49, LCA56, and LCA24; multiplex II included LCA65, CVRL01, VOLP32, LCA37, LCA19, LCA99, and LCA66; and multiplex III included YWLL08, CVLR05, LCA8, and VOLP3.

Multiplex PCR reactions were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems). PCR reactions were performed using a total volume of 25 µl, containing 10 ng of DNA template, 5 pmol of each primer pair, and 12.5 µl of AmpliTaq Gold™ 360 Master Mix (Applied biosystems, 4398881/USA).

The following thermal cycler programme was used: initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of 30 seconds at 95 °C, 55 °C (for multiplex I), 58 °C (for multiplex II), and 60 °C (for multiplex III) for 40 seconds for annealing, 72 °C for 1 min for extension and a final extension at 72 °C for 60 minutes. Samples were maintained at 4 °C after program completion. Fragments were mixed with Hi-Di Formamide (Applied Biosystems, USA) and a Genescan Liz-600 size standard (Applied Biosystems, USA). The mixture was separated by capillary electrophoresis, on an ABI 3500 Genetic Analyser (Applied Biosystems). Finally, the fragments were analysed using GeneMapper® Software 5 (Applied Biosystems).

Statistical analysis

The number of alleles per locus (N_o), the effective number of alleles (N_e), the allelic frequencies for each locus, the observed heterozygosity (H_o), and the expected heterozygosity (H_e) were calculated using GENALEX, version 6 software (Peakall and Smouse, 2005).

Polymorphic information content (PIC) was calculated using CERVUS, version 3 software (Marshall *et al,* 1998). The Hardy-Weinberg Equilibrium (*HWE*) was estimated using GENEPOP, version 1.2 software (Rousset, 1995).

The likelihood ratio of assigning the correct parentage to a given set of parents with significant confidence was determined using CERVUS, version 3 software (Marshall *et al,* 1998). For each offspring tested, the parentage analysis module calculated likelihood ratio (LOD) scores for each candidate parent, identified the two most likely parents, calculated a corresponding delta score (Δ), as the difference between the LOD scores of the first and second most likely candidate parents, and awarded parentage, with 95% confidence. Three general probability formulae exist for parentage exclusion (PE) (Jamieson and Taylor, 1997): PE1 estimates the probability of exclusion when the genotypes of both parents are known; PE2 estimates the probability of exclusion when the genotype of only one parent is known; and PE3 estimates the probability of excluding two putative parents.

The probability of identity, sibling identity, and combined power of exclusion (CPE) values for the 20 studied loci were calculated according to allele frequencies, using GENALEX, version 6 software (Peakall and Smouse, 2005).

Results

Population structure

The genotypes of 297 individual dromedary camels were successfully generated, using 20 camelid microsatellite markers (Table 1). Fifteen loci were found to be polymorphic, and the remaining five loci (LCA19, LCA24, LCA77, VOLP59, and YWLL36) were monomorphic, among the studied group. Summary statistics for the polymorphic microsatellites are presented in Table 3. The number of alleles per polymorphic locus ranged from 2, in LCA8, LCA65, and YWLL29, to 21, in CVRL01 (Table 3), with a mean of 8.13 per locus. Ne values varied from 1.128, in LCA65, to 7.44, in LCA99, with a mean value of 3.12.

H_o values varied from 0.114 (LCA65) to 0.889 (LCA99), with a mean value of 0.555 for polymorphic loci, whereas H_e values ranged from 0.114 (LCA65) to 0.867 (LCA99), with a mean value of 0.562. PIC values ranged from 0.107 (LCA65) to 0.852 (LCA99), with a mean value of 0.516. Nine microsatellites showed increased degrees of polymorphism compared with the others, including LCA66, LCA99, LGU49, VOLP3, YWLL08, YWLL44, CVRL01, CVRL04, and CVRL05, with PIC values that ranged from 0.520 to 0.852. Four loci, LCA8, LCA37, LCA56, and YWLL29, showed intermediate PIC values (0.241–0.368). Two loci, LCA65 and VOLP32, exhibited low degrees of polymorphism, and their PIC values were 0.107 and 0.225, respectively. Eight of the 15 microsatellite markers, including YWLL44, LCA56, VOLP3, LCA8, LCA37, YWLL29, VOLP32, and LCA65, had one allele with a relatively high frequency (0.6 to 0.94), which affected their polymorphic values, as shown in Table 3.

Furthermore, the combined probability of identity (CPI) values that two randomly chosen animals would show identical genotypes were estimated to be 2.10×10^{-11}, 1.10×10^{-9} and 2.00×10^{-8} for the 15, 9, and 7 loci, respectively (Table 4). In extreme situations, such as when all individuals are full siblings, the CPI values were 4.92×10^{-5}, 3.623×10^{-4}, and 1.369×10^{-3} for the 15, 9, and 7 loci, respectively. Only one locus (LCA56) deviated significantly from the *HWE* ($P < 0.001$), whereas the other loci were consistent with the *HWE*, after Bonferroni corrections. In addition, the null-allele frequency values ranged from -0.039 (LCA66) to 0.1717 (LCA56), with an average value of 0.012 (Table 3).

Parentage analysis

The exclusion probability and the high genetic variability observed among the tested markers demonstrated the efficiency of these markers for use during paternity testing. The PE1 values of the 15 loci

Table 1. Characteristics of the 20 microsatellite loci used in this study.

Locus	Primer sequences (5′ – 3′)	Range[a] (bp)	Accession/ reference
LCA19	F: TAAGTCCAGCCCCACACTCA R: GGTGAAGGGGCTTGATCTTC	75-85	(Penedo *et al*, 1998)
LCA24	F: ACTCACGGGTGACATACAGTG R: GAGCAGTGTTTGGTTTGCATT	99-109	AF060101
YWLL36	F: AGTCTTGGTGTGGTGGTAGAA R: TGCCAGGATACTGACAGTGAT	131-141	(Lang *et al*, 1996)
VOLP59	F: CCTTCCTCAGAATCCGCCACC R: CCCGCGCACCAAGCAG	100–137	(Paredes *et al*, 2014)
YWLL08	F: ATCAAGTTTGAGGTGCTTTCC R: CCATGGCATTGTGTTGAAGAC	127-177	(Lang *et al*, 1996)
LCA8	F: GCTGAACCACAATGCAAAGA R: AATGCAGATGTGCCTCAGTT	228-240	(Penedo *et al*, 1998)
YWLL44	F: CTCAACAATGCTAGACCTTGG R: GAGAACACAGGCTGGTGAATA	85-119	(Lang *et al*, 1996)
LCA37	F: AAACCTAATTACCTCCCCCA R: CCATGTAGTTGCAGGACACG	129-141	AF060105
VOLP3	F: AGACGGTTGGGAAGGTGGTA R: CGACAGCAAGGCACAGGA	141-183	AF305228
YWLL29	F: GAAGGCAGGAGAAAAGGTAG R: CAGAGGCTTAATAACTTGCAG	203-215	(Mehta *et al*, 2007)
LCA77	F:TGTTGACTAGAGCCTTTTCTTCTTT R: GGGCAAGAGAGACTGACTGG	228-238	(Penedo *et al*, 1999)
VOLP32	F: GTGATCGGAATGGCTTGAAA R: CAGCGAGCACCTGAAAGAA	250-266	(Obreque *et al*, 1998)
LCA56	F: ATGGTGTTTACAGGGCGTTG R: GCATTACTGAAAAGCCCAGG	125-139	AF091122
CVLR05	F: CCTTGGACCTCCTTGCTCTG R: GCCACTGGTCCCTGTCATT	151-183	AF217605
LCA66	F: GTGCAGCGTCCAAATAGTCA R: CCAGCATCGTCCAGTATTCA	231-255	(Penedo *et al*, 1998)
LCA65	F: TTTTTCCCCTGTGGTTGAAT R: AACTCAGCTGTTGTCAGGGG	160-172	AF091124
CVRL04	F: CCCTACCTCTGGACTTTG R: CCTTTTTGGGTATTTTCAG	152-180	AF217604
CVRL01	F: GAAGAGGTTGGGGCACTAC R: CAGGCAGATATCCATTGAA	198-252	AF217601
LGU49	F: TCTAGGTCCATCCCTGTTGC R: GTGCTGGAATAGTGCCCAGT	214-262	(Sarno *et al*, 2000)
LCA99	F: CAGGTATCAGGAGACGGGCT R: AGCATTTATCAAGGAACACCAGC	232-334	(La Manna *et al*, 2011)

[a] Ranges differ from the original publications as per this study.

Table 2. Parentage parameters used in CERVUS parentage analysis, following allele frequency estimations and simulations.

Parameter	Value
Percentage of candidate parents typed	100%
Percentage of loci typed	100%
Error rate in likelihood calculations	0 and 1%
Number of tests performed	10000
Strict confidence level of parentage assignment	95%
Relaxed confidence level of parentage assignment	80%

Table 3. Polymorphism statistics of microsatellite loci.

Locus	N_o	N_e	FNA	H_o	H_e	PIC	PE1	PE2	F(Null)	HW
LCA08	2	1.852	0.641	0.468	0.461	0.354	0.106	0.177	-0.0086	NS
LCA37	3	1.505	0.793	0.323	0.336	0.29	0.056	0.152	0.0216	NS
LCA56	3	1.904	0.623	0.337	0.476	0.368	0.113	0.188	0.1717	***
LCA65	2	1.128	0.939	0.114	0.114	0.107	0.006	0.054	-0.0027	ND
LCA66	7	4.125	0.32	0.818	0.759	0.716	0.349	0.527	-0.0392	NS
YWLL29	2	1.389	0.832	0.269	0.28	0.241	0.039	0.12	0.0194	NS
YWLL44	8	2.331	0.601	0.525	0.572	0.52	0.172	0.328	0.0396	NS
LCA99	17	7.435	0.229	0.889	0.867	0.852	0.578	0.734	-0.0138	NS
LGU49	13	4.75	0.268	0.795	0.791	0.758	0.413	0.59	-0.0034	NS
VOLP3	10	2.421	0.616	0.579	0.588	0.561	0.206	0.388	0.0074	NS
VOLP32	3	1.347	0.848	0.263	0.258	0.225	0.033	0.113	-0.0103	NS
YWLL08	16	5.266	0.303	0.828	0.811	0.787	0.464	0.638	-0.0119	NS
CVRL01	21	5.661	0.35	0.838	0.825	0.808	0.505	0.675	-0.0107	NS
CVRL04	4	2.595	0.455	0.613	0.616	0.535	0.191	0.326	0.0038	NS
CVRL05	11	3.088	0.46	0.66	0.677	0.624	0.262	0.429	0.0096	NS
Mean±SD	8.13±6.278	3.12±1.89	0.552±0.23	0.555±0.251	0.562±0.235	0.516±0.241	0.233±0.187	0.363±0.227	0.0115±0.048	

Table 4. The cumulative exclusion probability and Multi-locus probability of identity estimations for 3 tested combination of markers.

	Excluded markers	Cumulative probability of exclusion			Overall probability of identity	
		PE1	PE2	PE3	PI	PI-Sib
15 marker set	None	0.98838	0.99959	0.99999	2.10E-11	4.92E-05
9 marker set	LCA08, LCA65, YWLL29, LCA37, LCA56, VOLP32	0.98319	0.99904	0.99999	1.10E-09	3.62E-04
7 marker set	LCA08, LCA65, YWLL29, LCA37, LCA56, VOLP32, YWLL44, VOLP3	0.97443	0.9977	0.99997	2.00E-08	1.37E-03

ranged from 0.006 (LCA65) to 0.578 (LCA99), with an average value of 0.233 (Table 3). The mean value for PE2 was 0.363 for 15 loci, and these values ranged from 0.054 (LCA65) to 0.734 (LCA99). PE1 was larger than PE2, likely because extra maternal information facilitates the exclusion of false sires. Fig 1 shows how PE values change as the number of loci increases. A very high PE level can be achieved using 7–9 of the 15 loci. The cumulative values for PE2 and PE3 were higher than 0.999, regardless of whether all 15 loci or only 9 loci were considered (Table 4). When using the 7-marker set of loci, the cumulative PE2 and PE3 values declined to 0.9977 and 0.99997, respectively. However, in cases where the genotypes of both parents were known (PE1), the use of 15 loci showed a relatively increased cumulative PE value (0.988) than that observed for the combination of both the nine- (0.983) and seven-marker sets (0.974).

CERVUS was used to estimate the success rate of parentage assignments at both a strict confidence level (95%) and a relaxed confidence level (80%) (Table 2). Parentage analyses across the 15

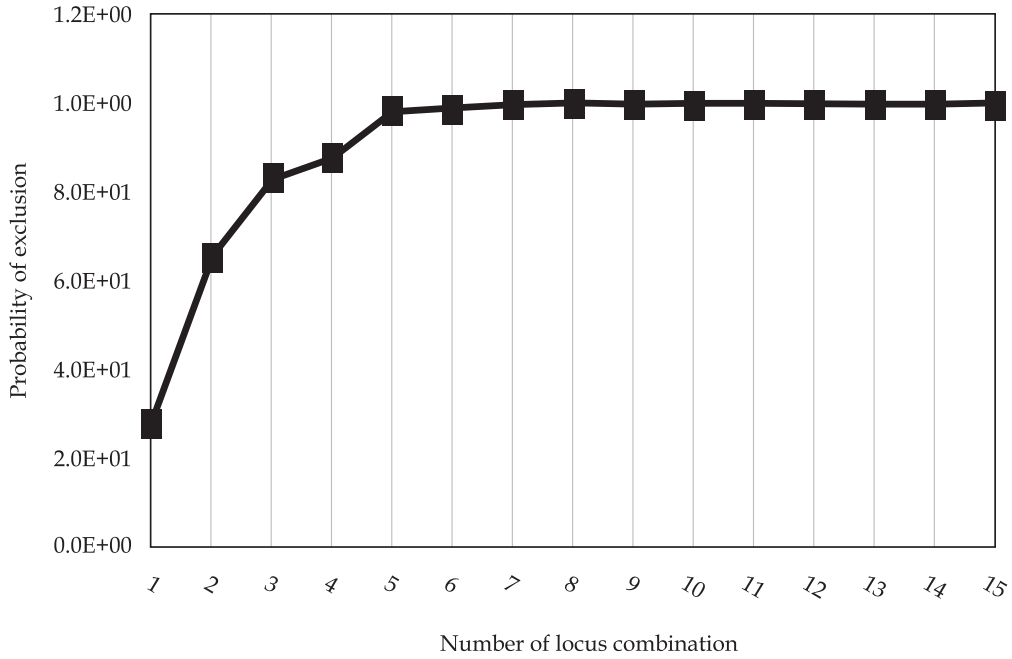

Fig 1. Probability of exclusion as a function of numbers of 15 microsatellite loci for the twenty seven bull camel families.

microsatellite markers depended on LOD and delta scores.

For maternity, the LOD values for microsatellite pairs ranged from 0.243 to 14.47, whereas the delta values for microsatellite pairs ranged from 0.243 to 14.47, which revealed the correct assignment of mothers. For paternity, the LOD values for microsatellite pairs ranged from 0.522 to 14.1, whereas the delta values for microsatellite pairs ranged from 0.522 to 14.1, denoting the correct assignment of offspring to their fathers. Moreover, the combined non-exclusion probability for a parent pair approached zero (0.0000017), providing a reliable method for correctly matching offspring with their sires and dams. However, a mismatch between 10 young calves and their candidate sires occurred during this study, likely due to mutations or null alleles in the LCA56 locus. In addition, seven offspring failed at the same loci as their corresponding candidate dams.

Discussion

DNA profiling facilitates the individual identification, parentage testing, and verification of genetic relationships among animals, helping breeders achieve their breeding goals. The parentage testing of racing camels can, therefore, enhance the efficiency of a selective breeding program.

As measures of polymorphisms, the mean number of alleles and the H_e, and PIC values were extensively studied. In the current study, which examined the Qatari racing camel population, the mean number of alleles (8.13) was comparatively higher than those reported for Omani (5.4), Pakistani (3.9) and Majaheim (7.3) dromedaries, similar to the results previously reported by Hashim *et al* (2014) for a small population containing different subtypes of Qatari camels. The mean H_o value for the Qatari dromedary population (0.555) was higher than those for Australian (0.45) (Spencer and Woolnough, 2010) and Tunisian (0.46) (Ahmed *et al,* 2010) dromedaries, and lower than those reported for Saudi Arabian (0.66) (Mahmoud *et al,* 2012) and Iranian (0.74) dromedaries (Hedayat-Evrigh *et al,* 2018) and Bactrian camels (0.676) (Ming *et al,* 2019).

The estimated mean H_e (0.562) value for the current population was lower than those reported for Sudanese (0.68) and South African camels (0.608) (Nolte *et al,* 2005). However, Kenyan (0.538) and Australian (0.544) dromedaries, studied by Spencer and Woolnough (2010), were found to have similar results. The average PIC value across the 15 loci was nearly equivalent to that reported for dromedary racing camels among 17 microsatellite loci, which was investigated by Spencer *et al* (2010).

In contrast, LCA19, LCA24, LCA77, VOLP59, and YWLL36 showed no allelic variations, similar to the results reported for Indian camel breeds, by Mehta (2014), and for a small population of dromedary

racing camels (Sasse *et al,* 2000). The monomorphic pattern observed for these 5 microsatellite loci may be attributed to the presence of higher degrees of genetic homozygosity among the studied dromedaries in Qatar. These monomorphic alleles behaved differently in Southern African (Nolte *et al,* 2005) and Australian camels (Spencer and Woolnough, 2010), where LCA77 was found to have 15 and 6 alleles, respectively.

To our knowledge, this is the first report examining the genetic indices associated with LCA99 and LGU49 among dromedary camels. The LCA99 locus produced 17 alleles, with a H_e value of 0.852, in Qatari dromedaries, whereas in Alpaca, this locus produced 11 alleles, with sizes ranging from 263-297 bp and a H_e value of 0.75 (La Manna *et al,* 2011). However, for the LGU49 marker, the N_o, H_o and H_e values in Alpaca (Sarno *et al,* 2000) were 9, 0.9 and 0.86, respectively, compared with the values found in the current study for dromedary camels, which were 13, 0.795 and 0.791, respectively. These high polymorphic indices indicated that this locus may serve as an effective marker for parentage verification in dromedary camels.

The number of detectable alleles, the number of efficient alleles, the heterozygosity and the PIC not only affect the paternity confirmation system but also affect the exclusion probability. The CPE values for our set of 15 microsatellite loci, in cases where one putative parent was excluded (0.9996), were higher than those reported for Emirati (0.9961), Australian (0.9962) and African (0.9975) dromedaries using 17 microsatellite loci, as reported by Spencer *et al* (2010). The cumulative probability for reported in that study for excluding a parent pair (0.99999) was similar to that reported by Spencer *et al* (2010).

Moreover, the 15 examined polymorphic loci conformed with *HWE* expectations, except for LCA56 (Table 3), which may be due to genotyping errors and reduced heterozygosity. The frequency of null alleles was relatively low among all loci, except for LCA56. Hence, LCA56 should be excluded from parentage verification in closely related, small populations, as suggested by Marshall *et al* (1998).

The H_o, H_e, and PIC values are inversely affected by allele frequencies larger than 0.5, according to Marshall *et al* (1998). Although YWLL44 and VOLP3 each showed one allele with a frequency exceeding 0.5, they still exhibited high degrees of polymorphism. The CPE1 and CPE2 values decreased by approximately 0.8% and 0.2%, respectively, when these two markers were excluded from the panel.

Recently, Ming *et al* (2019) identify a set of 14 polymorphic microsatellites in Bactrian camels, with an exclusion probability of 0.9999. In addition, Nouaïria *et al* (2018) stated that 12 microsatellite loci were necessary to achieve maximum exclusion in dromedary camels; however, this study found that fewer loci can provide a relatively high exclusion power. Six markers, LCA08, LCA65, YWLL29, LCA37, LCA56, and VOLP32, can easily be excluded from parentage testing, without significant reductions in exclusion power. High numbers of alleles and PIC values were observed for 9 loci, LCA66, LCA99, LGU49, VOLP3, YWLL08, YWLL44, CVRL01, CVRL04, and CVRL05, indicating that these were the most informative markers among the tested loci in this study. The use of an increasing number of loci consequently increased PE values and decreased probability of identity (PI) values. The maximum value obtained when using all 15 STRs, thus, represents an effective tool for confirming lineage.

In summary, the LCA56 locus should be interpreted with caution and should be analysed in future studies among different populations to determine whether relationships exist between this locus and any apparent traits or whether this locus is especially prone to genotyping errors. Due to the high PIC and allele frequency distribution values observed, the use of 9 loci (LCA66, LCA99, LGU49, YWLL08, YWLL44, VOLP3, CVRL01, CVRL04, and CVRL05) out of 20 markers resulted in a high degree of precision for individual identification and paternity assignment among dromedary camels within the studied population. All offspring from the 27 sire families were successfully assigned to the correct sires, dams, and parent pairs. For closely related families, and in cases where one putative parent has been excluded, increasing the number of microsatellite loci to more than the 20 ISAG-recommended loci may be necessary to increase the reliability of parentage assignment. The microsatellite analysis described in the present paper can be an efficient tool for constructing accurate breeding programs and determining the genetic merit of camel populations.

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