Genetic diversity of the Colombian Creole donkey in the Department of Sucre



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Abstract

The objective of this study was to evaluate the genetic diversity of the Colombian Creole donkey in the Department of Sucre using Random Amplified Microsatellites (RAM) molecular markers. In 100 individuals from the five subregions of the department, DNA was extracted and five RAM primers were amplified by PCR. In all, 291 bands were found, on average 11.96±1.45 per primer, the highest value in CCA (18±2.23) and the lowest in TG and GT (8.8±0.44). CA was the most polymorphic primer (88.09±10.91%) with the highest heterozygosity value (He) (0.376±0.021), while the lowest was GT (0.341±0.076 and 0.101±0.040, respectively). Intrapopulation analysis showed an average of 66.50±1.72 bands, of which 89.86±24.04% were polymorphic. The highest number of bands (63±3.84) was found in the Gulf of Morrosquillo (GO) subpopulation, and the lowest in Mojana (MO) (48±2.88); however, the highest value of polymorphic *loci* (81.16%) and He (0.335±0.022) were found in the Montes de María (MM) subpopulation, making it the most diverse. The average genetic diversity for the entire population was 0.351±0.021 bands. The population structure analysis showed a 10% variation between subpopulations, with an F_{ST} value of 0.17±0.01 (P<0.05). Genetic distances between subpopulations showed that MO and GO were the most distant. The RAM markers are effective in assessing the genetic diversity of the Creole donkey, which has high values of genetic diversity, particularly the MM subpopulation. The genetic revealed structure could be the result of natural geographical barriers between the subregions.

Key words: *endangered breeds; genetic diversity; genetic resources*

Introduction

The arrival of the donkey to the American continent dates from the time of colonization, as both the donkey and the horse made the same journey (Jordana et al., 2016). Since then, the Colombian Creole Donkey (CCD) has contributed to the agricultural and social development of several regions in

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Colombia. The CCD is rustic, adapts to climatic conditions, can be fed with forage of low nutritional quality, is resistant to diseases and ectoparasites, does not require special handling practices, has low-cost maintenance, and great strength (20-30 Nw in animals of 80 to 120 kg). In addition, it is very docile. This has made it an ally in the agricultural work of the country's farmers (Silva-Gómez et al., 2017).

Despite the above, and as in other Creole species of Colombia, it is possible that the CCD is at risk of extinction. Between 1995 and 2013, the population census decreased by 80% (MADR, 2014), and the country's current astral inventory is unknown. Two possible reasons that explain this alarming decrease in the number of donkeys are the increase in the use of alternative means of transport, such as motorcycle, and commerce for illegal slaughter to obtain their hide and meat (Silva-Gómez et al., 2017).

The genetic diversity of domestic species is considered an important component of global biodiversity (Boettcher et al., 2010), especially the variability of animal genetic resources. This is considered a key element in all production systems, as it provides the raw material for genetic improvement and adaptation to environmental conditions, with Creole breeds representing a natural and irreplaceable reservoir of genetic variability (Correa et al., 2015). Thus, this knowledge is key to the possible planning of long-term, sustainable conservation strategies (Rosenberg and Jonathan, 2015).

Molecular markers are a tool that allows for the understanding of how the genes of a population are established. The different types of markers used in population studies are distinguished by their ability to detect polymorphisms in single or multiple *loci* and whether they are dominant or codominant (Jordana et al., 2017). Some techniques available to study genetic diversity at the DNA level include RAPD (Random Amplified Polymorphic DNA) randomized amplified polymorphism, AFLP (amplified fragment length polymorphism) length polymorphism of amplified fragments, microsatellites, and random amplified microsatellites RAM (Random Amplified Microsatellites) (Muñoz et al., 2008).

RAM is a dominant type marker, using primers with an approximate length of 18 base pairs that include a degenerate 5` end of three nucleotides, which ensures the anchoring of the primer to the microsatellite and a repeated motif of between two and three nucleotides. RAM is based on the polymerase chain reaction (PCR), the method is widely reproducible and allows for the detection of intra and inter specific DNA polymorphisms (Muñoz et al., 2008). RAM has been used to evaluate the genetic diversity in pigs (Oslinger et al., 2008), cattle (Piedrahita et al., 2005, 2008) fish (Caetano et al., 2012; Hernández et al., 2017), ducks (Hernández et al., 2007), plants (Muñoz et al., 2008) and fungi (Hantula et al., 1996).

Microsatellites have been widely used as molecular markers in population genetics studies since they allow for estimation of the levels of genetic variability within populations. This is neutral, by virtue of its high degree of polymorphism, and in most cases its evolutionary pattern, (Patiño-Montoya and Giraldo, 2017).

Studies of genetic diversity in the CCD are limited. In 30 animals from southwest Colombia, moderate genetic diversity was examined using microsatellites (He = 0.563) with an absence of Hardy-Weinberg equilibrium (F_{IS}=0.071, P<0.05) (Jordana et al., 2016). There are no reports for the Colombian Caribbean region or the use of RAM markers in this species. Therefore, the objective of this study was to evaluate the genetic diversity of CCD

in the Department of Sucre using RAM molecular markers.

Materials and methods

Populations, blood collection and DNA extraction

Peripheral blood samples were taken in tubes with anticoagulant (EDTA 7.2 mg) from a total of 100 individuals, *i.e.* 20 individuals from each of the subregions: Montes de María (MM), Sabanas (SA), Gulf of Morrosquillo (GO), San Jorge (SJ) and Mojana (MO). The inclusion criteria of animals were: older than 2 years of age, clinically healthy, lower degree of kinship and possessing the morphological characteristics of Creole donkeys (Jiménez et al., 2016). All procedures for sample collection, handling and conservation, ethical, technical, scientific and administrative standards for research in animals contained in Law 84 (National Congress of Colombia, 1989) were followed. From the blood sample, the DNA was extracted using the QIAamp DNA Mini Kit 250 extraction kit, according to the manufacturer's instructions. The DNA was quantitatively and qualitatively quantified using the NanoDrop 2000TM (Thermo Fisher Scientific) spectrophotometer and diluted to 10 ng/μL.

Table 1. Primer sequences used (Hernández et al., 2017)

Primer	Sequence 5`-3`	ng/DNA
CCA	*DDB(CCA)$_5$	20
GT	VHV(GT)$_7$G	30
AG	HBB(AG)$_7$[a]	20
TG	VHV(TG)$_7$T	20
CA	DBDA(CA)$_7$	25

*Designation of degenerated sites: H (A o T o C), B (G o T o C), V (G o A o C) y D (G o A o T).

Amplification and genotyping of RAM markers

Five of seven RAM markers described in Table 1 were amplified, and the remaining two primers, CGA and CT, were discarded because they were monomorphic. Amplification reactions were performed in a final volume of 12.5 μL containing between 20 and 40 ng/μL DNA, 250 mM of each primer and 1X MangoMixTM super mix (Bioline©). The amplifications were performed in an Eppendorf® MasterCycler Nexus Gradient thermal cycler, the amplification programs are detailed in Table 2.

Table 2. The amplification programs used

Steps		Temperature (ºC)	Time
Initial denaturation		95	5 minutes
Amplification cycles: 37			
Denaturation		95	30 seconds
Hybridization	CA	54	45 seconds
	AG	50	
	CCA		
	TG		
	GT		
Extension		72	2 minutes
Final extension		72	7 minutes

The PCR products were mixed in a 3:1 ratio with EZ-VISION I DNA dye (ambresco®) as a loading buffer and subjected to vertical electrophoresis in gels (170 x 86 x 1.2 mm) of 8% polyacrylamide (37:1 acrylamide: 38% bisacrylamide), for 60 minutes at 160 volts. The gels were photographed under ultraviolet light on a Benchtop UV Transilluminators: Single UV variable intensity UV transilluminator. A 100 bp molecular weight marker (GelPilot 100 bp Plus Ladder, Quiagen®) was used as a guide for reading the bands.

Data analysis

Statistical analyses had two aims, first to know the effectiveness of the RAM technique in assessing the genetic diversity of the CCD, and second to know the diversity and genetic structure of the subpopulations and the entire population. For this purpose, the gels were read and transformed into a binary matrix (1=presence, 0=absence of band). In each analysis, the number of *loci*, percentage of polymorphic *loci*, number of *loci* with few frequent alleles (<1%) and the expected heterozygosity (He) were determined, while molecular variance between the subpopulations to estimate F_{ST} was analysed using the GenAlex program ver. 6.5 (Peakall and Smouse, 2012). Nei genetic distances calculated between subpopulations were plotted with the UPGMA method using the MEGA 7 program (Kumar et al., 2016).

Results

The effectiveness of the RAM technique in assessing the genetic diversity of the CCD is presented in Table 3. All RAM primers generated a total of 69 bands. The average number of *loci* found was 11.96±1.45 with the highest value in the CCA primer and the lowest in TG and GT. Of the *loci*, 70.24±11.18% were polymorphic. This value was maximized in the CA primer and had the lowest value in the GT primer. Only in this last primer were *loci* found with frequencies below 1%. The CA and GT primers presented the highest and lowest value of He, respectively. The average value of He of genetic diversity for all primers was 0.278±0.0376.

At the subpopulation level, 291 *loci* were found, on average 58.20±6.01 *loci* per subpopulation, with the highest number of bands in the GO subpopulation and the lowest in MO (Table 4). In all, 89.86±24.04% of *loci* were polymorphic, where the MM subpopulation presented the highest value for this indicator of genetic diversity, followed by SA, GO and SJ. The MO subpopulation presented only 24.64±5.26% of polymorphic *loci*. In three of the five subpopulations evaluated, no rare *loci* were found, these were found only in the subpopulations GO and SJ, corresponding to 1.03% of the total *loci* found. The estimated average He for the entire population was 0.351±0.021, where the highest value (0.335±0.022)

Table 3. Indices of genetic diversity for primers in CCD of Sucre (Mean ± SE)

Primer	No. *loci*	%polymorphic *loci*	Uncommon *loci*	He
CA	10.4 ± 1.34	88.09 ± 10.91%	0	0.376 ± 0.021
AG	12.2 ±1.09	67.69 ± 5.65%	0	0.273 ± 0.057
CCA	18 ± 2.23	76.84 ± 19.31%	0	0.305 ± 0.022
GT	8.8 ± 2.16	34.12 ± 7.06%	3	0.101 ± 0.040
TG	8.8 ± 0.447	84.44 ± 12.96%	0	0.335 ± 0.0482
Mean	11.96 ± 1.45	70.24 ± 11.18%	0	0.278 ± 0.0376

was found in the MM subpopulation and the lowest value (0.102±0.022) in the MO subpopulation. In the other three subpopulations, the values varied between 0.276 and 0.312.

The analysis of molecular variance showed a between subpopulation variation of 10% and within subpopulation variation of 90%, obtaining an F_{ST} value of 0.17 ± 0.01 (P<0.05). The genetic distances between subpopulations showed that the greatest genetic distance was between the MO subpopulation and the other subpopulations, with a higher value between MO-GO. Likewise, the lowest distance was found between SA and MM, the other distance values varied between 0.0916 and 0.1594 (Table 5). Figure 1 shows a dendrogram of genetic distance between subpopulations based on the UPGMA method.

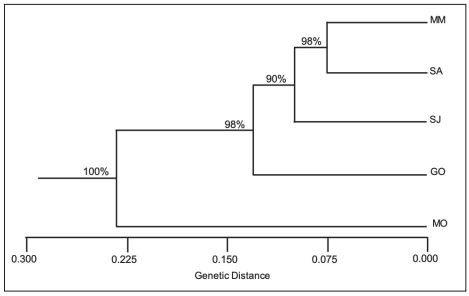


Figure 1. Dendrogram of genetic distances of Nei between subpopulations of CCD made from the UPGMA method (bootstrapping values appear in the nodes)

Discussion

There are no reports of the use of dominant markers for the study of genetic diversity in any donkey race in the world, making this the first report. RAMs have been developed to assess genetic diversity in fungi (Hantula et al., 1996); however, their use has since been extended to animal and plant species (Muñoz et al., 2008).

In cattle, CGA and CT markers were found to be monomorphic, supporting the data presented here, and the CCA, GT, AG, TG and CA markers generated

Table 4. Indices of genetic diversity for each subpopulation and for the CCD of Sucre (Mean ± SE)

Population	No. *loci*	%polymorphic *loci*	Uncommon *loci*	He
MM	62 ± 3.97	81.16 ± 12.16%	0	0.335 ± 0.022
SA	58 ± 4.87	78.26 ± 12.18%	0	0.312 ± 0.023
GO	63 ± 3.84	76.81 ± 12.17%	2	0.296 ± 0.024
SJ	60 ± 4.34	76.81 ± 12.17%	1	0.276 ± 0.023
MO	48 ± 2.88	24.64 ± 5.26%	0	0.102 ± 0.022
CCD	291 ± 3.91	89.86 ± 24.04%	3	0.351 ± 0.021

Table 5. Genetic distances of Nei among the CCD subpopulations of Sucre

Subpopulation	MM	SA	GO	SJ	MO
MM	-----				
SA	0.0745	-----			
GO	0.0916	0.1367	-----		
SJ	0.0991	0.0980	0.1594	-----	
MO	0.2306	0.2409	0.2698	0.1782	-----

52 bands (Piedrahita et al., 2005, 2008). In pigs, 46 *loci* were found using the primers CCA, CT and CGA, and the markers AG and CA were reported as monomorphic (Oslinger et al., 2006). In the fish bocachico (*Prochilodus magdalenae*), the seven markers described here were polymorphic, reporting 106 bands, where the CCA marker was the largest contributor to this indicator (Hernández et al., 2017), which is consistent with our results. In Creole ducks (*Cairina moschata*), only three primers are reported as polymorphic (CA, CCA and CGA), but with 116 *loci* found a higher value than presented here (Hernández et al., 2007).

The percentage of polymorphic *loci* found in the bocachico (95.40%) was higher than that found in the CCD. In the bocachico, the AG marker presented the highest value (99.17%) of the percentage of polymorphic *loci*, while CA had the lowest (93.33%) (Hernández et al., 2017). Likewise, the percentage of polymorphic *loci* found in the Creole duck (61.20%) was lower than reported here, and the CA marker (76.19%) gave the greatest contribution to this indicator, while the CCA marker (50%) contributed less (Hernández et al., 2007). Our results agree with the latter study, in which the CA marker had the highest value.

The expected heterozygosity value (He) can be considered the best indicator of genetic diversity (Rincon et al., 2013). In this regard, the highest value in cattle was found with the GT primer (0.35) and the lowest with CA (0.20) (Piedrahita et al., 2005, 2008). The latter differs from what is presented here because in the studied population of asses, the GT marker had the lowest value of He. In pigs, the highest value of He was for CCA (0.22±0.07) and the lowest for CT (0.19±0.07) (Oslinger et al., 2006), while in the present study, CT was monomorphic. In the bocachico marker, AG presented the highest He (0.469±0.005) and CCA the lowest value (0.321±0.01) (Hernández et al., 2017). The CGA marker in Creole ducks had the highest value of He (0.23) and the lowest was found in CCA (0.16) (Hernández et al., 2007), while CGA was monomorphic in the studied population of Creole donkeys.

The value of genetic diversity in this study was higher than those reported in studies using RAMs in different species such as in pigs (Oslinger et al., 2006), cattle (Piedrahita et al., 2005, 2008), birds (Hernández et al., 2007) or fish (Caetano et al., 2012). The only value that was above that reported here was that of the fish *Prochilodus magdalenae* with He=0.394 (Hernández et al., 2017).

The values found suggest that the Montes de María subpopulation (MM) has the greatest genetic diversity, while the Mojana subpopulation (MO) has the lowest genetic diversity. In the MM subpopulation, this high diversity may be due to the level of use of the donkey, its geographical location and the location of the subregion in the Department of Sucre. The MM subpopulation is characterized by their agricultural vocation, it is here that the producers use the Creole donkey as a means of transport and cargo for products, food and supplies. As for its geography, this subregion is mountainous and it is located in the centre of the Department of Sucre, allowing for interconnection with the most important cities of the department, sub-regions and other departments. This justifies a large inventory of animals and their wide movement, with higher frequencies of crossings between individuals from different regions, and thus the high values of genetic diversity would be expected. On the other hand, the MO subregion is characterized by producing rice on an industrialized scale and fishing. These economic activities mean that the level of use of the Creole donkey in this area is lower and accordingly, there is also a smaller population of donkeys. As for geography, it is a subregion dominated

by marshes, flooded forests, and beaches, thereby reducing gene flow, which would explain the low genetic diversity of the MO subpopulation. This result is consistent with that reported by Jordana et al. (2016) who show greater genetic diversity in asses of the Caribbean compared to those in the southern United States. The geographical location of Colombia within the continent makes it a possible point of genetic convergence, which would explain its high genetic diversity (Boettcher et al., 2010).

Other studies of genetic diversity of donkeys in breeds of the world have been based on the use of microsatellite-type codominant molecular markers (Aranguren et al., 2001; Jordana et al., 2001; Ivankovic et al., 2002; Jordana et al., 2016; Lara et al., 2015). Although microsatellites and RAMs are not methodologically comparable, since the latter is the dominant technique, the above authors reported He values ranging from 0.712 in the Catalan race of Spain to 0.452 in the Creole race of Uruguay.

The only report for Colombian donkeys (Jordana et al., 2016) reports a value of 0.563, 37% higher than the one found here (0.351). This can be explained in part by the use of different genotyping methods, and by sample size (n=30) and different animal origin. Jordana et al. (2016) analysed animals from the southwest of the country, mainly Valle del Cauca, Cauca and Nariño, while in this study, animals were from the Caribbean, only from the Department of Sucre.

The F_{ST} value (0.17±0.01, P<0.05) indicates large genetic differentiation between the studied subpopulations (Wright, 1951). This implies low gene flow (crosses between individuals from different regions) between the subregions of the department, and implies that the astral population of Sucre is composed of at least two populations (Rincon et al., 2013). It is possible that geographical isolation contributes to the complete lack of crossings between the animals of these subpopulations. The F_{ST} values reported in races of the world show variations from 0.271 in the Marchador Brasileiro race (Lara et al., 2015), to a minimum of 0.003 in the Istria race of Croatia (Ivankovic et al., 2002). For its part, Jordana et al. (2016) reported a lower value (0.071) in the CCD than found here, indicating a moderate genetic differentiation.

The low genetic distance between MM and SA can be explained by the geographical proximity of the regions, and by the similar use of the donkey in each region. The SJ subpopulation was intermediate amongst the subpopulations and is geographically located in the centre of the department. Likewise, the GO and MO subpopulations were genetically isolated. This could be explained in part by the geographical locations, where genetic distance is directly proportional to geographical location (Correa et al., 2015), its agroclimatic conditions and the particular use of the donkey in each region. Additionally, the variation shown by the distance model (Nei) is mainly attributed to the action of mutation and genetic drift, assuming a balance between the two. However, as these are subpopulations of the same species and with relative geographical proximity, mutational phenomena are not taken into account, and therefore, gene drift is considered the main cause of differentiation between populations (Correa et al., 2015).

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Genetska raznolikost kolumbijskog kreolskog magaraca u odjelu Sucre

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Cilj ove studije bio je procijeniti genetsku raznolikost kolumbijskog kreolskog magaraca iz odjela Sucre koristeći molekularne markere tipa RAM-a (engl. *Random Amplified Microsatellites*). U 100 jedinki iz pet podregija odjela izlučeno je DNK, a pomoću PCR-a amplificirano je pet primera RAM-a. Pronađene su 291 trake, prosječno 11,96±1,45 po primeru, najveća vrijednost u CCA (18±2,23), a najniža u TG i GT (8,8±0,44). Najviši polimorfni primer (88,09±10,91 %) i s najvišim He (engl. *heterozygosity value*) (0,376±0,021) bio je CA, dok je najniži bio GT (0,341±0,076 i 0,101±0,040). Intrapopulacijska analiza pokazala je prosječno 66,50±1,72 pojasa, od kojih je 89,86±24,04 % bilo polimorfno. Subpopulacija Gulf of Morrosquillo (GO) imala je najveći broj opsega (63±3,84), a najmanju je pronašla u Mojana (MO) (48±2,88), međutim, najveću vrijednost polimorfnih lokusa (81,16 %) i He (0,335±0,022) pronašli su u subpopulaciji Montes de María (MM), koja je najraznolikija. Ustvrđena prosječna genetska raznolikost iznosila je 0,351±0,021 za cijelu populaciju. Analiza populacijske strukture pokazala je 10 % odstupanja između subpopulacija, s vrijednosti FST-a od 0,17±0,01 (P<0,05). Genetske udaljenosti između subpopulacija pokazale su da su MO i GO najdalje. RAM markeri učinkoviti su za procjenu genetske raznolikosti kreolskog magaraca, ova kreolska pasmina ima visoke vrijednosti genetske raznolikosti, MM subpopulacija je najraznolikija, pronađena genetska struktura može biti rezultat prirodnih geografskih barijera između podregija.

Ključne riječi: *ugrožene pasmine, genetska raznolikost, genetski resursi*