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# Analysis of the Efficiency for the Pig-Tailing Technique to Dissect the Molecular Parameters Based on Microsatellite Markers: Camels (*Camelus dromedaries*) as Model

Research Article

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## ABSTRACT

Microsatellite markers historically have been one of the most popular tools for assessing the genetic diversity of livestock due to their nature and polymorphism with their motifs. A challenging problem that arises in this domain is the occurrence of errors in the genotyping process. Additionally, errors in genotyping and allele size determination occur when the genotype determined by the molecular technique does not match the actual genotype in the individual's genome. Furthermore, errors have a direct impact on the ability to differentiate between individuals in a population, and incorrect conclusions and mislead breeders in decisions. With this motivation, the present report aims to evaluate the efficiency of the pig-tailing technique on the parameters of molecular differentiation based on microsatellites in a population of camels. For this purpose, a total of 20 unrelated camel individuals from different regions of Yazd province were collected, and then genomic DNA was extracted using routine salting out methods. Then, five microsatellite polymorphic sites were selected according to Food and Agriculture Organization (FAO), and two groups of common primers and pig-tailed primers (GTTTCTT nucleotide sequence added to 5' reverse primer) were fluorescently candidates for in-population differentiation and diversity. Clustering and principal component analysis (PCA) was performed using the R Package (version 4.2.0), MEGA, and PRCOMP package under the R environment. Interestingly, the results of genotyping showed that the pig-tailing technique tends to reduce the false band patterns (stutter) and also significantly reduce the chance of error in di-motif microsatellite type. On this basis, we can conclude that the pig-tailing strategy in microsatellite genotyping in camel's genetic diversity, seems to be a suitable method.

KEY WORDS alleles, camel, genetic diversity, pig-tailing, polymorphism.

## INTRODUCTION

Over the past century, Camelids have become a primary livelihood, a unique organism, and one of the most diverse mammalian species and highlighted as one of the most important genetic resources for countries experiencing arid, extreme environments, and high altitudes (Guliye *et al.* 2007). *Camelus dromedaries* or Arabic camel is a species

of camel that lives in North Africa and the Middle East (Hassanin *et al.* 2012; Proskuryakova *et al.* 2017).

Iran is a country with many deserts and currently is experiencing moderate drought conditions this factor is forcing farmers off their land to seek work in the cities (Ghasemi Meymandi *et al.* 2015) and therefore camel is a candidate livestock plays a vital role to improve the economic status and food security of villages taking place of arid and semi-arid areas and reducing the immigration rate of people to modern cities. Current report statistics of FAO showed the existence of 138,659 camels in Iran (Guerouali and Acharbane, 2004).

The main challenge faced by many researchers in the area is that genetic conservation issues for Camelids should be based on accurate knowledge of the genetic resources of specific breeds. There is evidence that knowledge about the population genetic structure is the primary step in conservation plays a crucial role in regulating the survivability of camel populations (Rout *et al.* 2008; Almathen *et al.* 2016). DNA markers that have high polymorphism, matching diversity, co-dominant inheritance, and invariable dispersion at the genome level can be a good candidate and a tool for genetic diversity programs (Mohandesan *et al.* 2017). One of these main markers is microsatellite markers. Microsatellites are specific, sequential nucleotide sequences with a pattern and motif. The variation of these motifs is from one to six. These markers are polymorphic in length in several eukaryotic genomes (Guerouali and Acharbane, 2004; Hale *et al.* 2012). The frequency of their occurrence (once every 50000-60000 bp), the high degree of polymorphism displayed, and their random distribution in the genome (Mburu *et al.* 2003; Schulz *et al.* 2005) make them potentially very useful as DNA markers in gene mapping studies. In addition, several microsatellites may be analyzed in genetic studies simultaneously (Hedayat-Evrigh *et al.* 2018). A notable feature is that polymorphisms can be described numerically and, therefore, the system tends to manage and analyze computer data. Uses of these markers in plants and livestock populations include population structure analysis, dispersion patterns, estimation of genetic diversity, inbreeding, paternal evaluation, pedigree, tracking alleles through a population and individual identity, and estimating the degree of dependence (Mahmoud *et al.* 2012). Estimation of genetic parameters is the most significant step toward managing genetic resources for conservation (Geng *et al.* 2003). The combination of inbreeding and loss of genetic diversity is a matter of animal conservation (Musthafa, 2015). Inbreeding increases the likelihood of substitution of a rare recessive genetic disease and decreases population adaptation. Populations with superior genetic diversity have more opportunities of surviving under new environmental pressures (Cherifi *et al.* 2017). Therefore, identifying the genomic characteristics of an animal species is essential for managing genetic resources in farm animals. Despite the significant advantages of microsatellite markers, there are disadvantages that, if not examined by researchers in their experimental design, can result in inaccurate results concerning demographic molecular parameters and practically interferes with the right decision-making (Arthofer *et al.* 2018). One of the major disadvantages of microsatellite markers is the production of stutter (artifact none specific amplicons).

The pig-tailing technique applied on specific microsatellite primers to solve the problem of stutter in microsatellites is an efficient method for removing the target and non-specific bands due to the addition of nucleotide A by the Taq enzyme (Ballard *et al.* 2002). It is more common in genotyping and electrophoresis and is applied by the capillary method in which a GTTCTT nucleotide sequence is added to the end of the primer (Brownlow *et al.* 2008). In this alternative method and strategy, GTTCTT is added to the end of the non-fluorescent primer and the second primer is colored to reproduce that position with fluorescent (Bouvet *et al.* 2020). As a result, in the pig band pattern of the sequestering device, the noise caused by the stutter and the non-uniform addition of adenine A nucleotide is reduced to a minimum due to enzyme function. Finally, the user will be able to accurately identify the true allele and heterozygous and homozygous genotype. The GTTTCTT sequence is added at the end of the 5 prime primers in the pig-tailing technique applied to specific microsatellite primers. One of the drawbacks related to parts amplified with fluorescent primers separated by horizontal or capillary electrophoresis is the formation of the double peak pattern due to the addition of adenine nucleotide (A) to a non-patterned and specific fragment resulting from the Tag enzyme (Shirk *et al.* 2013). This unusual structure is directly related to the nucleoside sequence in the reverse 5 prime of the primer (Ballard *et al.* 2002). The appearance of two bands or two peaks in the outputs is due to heterozygosity or a difference in the number of repetitions of the motif. The stutter band due to the similarity of the pattern to this state causes the pattern not to be read correctly (Shirk *et al.* 2013).

Approximately a quarter of markers with their dinucleotide motif are repeated and have a 30-65% addition of adenine nucleotide (A) to the end of a pseudo-non-target fragment. Finally, it shows the development of a potential source of genotypic error (Bouvet *et al.* 2020). Estimates show that about 50% of a challenge is created for scoring in microsatellite bands. Tetra motif-type microsatellite markers are also subjected to genotype error. They can also select any binary peak during these algorithms, which causes an incorrect judgment about the actual allele size and geno-

type (Ballard *et al.* 2002). There are persuasive strategies for designing non-specific starters and bands in terms of reproduction. First, increase the concentration of magnesium ions and reduce the bond temperature and increase the amplification cycle by a few more cycles and؛ Second, reduce the degree of reaction or stringency (i.e., some strategies that can increase the degree of reaction difficulty) like Hot-start PCR, it can help reduce starter and dimer and non-specific bands in PCR. Stringency is a specific term used to describe the hybridization of a target pattern primer that provides the ability to develop a non-specific band or pseudo product (Shirk *et al.* 2013).

The second strategy is to apply an enzyme with 3-exonuclease activity after Taq DNA polymerase as an additional manipulation after PCR. This method is successful, however, high cost and increased analysis time are disadvantages. The amount of enzyme should be carefully titrated for each marker to prevent degradation of the target product. Stringency-polymerase chain reaction is required under specified conditions of temperature, pH, salt concentration, etc. During nucleic acid hybridization or rebounding, the high degree of stringency reaction conditions requires pairing all bases of one polynucleotide with complementary bases on another strand (Brownlow *et al.* 2008). The low degree of stringency reaction conditions prevents some bases from pairing. A review of previous studies shows that the application of pig-tailing in the synthesis of microsatellite primers is an effective solution. It significantly reduces the error caused by genotype determination.

This study aimed to evaluate the efficiency of the Pig-tailing technique on the parameters of molecular differentiation based on a microsatellite in a population of camels, *Camelus dromedaries* in Yazd province based on these motivations.

## MATERIALS AND METHODS

### Sampling and DNA extraction

In this study, a total of 20 unrelated camels were collected from different parts of Yazd province. Blood collection and genomic DNA extraction were performed using routine salting out methods. Figure 1 displayed the distribution and geography of the sampled area for testing in this study and also Figure 2 described the phenotype and anatomical shape of a camel in Yazd province.

### A quantitative and qualitative examination of DNA samples

Electrophoresis and spectrophotometry methods were used to determine the quality and quantity of DNA in different stages of the experiment. NanoDrop 1000 (NanoDrop Technologies, Wilmington DE, USA) was used to quantify DNA extracted and its contamination with RNA and protein. Spectrophotometry of the sample was performed by UV irradiation by injecting 2 µL of DNA solution into a special ocular plate. The device reported the amount of light absorption at a distance of 220 to 350 nm of DNA as a graph and the ratio of light absorption at 260 to 280 and 230 wavelengths (indicating the degree of purity and pollution of DNA) and finally the concentration of DNA to ng/µL. Electrophoresis gel was used to evaluate the quality of DNA samples by examining the possibility of DNA damage and controlling its breakage and RNA contamination. This happened by loading 3 µL of DNA sample diluted with distilled water along with 3 µg of colored and weighting buffer in wells with a width of 5 mm in 1.5% agarose gel (Invitrogen California, USA).


**Figure 1** Distribution and geography of the sampled area for testing in this study

**Figure 2** Phenotype and anatomical shape of Arabic camel in Yazd province

### Amplification of microsatellite loci through the polymerase chain reaction

The PCR was implemented with 35 cycles under heat treatment by Touch down method. A German-made thermocycler was used for this purpose. The polymerase chain

reaction was performed in a volume of 50 µL (PCR Master kit of SINAGEN Company with 10 µmol of each specific primer and 50 ng of DNA extracted). Table 1 illustrated the specifications and summary of camel genome-specific microsatellite loci in this study. The GTTTCTT sequence is added at the end of the 5 prime of primer in the pig-tailed technique applied to specific microsatellite primers.

**Electrophoresis of PCR products**

After staining, 5 µL of PCR product was electrophoresed on 6% agarose gel to ensure that the desired segment is amplified and there have been no nonspecific bands. The quality and length of the amplified segment be checked after staining and evaluated by GelDoc (SINAGENE Cambridge, United Kingdom models). Agarose gel and ethidium bromide solution were prepared for staining before electrophoresis of the required solution (buffer X1 and X50) TAE. The electrophoresis tank was filled to a height of 2-3 mm on the gel with TAE buffer after the preparation of 1.5% agarose gel.

The PCR products were mixed in a ratio of 5:1 with a loading buffer made by Fermentas Company and poured into wells. The size indicator (Gene Ruler 100 bp Plus DNA Ladder) of Fermentas Company was used in the first well of each gel. In this study, a voltage of 100 v and a current of 80 mA were used for electrophoresis.

The system was cut off when the loading buffer dye run two-thirds of the gel length after about 40-30 minutes. The gel was placed inside ethidium bromide for staining (0.5 to 1 microgram per ml for 10-20 minutes depending on the percentage of gel and old and fresh color). It was placed on an ultraviolet (UVidoc) device after washing in water and immediately prepared by a specific camera and gel photo printer and the results were evaluated (Brookes *et al.* 2011).

**Fragmentation in sequence finder by capillary electrophoresis**

Different WellRED colors were used (sigma company) for fluorescent forward primers. D2, D3, and D4 were used to maintain equilibrium in the PCR reaction.

It also used 0.2 picomols of forward-labeled fluorescent primer. This act of mixing DNA was used in the dark as fluorescent primers are destroyed and inactivated near room light.

**Data analysis**

Clustering and principal component analysis were performed using POPGENE software, mega (version), and PRCOMP package under R (version 4.2.0).

## RESULTS AND DISCUSSION

Many theoretical studies on microsatellite markers have shown that there are certain unknown problems, the most important of which is the misunderstanding of the actual allele size.

These errors occur due to the slippage phenomenon. DNA polymerase enzyme slips in regions containing motifs and repeats during the replication process (Ballard *et al.* 2002; Brownlow *et al.* 2008). As a result, pseudo products of different sizes are formed, which differ in size by 1 to 5 bp of repetitive nucleotides from the main products (Brownlow *et al.* 2008). Such electrophoretic bands usually have a b and intensity and a lower concentration of the original amplified product that can usually be practically ignored. However, if these pseudo bands overlap with the product of a heterozygous individual then it becomes difficult to distinguish these patterns from real bands and products and pseudo slippage (Shirk *et al.* 2013). Slipping of the polymerase enzyme at the microsatellite loci of the dinucleotide motif often causes the adjacent alleles to overlap on electrophoresis.

The negative consequences are that these errors lead to a false increase in the observation of pseudo homozygotes in the population genetics (Ballard *et al.* 2002). Figure 3 shows the band pattern and observed genotypes of CVRL05 locus amplification without fluorescent and pig tailing. As viewed, non-specific bands are formed and it is not possible to read accurately. The size of the allele in CVRL05 was estimated to be between 140 and 155 bp.

**Table 1** Specifications and summary of camel genome-specific microsatellite loci in this study

| Locus | Motif | Primer sequence | Annealing | Accession | Expected bands |
|---|---|---|---|---|---|
| CVRL01 | (tg)n | GAAGAGGTTGGGGCACTAC<br>CAGGCAGATATCCATTGAA | 55 | AF217601 | 188-253B<br>196-253D |
| CVRL02 | (ta)n | TGTCACAAATGGCAAGAT<br>AGTGTACGTAGCAGCATTATTT | 55 | AF217602 | 206-216B<br>205-216D |
| CVRL05 | (tg)n | CCTTGGACCTCCTTGCTCTG<br>GCCACTGGTCCCTGTCATT | 60 | AF217602 | 148-174B<br>155-176D |
| CVRL06 | (ca)n | TTTTAAAAATTCTGACCAGGAGTCTG<br>CATAATAGCCAAAACATGGAAACAAC | 60 | AF217606 | 185-205B<br>196-203D |
| CVRL07 | (gt)n | AATACCCTAGTTGAAGCTCTGTCCT<br>GAGTGCCTTTATAAATATGGGTCTG | 55 | AF217607 | 255-263B<br>272-306D |

Mariasegaram *et al*. (2002).

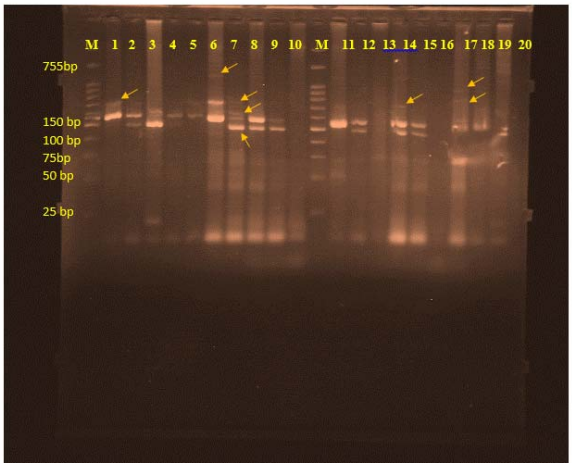


**Figure 3** Band pattern and observed genotypes of CVRL05 locus amplification without fluorescent and pig tailing. As viewed, non-specific bands are formed and it is not possible to read accurately. The size of the allele in CVRL05 was estimated to be between 140 and 155 bp

Figure 4 explained the band pattern and genotype of three CVRL05 (120-130) (green CVRL02 (200-210) (black) that had a starter in the upper row A because it is amplified with non-pig tail primers and the bottom row B is amplified by adding GTTTCTT in the pig-tailing technique applied on specific microsatellite primers at the end of 5´of primers. The occurrence of starter and false bands is reduced in the second case and as a result, the genotype is error-free.

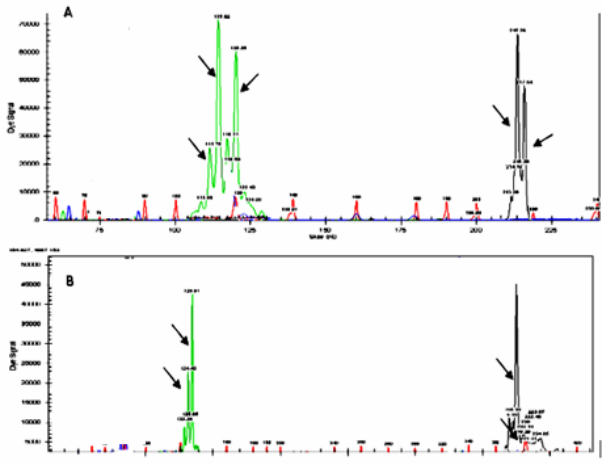


**Figure 4** The band pattern and genotype of three CVRL05 (120-130) (green CVRL02 (200-210) (black) that has a starter in the upper row A because it is amplified with non-pig tail primers and the bottom row B is amplified by adding GTTTCTT in the pig-tailing technique applied on specific microsatellite primers at the end of 5ʹof primers. The occurrence of starter and false bands is reduced in the second case and as a result, the genotype is error-free

One of the necessities of genetic diversity studies in livestock is the accurate determination of alleles to determine the lineage and exact size of alleles related to economic traits, especially the separation of wild and mutant alleles.

The incidence of error increases, usually with increasing fragment length and repetition size of microsatellites. The selection of loci with replication of four nucleotides (tetra motif) is one of the possible methods to eliminate these problems. Determination of alleles at these loci will be easier and less inadequate as the distance between alleles at these sites is greater. Another method is to use internucleotide sites with a band size of 120 bp. Therefore, these pre-selection strategies for loci and primers will increase the accuracy of allele determination. In these loci, the occurrence of errors is reduced and the small size of the alleles makes them easier to separate physically in the electrophoresis process. In addition, variability in microsatellite loci with the least genotype reading error is required in most genetic applications. Figure 5 illustrated Cluster pattern and dendrogram resulting from genetic differentiation analysis of 5 microsatellite loci in two modes: a normal primer (A) and pig primed tailed. Add GTTTCTT on the end of 5 primers in the pig-tailing technique applied to specific microsatellite primers.

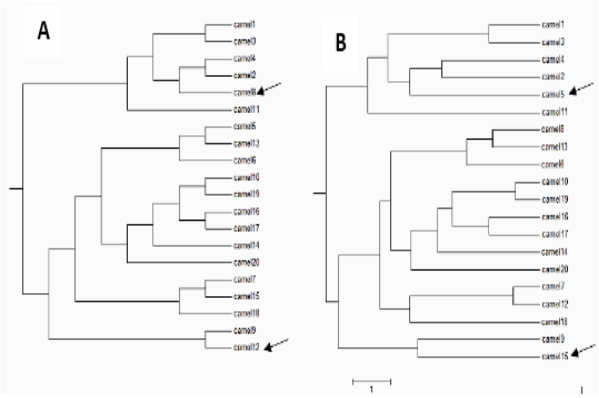


**Figure 5** Cluster pattern and dendrogram resulting from genetic differentiation analysis of 5 microsatellite loci in two modes: a normal primer (A) and pig primed tailed. Add GTTTCTT on the end of 5 primers in the pig-tailing technique applied to specific microsatellite primers. As viewed, due to the difference in raw data and the elimination of genotype errors in comparing the two outputs of some individuals in terms of allele similarity or genotype, their rank and position change, and sometimes the answer is different

The raw results of genotyping in the two primer groups showed that the pig-tailing technique tends to reduce the false band patterns (starter) and reduce the number of bands of more than 2 to the number of one or two peaks. Also, the probability of starter occurrence and error in genotype reading increases, if this technique is not used, especially in a site with a dinucleotide motif thus, causes the error of genetic differentiation outputs. Finally, the use of the pig-tailing technique is appropriate to determine the genotype of microsatellite loci in domestic livestock, including cam-

els. Figure 6 displays the Output view as a result of the Principal Coordinate analysis of variance components obtained from genetic differentiation analysis of 5 microsatellite sites in two typical primer modes.

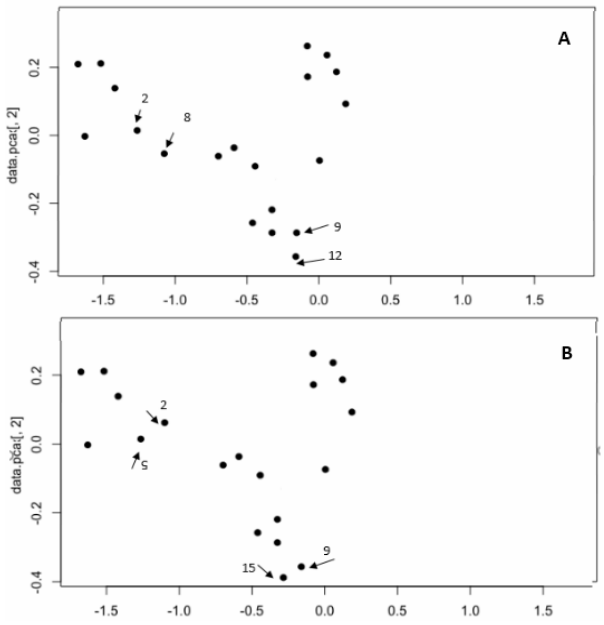


**Figure 6** Output view as a result of analysis of variance components obtained from genetic differentiation analysis of 5 microsatellite sites in two typical primer modes (A) and Pig-tailed primer. Add GTTTCTT on the end of 5of primers in the pig-tailing technique applied to specific microsatellite primers. As viewed, the results of the cluster analysis here are also consistent with the two-dimensional PCA results. Some individuals change in allele similarity or genotype and their position together due to differences in raw data and correction of genotype error in comparing the two outputs and sometimes the answer is different

The limitations of the present studies naturally include a limited type of SSR-specific loci for the study genome behavior of camel and also low sample size and deficiency of different populations for the study of pig tailing strategy on genotyping and estimation of actual allele size per locus. From this standpoint, pig tailing strategy can be considered a proper tool for the minimization of errors that occurs during genotyping process and our results demonstrate that this is necessarily true and subsequent statistical analysis for genetic diversity and QTL mapping substantially depend on the observed true actual size of alleles per loci. To our knowledge, this is the first report on the influence of pig tailing on the estimation of parameters and used camel material as a model. Future investigations are necessary to validate the kinds of conclusions that can be drawn from this study.

## CONCLUSION

The most important step in managing genetic resources for conservation and tolerable use is to estimate genetic parameters. The combination of inbreeding and damage to genetic diversity is a matter of animal conservation. Microsatellite markers are important and useful in assessing the genetic diversity of domestic animals, but they are always prone to errors in genotype determination due to their specific nature and repetitive motifs. Usually, when the genotype determined by the molecular technique does not match the actual genotype in the individual's genome, an error occurs in reading the genotype and determining the size of the allele. Errors have a direct effect on the ability to differentiate between individuals in a population and alter frequency changes. Overestimation of inbreeding distorts population size estimates and affects inter-parental and offspring analyzes, as well as erroneous and false conclusions. The raw results of genotyping in the two primer groups showed that the pig-tailing technique tends to reduce the false band patterns (stutter) and reduce the number of bands more than 2 to the number of one or two peaks. The probability of starter occurrence and error in genotype reading increases if this technique is not used, especially in a position with a dinucleotide motif and genetic differentiation outputs are associated with the error. The error rate increases with increasing fragment length and repetition size of microsatellites. The selection of sites with replication of four nucleotides (tetra motif) is one of the possible methods to solve these problems. Determination of alleles at these loci will be easier and less inadequate as the distance between alleles at these sites is greater. Another method is to use inter-nucleotide sites with a band size of 120 bp. Therefore, these pre-selection strategies for loci and primers will increase the accuracy of allele determination. In these loci, the occurrence of errors is reduced and the small size of the alleles makes them easier to separate physically in the electrophoresis process. In addition, variability in microsatellite loci with the least genotype reading error is required in most genetic applications. Finally, the use of the pig-tailing technique is appropriate to determine the genotype of microsatellite loci in domestic livestock, including camels.

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