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Full Length Research Paper

DNA-based population density estimation of black bear at northern Mexico: A preliminary study

Ávalos-Ramírez, R.[1], Mijangos-Araujo José, L.[1], Zarate-Ramos, J.J, Martinez-Muñoz, A.[2], Salinas-Meléndez, J. A.[1], Chávez-Briones María De L.[3] and Riojas-Valdés Victor M.[1]*

[1]Universidad Autónoma de Nuevo León, U.A.N.L., Fac. de Medicina Veterinaria y Zootecnia, Av. Universidad S/N, Cd. Universitaria, San Nicolás de los Garza, Nuevo León, Mexico.
[2]Universidad Autónoma de Nuevo León, U.A.N.L., Fac. de C. Forestales, Av. Universidad S/N, Cd. Universitaria, San Nicolás de los Garza, Nuevo León, Mexico.
[3]Universidad Autónoma de Nuevo León, U.A.N.L., Fac. de Medicina, Av. Universidad S/N, Cd. Universitaria, San Nicolás de los Garza, Nuevo León, Mexico.

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The analysis of deoxyribonucleic acid (DNA) microsatellites from hair samples obtained by the non-invasive method of traps was used to estimate the population density of black bears (*Ursus americanus eremicus*) in a mountain located at the county of Lampazos, Nuevo Leon, Mexico. The genotyping of bears was performed by multiplex polymerase chain reaction (PCR) using an average of two hairs for each animal. Samples were obtained with barbed wire placed at the traps, which contained food as bait. Multiplex PCR was performed with the GenomiPhi™, G.E. kit and genotyping with an automated DNA sequencing machine (ABI 310 System). Allelic frequency, heterozygosis and exclusion probability of seven DNA microsatellites were calculated and analyzed with computer programs to determine the population density. Three of the microsatellites had a heterozygosis higher than 0.7 and the population density was calculated in at least 1 bear/km^2.

Key words: Black bear, *Ursus americanus*, population size, DNA microsatellite, Mexico.

INTRODUCTION

The black bear *(Ursus americanus)* is considered to be the most common bear in North America, with population estimates ranging from 400 000 to over 680 000, including Alaska, the U.S.A. and northern Mexico. However, some populations of this bear declined very much around 1940, and it was declared as an endangered species (Doan-Crider, 1995; Doan-Crider and Hewitt, 2005, Martínez-Muñoz 2001). In Mexico, three of the subspecies of black bear can be found (*U. a. amblyceps*, *U. a. eremicus* and *U. a. machetes*), which are distributed at the north and central regions. Of these, only *U. a. eremicus* is considered endangered, although little information exists on the population density of this species. Therefore, the need to obtain data on their population number for conservation studies (Martínez-Muñoz, 2001; Onorato et al., 2004).

Some studies suggest that black bear population is showing a natural come back at some regions such as the southeast of the big bend national park of Texas, U.S.A. and that this populations emigrated from the Mexican state of Coahuila through the called “Sierra del Carmen” (Carmen mountains), which communicates both states. Also in Coahuila there is a region called “Serrania del Burro” (Donkey mountains), located next to the state of Nuevo Leon, which is where the present study was conducted. Studies performed in this place from 1991 to 1994 indicate that the subspecies *eremicus* is very abundant and the estimated population density was as high as 0.72 bears /km^2, although other studies using a different methodology calculated this value in 0.31 bears/km^2 (Doan-Crider, 1995; Doan-Crider and Hewitt,

*Corresponding author. E-mail: vriojas@hotmail.com. Tel: 8329-4050.

2005; Martínez-Muñoz, 2001).

On the other hand, in recent years and especially during drought times, many sightings are reported by the news media of black bears coming down to mountain-side neighborhoods located at the Nuevo Leon capital city, searching for water and food. These reports could indicate a current high population density. Some previous studies performed in California, U.S.A. and Coahuila, Mexico postulate populations densities ranging from 0.77 to 0.62 bears/km^2, whereas many other papers have informed values of 0.62 to 0.06 bears/km^2 at different states of the U.S.A. (Doan-Crider, 1995; Doan-Crider and Hewitt, 2005). A governmental study containing a literature review table on work realized at several states of the U.S.A. reports densities ranging from 0.06 (Arizona) to 0.77 (California) (Martínez-Muñoz, 2001).

One way to estimate the population of wild species is by non-invasive methods, such as collecting hair and feces samples, which are considered as captures by genotyping the DNA extracted from them. This is achieved by analyzing molecular markers called microsatellites, which can give the identification of individual animals with a very high degree of confidence (99.99%) (De Barba et al., 2010; Kim et al., 2011). In order to achieve this goal, it is necessary to determine the number of alleles and the heterozygosis of several microsatellites, which in turn can be used to determine their polymorphism information content (PIC), the probability of exclusion (PE) and the probability of identification (PI). The PI is the probability that two individuals selected at random have the same genotype (Waits and Leberg, 2000). Generally, 7 to 15 different microsatellite loci are used. This measurement allows parentage verification and identification of individual animals in order to calculate the population density in a determined geographical area.

Genotyping can be difficult when only small amounts of DNA can be obtained from the samples, as is the case with hair samples, leading to an overestimation of the population size. Therefore, in the present study, statistical methods were applied in order to correct these possible mistakes (allelic drop-out, false allele, and null allele (Frantz et al., 2003; Broquel et al., 2007; Walsh et al., 1996).

Some additional considerations for the genotype-based population studies are the risk that two individuals have the same genotype, mostly in small and inbreed populations, leading to an underestimation of the population size, called the shadow effect. In this case the acceptance of the genotypes belonging to different individuals is determined by using the upper limit as the probability of identification that exists among brothers (PIh), assuming that the population is in Hardy-Weinberg equilibrium (allele frequency is independent among alleles from a locus and between different loci), as happens in natural populations (Waits et al., 2001).

If a determining factor causes by a natural population is not in equilibrium, the observed PI will be higher than the theoretical one; furthermore, the population size is determined based in genotyping to have a good degree of confidence, it is important a high heterozygosis of the genetic markers is utilized, between 0.6 and 0.8 (Taberlet and Luikart, 1999*).* In order to determine the population size using the genotyping data by non-statisticians, computer programs already standardized, such as the MARK program (White and Burnham, 1999) can be used. Thus, this study was aimed at determining the population density by the genotyping methodology and using a sample collection technique that does not disturb the animals, in this case the population of black bear located at the mountains of northern Mexico.

MATERIALS AND METHODS

Samples were collected from the "Santa María" ranch, with an extension of 50 km^2 at the county of Lampazos, Nuevo Leon, Mexico (between 27°01' and 27°08' north latitude; 100° 42' and 100° 57' west longitude) at a plain of altitude of 350 m above sea level. Sampling time lasted 3 months (October to December). The ranch is located between the "Pájaros Azules" and "Mesa" mountains, of altitude of 1750 m above sea level, run from west to east and have a dry-hot weather. Average annual pluvial precipitation is 445.9 mm. (Martinez-Muñoz, 2001). The ranch was divided in two quadrants (5 × 5 km each) and each quadrant was subdivided into 25 sub-quadrants of 1 km^2. Five sub-quadrants were chosen at random from each quadrant to place hair traps (formed with barbed wire around the bait), which were doted with rotten meat (Figure 1). A total of four sampling sessions were performed, each lasting 12 days. For each session the traps were placed at a different location and as far as possible from each other.

In order to have a positive control, DNA was extracted from blood of a black bear located at the local Zoo. DNA from the hair samples was obtained with a commercial kit (BloodPrepTM, Applied Biosystems or Maxwell 16TM, Promega) from a total of 59 samples containing at least two hair roots. PCR of the chosen microsatellites was performed with the commercial kit GenomiPhi V2^{TM}, General Electric Healthcare. This method allows the multiple amplification by displacement analysis, using random hexamers as primers and requires the least DNA amount to obtain a high fidelity of amplification. In order to concentrate the extracted DNA, it was vaporized into a DNA vacuum-centrifuge, followed by addition of 1 µL of PEG 400 (99%) and 9 µL of sample buffer. A PCR master mix was made with the DNA pellet, 9 µL of reaction buffer and 1 µL enzyme. For the PCR, the DNA denaturalization step was omitted; the master mix was incubated at 30°C by 90 min. and was inactivated at 65°C by 10 min. To the final amplification product was added 20 µL of TE 1X buffer. All PCR reaction included a negative control, which contained all ingredients of the reaction mix except DNA.

For the identification and differentiation of animals, the PCR primers for seven microsatellites described by Peacock (2004) were used, labeled with fluorescent dyes at their 5'-end (Table 1). Microsatellite PCR reactions were done separately in a total volume of 10 µL per reaction as follows: 5 000 ng bovine albumin serum, 1.25 M each primer (12.5 pmols), 1 µL DNA, 1 µL ultra pure water and 5 µL Amplitaq Gold PCR DNA polymerase ^{TM} (Applied Biosystems, contains 0.05 U/µL AmpliTaq Gold DNA polymerase ^{TM}, 30 mM Tris/HCL, 100 mM KCl, 400 µM dNTP of each base, 5 mM $MgCl_2$). PCR program consisted of a starting incubation at 95°C for 30 s, 35 cycles of denaturing at 95°C for 30 s, annealing

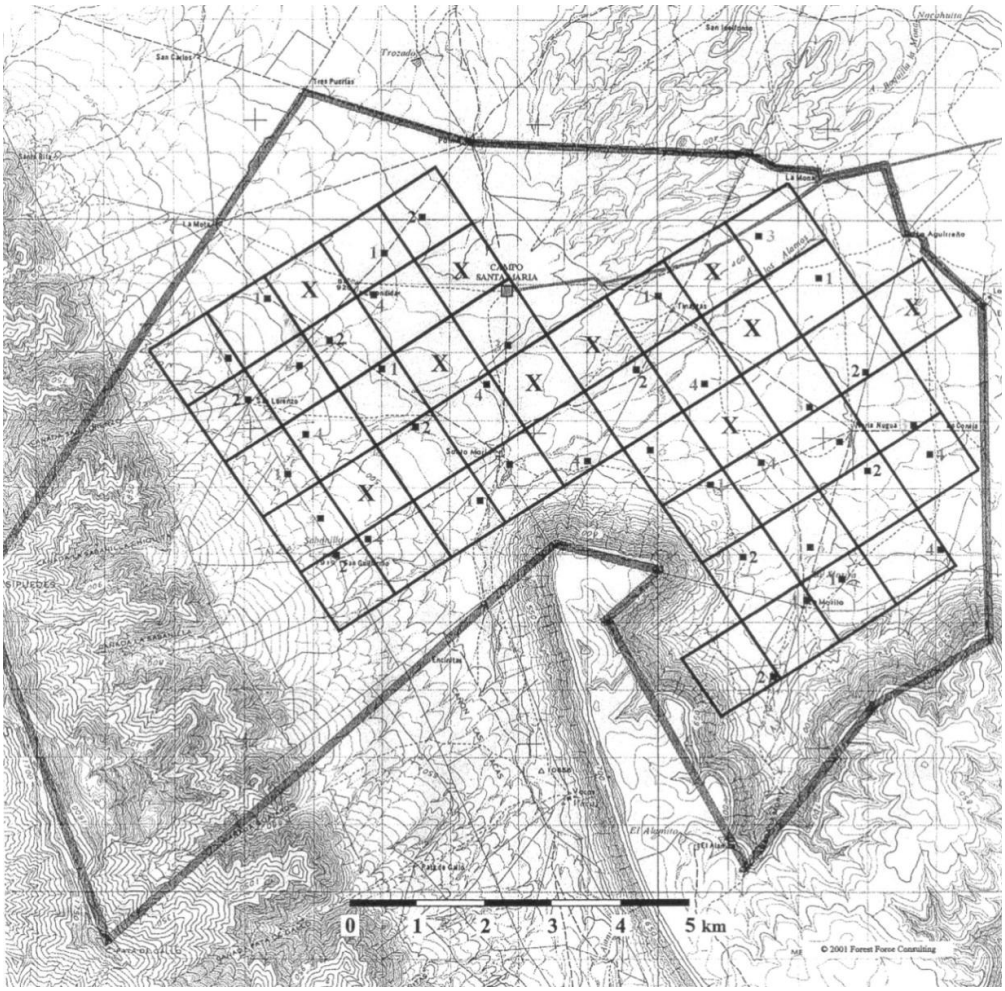

Figure 1. Sampling area.

for 30 s at specific temperature for each primer (Table 1), and extension at 72°C for 1 min; the final step consisted of a final extension at 72°C for 30 min. Microsatellite genotyping was performed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and the allele size was determined with the Gene-mapper ID 3.1^{TM} computer software. For these purpose, two PCR products combinations were done (addition of 1 µL of each product); the first combination contained microsatellites A, B, C and D and the second E, F and G. For the microsatellite analysis, a master mix containing 0.3 µL of Liz 500 (size standard) and 12 µL formamide HiDi^{TM} was made; 12 µL of this mix and 1 µL of each microsatellite combination were combined to be run in the Genetic Analyzer for 23 min as far as the 350 bp of the size standard.

Allele interpretation was done having in consideration diverse factors that can have an influence on the results, such as the presence of shadow bands (Walsh et al., 1996; Wattier et al., 1998), detection of null alleles and false homozygous with Micro-Checker (Van Oosterhout et al., 2004) and Cervus (Kalinowski et al., 2007) computer programs, which give null allele frequency estimation based on frequency differences among homozygous (Bayard et al., 2005). Furthermore, in order to increase the results accuracy, repetitions of PCR reactions was performed, as well as grouping of genotypes with the Gimlet software (Taberlet et al., 1996; Franz et al., 2003; Paetkau, 2003). False homozygous index, taking into account only heterozygous genotypes, were determined for each locus and for all loci according to the following equations (Broquet et al., 2007):

$$f_j = \frac{F_j}{A_j}$$

Where, fj= probability for a false allele per locus to happen; Fj= number of amplifications with false allele; Aj= number of positive amplifications, whether homozygous or heterozygous, per locus.

$$f = \bar{f}_w = \frac{\sum_{j=1}^{L} F_j}{\sum_{j=1}^{L} A_j}$$

Calculation of weighted mean (fw) was done to determine f for all loci. Calculation of false allele was done taking into account both

Table 1. PCR primers of analyzed loci.

Locus	GenBank identity number	Forward primer	Reverse primer	Dye	Tm (°C)
+A	U22090	CCTTGGCTACCTCAGATGG	GCTTCTAATCCAAAGATGCATAAAGG	FAM	61
+B	U22087	GCTTTTGTGTGTGTTTTTGC	GGATAACCCCTCACACTCC	HEX	58
+C	U22093	CCCCTGGTAACCACAAATCTCT	GCTTCTTCAGTTATCTGTGAAATCAAAA	PET	62
+D	U22094	GATCTGTGGGTTTATAGGTTACA	CTACTCTTCCTACTCTTTAAAGAG	NED	54
+E	U22088	GTACTGATTTAATTCACATTTCCC	GAAGATACAGAAACCTACCCATGC	FAM	59
+F	U22085	AAAGCAGAAGGCCTTGATTTCCCTG	GTTTGTGGACATAAACACCGAGACAGC	HEX	68
+G	U22089	TTCCCCTCATCGTAGGTTGTA	GATCATGTGTTTCCAAATAAT	NED	60
*SE47/48	-	CAGCCAAACCTCCCTCTGC	CCCGCTTGGTCTTGTCTGTTGGC	-	62

[+]Ennis, et al. (1994); *Peacock, 2004; Tm, melting temperature.

Table 2. Allele number, size and heterozygosis of amplified microsatellites.

Locus	Allele number	Size rank	Heterozygosis
A	21	105-191	0.92
B	3	70-74	0.35
C	7	145-171	0.82
E	11	126-159	0.79
Average		0.72	

homozygous and heterozygous genotypes:

$$p_j = \frac{D_j}{A_{het_j}}$$

Where, Pj=false homozygous index per locus; Dj= number of false homozygous per locus; Ahetj= number of positive amplifications of heterozygous per locus.

$$p = \bar{p}_w = \frac{\sum_{j=1}^{L} D_j}{\sum_{j=1}^{L} A_{het_j}}$$

Calculation of weighted mean (pw) was done to determine p for all loci.

Allele frequency, heterozygosis and probability of identity among non-related individuals (PI) and among brothers and sisters (PIh) were estimated with the Gimlet computer program and population size was calculated with the Mark program (White and Burnham, 1999).

RESULTS AND DISCUSION

A total of 56 samples containing more than two hair roots were analyzed. Only the alleles of microsatellites A, B, C and E were successfully amplified by PCR and genotyped. Three of the analyzed loci were highly polymorphic (Table 2), and the PI per sample was of less than 0.01, which allowed the conclusion that no two animals shared the same genotype. Because only 3 loci were used to obtain the results, the presence was considered as a preliminary study. Microsatellites A, C and E showed heterozygosis higher than the considered ideal by Taberlet and Luikart (1999) to differentiate among individuals (0.6 to 0.8), which means that each individual can be successfully differentiated. Heterozygosis values were similar to the information previously for North-American bear populations (De Barba et al., 2010; Paetkau, 2003). Locus A was among the loci with the higher allele number known.

Allele frequencies found are shown in Table 3.

Table 3. Allele frequencies of analyzed loci.

Locus	Allele	Frequency
	105	0.009 (1/112)
	107	0.036 (4/112)
	109	0.071 (8/112)
	111	0.089 (10/112)
	113	0.036 (4/112)
	115	0.143 (16/112)
	117	0.125 (14/112)
	119	0.089 (10/112)
	121	0.054 (6/112)
	127	0.054 (6/112)
A	129	0.036 (4/112)
	131	0.009 (1/112)
	135	0.009 (1/112)
	158	0.018 (2/112)
	160	0.027 (3/112)
	171	0.009 (1/112)
	173	0.009 (1/112)
	177	0.125 (14(112)
	187	0.009 (1/112)
	189	0.027 (3/112)
	191	0.018 (2(112)
	70	0.214 (24/112)
B	72	0.777 (87/112)
	74	0.009 (1/112)
	145	0.143 (2/14)
	155	0.071 (1(14)
	161	0.071 (1/14)
C	165	0.071 (1/14)
	167	0.286 (4/14)
	169	0.143 (2/14)
	171	0.214 (3/14)
	126	0.011 (1/94)
	130	0.106 (10/94)
	132	0.383 (36/94)
	134	0.074 (7/94)
	136	0.17 (16/94)
E	148	0.021 (2/94)
	150	0.064 (6/94)
	152	0.011 (1/94)
	155	0.032 (3/94)
	157	0.064 (6/94)
	159	0.064 (6/94)

From the 56 samples taken, 51 different genotypes were recognized; therefore, there were five recaptures. The obtained results demonstrate that it is possible to obtain a correct genotype using two hairs. The obtained data was fed to the MARK program and the results indicate that at least 51 bears inhabit the studied area of 50 km^2, which translates to 1 bear per km^2. Therefore, the estimated population density in the present paper seems to be higher than that previous reported. Some previous studies performed in California, U.S.A. and Coahuila, Mexico postulate populations densities ranging from 0.77 to 0.62 bears/km^2 whereas many other papers have informed values of 0.62 to 0.06 bears/km^2 at different states of U.S.A. (Doan-Crider, 1995; Doan-Crider and Hewitt, 2005; Onorato et al., 2004). Therefore, our estimation of population size seems to be higher that any other reported before. One possible reason for the obtained results could be the presence of null alleles, false homozygous or genotyping mistakes caused by the presence of shadow bands. However, the use of the Micro-Checker program (Van Oosterhout et al., 2004) in the present study did not showed any of the above mentioned factors as a possible source for mistakes.

In conclusion, the present paper postulates that primer selection and PCR optimization were able to give successful genotyping results and that the population density found (1 bear/km^2) was higher but close to the highest density reported before.

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