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RESEARCH ARTICLE

Characterization of the genetic diversity of a population of *Odocoileus virginianus veraecrucis* in captivity using microsatellite markers

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Abstract

The genetic diversity and effective population size (N_e) of a population of *Odocoileus virginianus veraecrucis* in captivity were characterized in the Wildlife Management Unit "El Pochote", located in Ixtaczoquitlán, Veracruz, Mexico. Blood tissue was collected from 20 individuals of the reproductive nucleus, its genomic DNA was extracted, and genetic diversity was characterized by six microsatellites amplified by PCR and visualized in polyacrylamide gels. With four polymorphic microsatellites, 66.7% of the population's genetic variation was explained, which was characterized by an allelic diversity that fluctuated between 9 and 28 alleles (18 average alleles), suggesting a mean allelic diversity (Shannon index = 2.6 ± 0.25), but only 12 ± 2.9 effective alleles would be fixed in the next generation. The heterozygosity observed (H_o= 0.81) exceeded that expected (H_e= 0.79) and these were significantly

different (P> 0.05), as a result of a low genetic structure in the population (fixation index F = -0.112 ± 0.03), due to the genetic heterogeneity that each sample contributed, since the specimens came from different geographical regions. The Ne was 625 individuals and a 1:25 male:female ratio, with which 100% of the genetic diversity observed can be maintained for 100 years. The information obtained in the study can help in the design of a reproductive management program to maintain the present genetic diversity, without risk of losses due to genetic drift and inbreeding.

Keywords
allelic diversity, conservation, effective population size, Veracruz white-tail deer

Introduction

Populations in nature, isolated or fragmented, are subject to the constant process of adaptation to different extreme environmental conditions and to the effects of anthropogenic nature, which can reduce genetic diversity. In addition to recombination, changes in genetic diversity derive from mutations in deoxyribonucleic acid (DNA) and their variation (polymorphism) determines the phylogenetic and evolutionary relationship of species (Frankham 2005). However, in captive populations, the small effective population size (N_e) resulting from the founding effect can lead to inbreeding depression (Rocha and Gasca 2007).

In Mexico, an alternative for the conservation and utilization of wild populations is carried out in a productive diversification system called the Wildlife Conservation Management Unit (Unidad de Manejo para la Conservación de la Vida Silvestre; UMA in Spanish), where an alternative is the management of populations in captive conditions (INE 1997; 2000). In UMAs, the study of genetic diversity using tools such as molecular markers provides useful information to identify inbreeding problems (Serna-Lagunes et al. 2012) and reduces the risk of loss of genetic diversity due to a N_e small (Serna-Lagunes and Díaz-Rivera 2011), particularly in UMAs where the record of the genealogy of the reproductive nucleus is low or unavailable. In this regard, microsatellite markers (SSR) are short tandem repeated sequences of 1-6 nucleotides distributed throughout the genome (Avise 1994; Dakin and Avise 2004), and have been widely used to identify kinship relationships (Armstrong et al. 2011), migration rates, N_e and mating system (Selkoe and Toonen 2006); priority information for the conservation of genetic diversity (Avise 1996).

Of the cervids with distribution in Mexico, White-Tailed deer (*Odocoileus virginianus* Zimmermann, 1780) is the species of deer with the highest capacity to adapt to the environment (Sheffield et al. 1985) and has the ability to tolerate the effects of human activities on their populations and their habitat (Serna-Lagunes 2016). Over the past decade, *O. virginianus* has become a focal species of importance for its reproduction and conservation under the scheme of UMAs, in which it is required to minimize the impact generated by captivity on genetic diversity to achieve a viable population in space and time (Buenrostro and García-Grajales

2016). Normally, to constitute a reproductive nucleus of *O. virginianus* in a UMA, individuals with different geographical origin and different lineages are selected; as they do not have information on this genetic diversity and with the unplanned crossing, genetic diversity would be at risk; therefore, genetic characterization studies can provide supporting information to conserve existing genetic diversity (Hernández-Mendoza et al. 2014).

The assessment of the genetic status of populations of *O. virginianus* has been useful for implementing risk prevention measures of a genetic nature; for example: multiple paternity (DeYoung et al. 2002), kinship relations (Anderson et al. 2002), and mating system (DeYoung et al. 2009) and social dominance (DeYoung et al. 2006). This information is essential for the conservation of genetic variability in which microsatellite molecular markers are the appropriate tool for basic studies of the characterization of genetic diversity (Godoy 2009).

In Mexico, *O. virginianus* management plans in UMAs, are mainly based on habitat management, but in captivity or *ex situ* UMA, do not contemplate programs of conservation of genetic diversity to reduce the risks caused by a N_e small or a few founders. In this sense, the objective of this study was to describe the characteristics of the genetic diversity of a population of *O. v. veraecrucis*, kept in a hatcheries (UMA) located in the municipality of Ixtaczoquitlán, Veracruz, Mexico. Captivity is highlighted as a means of conserving genetic diversity in an *ex situ* system.

Methods

Biological material and amplification of microsatellites

Between March-September 2017, 20 individuals from the reproductive nucleus of the population of *O. v. veraecrucis* managed in the UMA "El Pochote", were sedated (Xilacina of 0.5-1.25 ml per 25 kg of live weight) to extract by puncture in the jugular vein between 2 and 3 ml of blood with Vaccutainer® equipment with 4 g of EDTA as anticoagulant. Blood samples were stored in refrigeration at 15 °C (Serna-Lagunes et al. 2012) and the DNA was extracted with PROMEGA E. Z. N. A. Tissue DNA KIT, following the Blood and Body Fluids protocol. To characterize the genetic diversity of the population of deer, six microsatellite markers were genotyped: BM203, BM4208, D, BM848, TGLA126 and MSTN01, which were initially described for genetic exclusion studies (DeYoung et al. 2003a) and have been tested in *O. v. veraecrucis* and other subspecies (De la Rosa-Reyna et al. 2012).

The microsatellites were amplified from the DNA of the specimens by PCR to a final volume of 25 µl with the following content: 2.5 µl of DNA (<250 ng), 1.25 µl of the primer Forward and 1.25 µl of the primer Reverse, both at a final concentration of 10 µM, 12.5 µl of Promega® Brand Master Mix 2X PCR (25 mM Tris-HCl pH 9, 25 mM NaCl, 2.5 mM MgCl2, 100 µM dNTPs (dATP, dGTP, dCTP, dTTP), 0.5 U of Taq DNA polymerase, 0.05 mg / ml BSA) and 7.5 µl of nuclease-free water. The PCR amplification program included the following stages: initial denaturation at 95

°C for 2 min, denaturation at 95 °C at 1 min, alignment at 54 °C for 30 s, extension at 72 °C for 1 min; final extension at 72 °C for 5 min, and at 4 °C until samples are withdrawn. These conditions were adapted based on those reported by De la Rosa-Reyna et al. (2012). The amplified PCR products were verified on 1.5% agarose gels; those microsatellites that did not amplify on a first occasion or where the amplification was not clear were subjected to PCR again until a reliable amplification was obtained.

The microsatellite PCR products were visualized on 7% polyacrylamide gel electrophoresis in 0.5X TBE buffer. 15 µl of the PCR product of each sample was loaded for each microsatellite. The electrophoresis was maintained at a constant voltage of 70 V for 24 h. The gels were stained with ethidium bromide and at the beginning of each gel, a molecular size marker of 500 bp was placed, with label fragments every 50 bp, which served as a reference to determine the size in bp of the observed alleles.

Analysis of genetic diversity

The size (in base pairs) of the amplified alleles was determined from each sample, based on the size of the molecular marker. In the Excel® program, a data matrix was built based on the design proposed by Peakall and Smouse (2006) for the analysis of genetic data derived from microsatellite markers. With the Structure software v. 2.3.4 (Pritchard et al. 2000) the mixture model was assumed and the analysis was performed considering the frequency model of independent and correlated alleles without prior information on the geographical location of the samples, which allows *K* to vary between one and the number of potential sources of individuals; this analysis was performed with 1 000 000 chain iterations of MCMC with 10 repetitions (Pritchard et al. 2000). According to Evanno et al. (2005), the ΔK statistic was calculated since this indicator accurately detects the upper hierarchical level of structure for the scenarios tested. To do this, we use the online Structure Harvester software to visualize the ΔK values of each genetic group (Earl and vonHoldt 2012).

The Hardy-Weinberg equilibrium test (HW) was applied to the microsatellite data matrix to determine the loci that were found or not in equilibrium; the test statistic was based on an X^2 test. According to De la Rosa-Reyna et al. (2012), the basic characteristics of genetic diversity were calculated as: allelic frequencies at 95% reliability (Aranguren-Méndez et al. 2005), number of different alleles per locus (N_a), effective number of alleles (N_e) [(1/(Sum p_i^2)], Shannon information index (I) [-1 * Sum (p_i * Ln (p_i))]; expected heterozygosity (H_e) [(1 - Sum p_i^2)] and observed (H_o) [(No. of heterozygotes / N)], expected impartial heterozygosity (uH_e) [((2N/(2N-1))*H_e)] and fixation index F [(H_e - H_o)/H_e = 1 - (H_o/H_e)]; these diversity indicators were calculated with the GenAlex software (Peakall and Smouse 2006). To determine if the H_o average is significantly different from the H_e average, a *t*-student test was applied. In view of the fact that four of the six genotyped microsatellites were polymorphic (see Results), the characteristics of the population's genetic diversity were calculated with only four microsatellites.

Estimating the effective population size (N_e) in captive populations is important to preserve the genetic diversity characterized by microsatellite markers (Wang 2005). In this sense, N_e was estimated with the following equation: $Ht/H_o = (1 - 1/2N_e)$ t ~ e –t /2N_e to retain 100% of the existing genetic diversity for 100 years (Wang 2005).

Results

The analysis of the mixture model showed three possible sources of genetic origin (cluster 1 = 0.335 and H_e = 0.9042; cluster 2 = 0.332 and H_e = 0.9048; cluster 3 = 0.333 and H_e = 0.9046; estimated probability of Ln of the data = - 753.2; mean value Ln likelihood = -593.8 ± 318.9; average alpha = 3.9565; Fig. 1) and the differentiation coincided with the results of the *ΔK* indicator (Fig. 2). The HW equilibrium test determined that of the six genotyped microsatellites, four were in HW equilibrium (BM203, BM848, MSTN01 and TGLA126) and two others turned out to be monomorphic (BM4208 and D), that is, they did not conform to the assumptions of HW balance (Table 1). In this sense, the four polymorphic microsatellites explained 66.67% (4/6) of the population's genetic diversity.

The allelic frequencies obtained from the four microsatellite *loci* are presented in Figure 3; the maximum number of alleles found ranged from 9 (BM848), 14 (BM203), 21 (TGLA126) and 28 (MSTN01) alleles; with a minimum and maximum value of 78 and 330 bp of the amplified alleles, respectively, recorded in the BM848 microsatellite.

Table 2 shows the characteristics of the existing genetic diversity for the population of *O. v. veraecrucis* of the UMA El Pochote. The N_a indicates that only 18 ± 4.1 alleles are unique in the population and 12 ± 2.9 are effective alleles and that they are alleles with the capacity to move on to the next generation. The average of the H_o = 0.99 ± 0.0004 exceeded the H_e = 0.901 ± 0.02; according to the *t*-test, there is a significant deviation between values of H_o and H_e (t-value = 4.0828, g.l. = 20.01, *P* = 0.0006). The fixation index showed negative values (average of *F* = -0.112 ± 0.03 alleles), an indicator of a low genetic structure among the individuals of the population, due to the genetic heterogeneity of each individual. The average Shannon diversity index (I= 2.6 ± 0.26), shows that the genetic diversity in the population is in a range of allelic diversity medium.

The effective population size (N_e) was developed as follows: Ht/H_o = 0.99 (to maintain 100% of the existing genetic diversity), *t* (average life span) = 8 years, then,

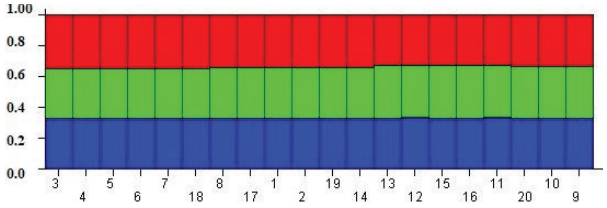

Figure 1. Classification of *K* putative populations of 20 individuals of *O. v. veraecrucis* at the UMA El Pochote, Ixtaczoquitlán, Veracruz, Mexico, which shows three possible sources of genetic origin.

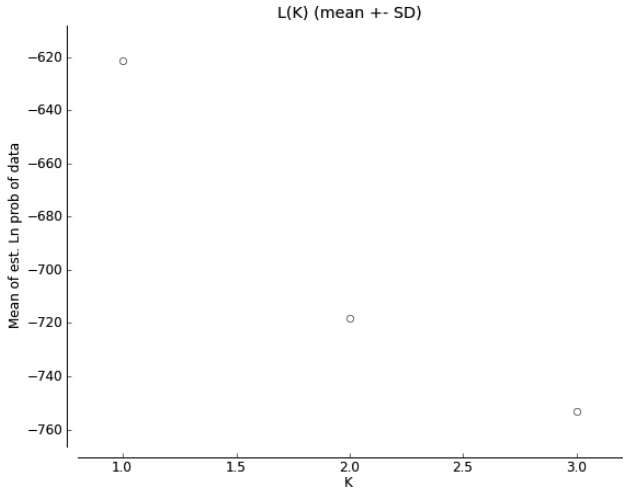

Figure 2. Average values for the three possible genetic sources of the reproductive nucleus of *O. v. veraecrucis* from the *ΔK* indicator.

Table 1. Hardy-Weinberg equilibrium test for the loci in the populations of O. *virginianus veraecrucis* in the UMA EL Pochote.

Locus	Degrees of freedom	Chi-square	Probability	Significance
BM203	91	95.000	0.366	Ns
BM848	36	34.833	0.524	Ns
MSTN01	378	380.000	0.461	Ns
TGLA126	210	198.333	0.708	Ns
BM4208	15	26.007	0.038	*
D	91	117.718	0.031	*

ns = not significant; * P<0.05, ** P<0.01, *** P<0.001

Table 2. Sample size (N), number of different alleles (N_a), effective number of alleles (*Ne*), information index Shannon (*I*), observed Heterozygosity (H_o) and expected Heterozygosity (H_e), Unbiased Expected Heterozygosity (uH_e) and fixation index (*F*) for four microsatellite loci of *O. v. veraecrucis*.

Locus	N	N_a	*Ne*	*I*	H_o	H_e	*uHe*	*F*
BM203	19	14	7.848	2.325	1.000	0.873	0.896	-0.146
BM848	11	9	6.541	2.035	1.000	0.847	0.887	-0.180
MSTN01	20	28	18.605	3.164	1.000	0.946	0.971	-0.057
TGLA126	20	21	16.000	2.907	0.999	0.938	0.962	-0.067
Media	17.5	18	12.248	2.608	0.999	0.901	0.929	-0.112
SE	2.17	4.14	2.98	0.26	0.0004	0.02	0.02	0.03

t = 100 / 8 = 12.5. Therefore, 0.98 = e - 12.5 / $2N_e$ = e - 6.25/N_e. Then, the natural logarithm (ln) of the equation was obtained and we established that Ln 0.99 = -6.25/ N_e; N_e = -6.25 / ln0.99 (-0.01); N_e = 625 individuals; that is, 625 reproductive animals are required to maintain 100% of genetic diversity, a number of animals greater than the current reproductive nucleus and that the UMA can sustain.

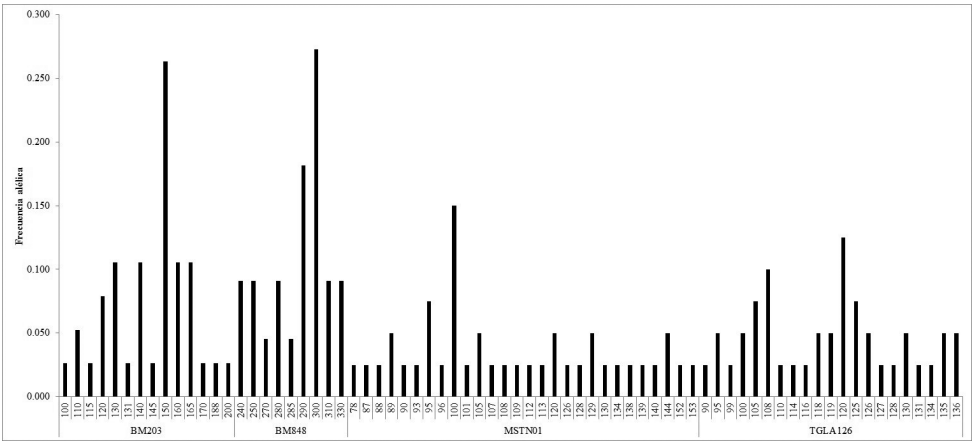

Figure 3. Allelic diversity for four microsatellite loci tested in 20 individuals of *O. v. veraecrucis* in the UMA El Pochote, Ixtaczoquitlán, Veracruz, Mexico.

Discussion

In this study, microsatellites genotyped in the *O. v. veraecrucis* DNA showed an estimated 67% polymorphic loci and 33% monomorphic loci; the two monomorphic microsatellites may result from the low number of alleles (N_a = 4.143) and, consequently, this may be affected by the founder effect. The presence of monomorphic loci reported in this work is also reported in other studies of wild populations of *O. virginianus* (DeYoung et al. 2003b). This can be explained in part by the aspect of the reproductive biology of the species: males usually inhabit adjacent areas to which they were born, while females colonize new habitats; therefore, the reduction of genetic variation is manifested in a higher frequency of homozygous alleles in the progeny (Engel et al. 1996). It is important to consider that the polymorphism detected (66.7%) may also be affected by the low number of sampled individuals and the number of loci analyzed (Breshears et al. 1988). In this sense, the results of this study should be considered as an approximation to the total genetic diversity of the population of *O. v. veraecrucis* at the UMA El Pochote, but the information obtained is relevant to propose conservation strategies for the observed genetic diversity.

The mixing model shows three possible potential sources of the population of *O. v. veraecrucis* analyzed in this study. As a fundamental effect, this may be the result of the heterogeneity of the geographical origin of the specimens with which the reproductive nucleus of the UMA El Pochote was founded, which enables us to know the degree of mixing and genetic independence between the analyzed deer that can help technicians in the planning of crossbreeding programs that guarantee the conservation of existing genetic diversity. On the other hand, the geographical origin of the deer studied corresponds to the geographical distribution area of *O. v. veraecrucis*, which also gives it some spatial genetic heterogeneity (Blanchong et al. 2006), since in Mexico the translocation of specimens of subspecies of *O. vir-*

ginianus outside its geographic range can have negative consequences due to the mixture of lineages (Galindo-Leal and Weber 1994; Hopken et al. 2015).

In terms of heterozygosity, the results indicate a relatively high level of genetic diversity in the captive population of *O. v. veraecrucis* at the UMA El Pochote. When analyzing heterozygosity values, it can be inferred that wild parents of the examined specimens have experienced bottlenecks and population expansion. Although these demographic processes were not tested in this study, it is possible that anthropic use (use for self-consumption, illegal trade, habitat fragmentation) and hunting use (controlled and clandestine hunting) that is exercised on the wild populations of the different subspecies of *O. virginianus* with geographic distribution in Mexico (Mandujano 2007), can change the population dynamics and with it the genetic diversity is affected (Hopken et al. 2015), particularly those of hunting interest such as *O. v. veraecrucis* (Logan-López et al. 2007).

DeYoung et al. (2009), described the allelic diversity of *O. virginianus* populations from a shelter (8.4 alleles), a private unit (9.4 alleles) and a research area (10.4 alleles); Brommer et al. (2015), determined an allelic diversity of 5 to 11 alleles in an isolated population of *O. virginianus* and was founded with few individuals. On the other hand, Hernández (2010), reports a maximum allelic diversity of 17 alleles for a population of *O. virginianus* in captivity; De la Rosa-Reyna et al. (2012) reported a maximum allelic diversity of 27 alleles for three subspecies of *O. virginianus* (including *O. v. veraecrucis*) obtained within its natural distribution range. A similarity pattern was found when comparing these allelic diversity reports with those obtained in this work - despite the low number of genotyped loci. That is, the allelic diversity in the population of *O. virginianus* in the UMA El Pochote is relatively similar to that reported for wild populations and overcrowded in other management systems (Hernández-Mendoza et al. 2014), which puts in evidence the importance of captivity in the conservation of genetic diversity.

Erickson (1979) and Kennedy et al. (1987) point out that in a population of *O. virginianus* its allelic frequencies are stable; this will confer positive genetic characteristics on the population, but it can also be interpreted as an indicator of a poor artificial selection. This was observed with the results of allelic diversity obtained for the population of *O. v. veraecrucis* from El Pochote UMA's, where rigorous reproductive management or a systematic crossbreeding program has not been implemented, although the difference in geographical origin of individuals has favored the maintenance of allelic diversity. Therefore, future studies should evaluate in the progeny, the genetic diversity to determine or not the loss of alleles, as it has been shown that it is possible to maintain the genetic diversity populations of *O. virginianus* in UMAs (Hernández-Mendoza et al. 2014).

In this study, it was found that H_o was significantly different from H_e. The deviation between these indicators is due to genetic differences between the individuals in the population and may be due to the variation in allele frequencies between the samples or to the independent mixture of genes (Vega and Gutiérrez 2015). In other populations of *O. virginianus*, Hernández (2010), in his work on space-time dynam-

ics of *O. virginianus*, compared different populations belonging to UMA, obtaining low heterozygosity values (H_o= 0.60, H_e= 0.82). In another study, De la Rosa-Reyna et al. (2012) when evaluating subspecies of *O. virginianus* from different geographic regions of Mexico, found that heterozygosity was different between subspecies and the H_o was less than H_e (H_o= 0.59, H_e= 0.76; H_o= 0.53, H_e= 0.85; H_o= 0.64, H_e= 0.78). Kollars et al. (2004) report a low H_o (0.19 - 0.22) for five populations of *O. virginianus* in the state of Tennessee, USA. It is possible that the characteristics of evolutionary life stories experienced by each subspecies and each population of *O. virginianus* are reflected in the levels of heterozygosity and genetic differentiation, since this species of deer has an evolutionary past that has promoted genetic variants throughout its geographical distribution in the Americas (Suárez et al. 2017).

The allelic diversity found in the population of *O. virginianus* studied, was present in a smaller range of alleles per locus than that found for many species of mammals. Cronin et al. (2006, 2008), establish that genetic diversity in deer in production systems where reproductive management and planned crossing is practiced requires between 10 to 15 generations to find a significant change in genetic diversity, due to the induced selection to tame the deer. In the UMA El Pochote, there is no planned crossbreeding program, so genetic diversity and the degree of deer mixing of this population should be considered in the design of a reproductive management program.

Frankham (2005), states that reproductive management plans help increase the genetic diversity of small populations, only if the individuals selected for mating have different alleles. A case that supports this was reported by Cronin et al. (2008), who indicate that closed populations of American red deer (*Cervus elaphus*) showed a H_o= 0.54 and N_a= 3.53, higher than those reported for wild populations (H_o= 0.429 and N_a= 2.16), due to the mating structure which is kept in confinement (ratio 1:25 male:female). This mating relationship can be implemented in the UMA El Pochote, to minimize the risk of loss of genetic diversity. However, the results of the N_e indicate that a population of 625 deer must be managed to maintain 100% of the genetic diversity over a period of at least 100 years, however, the current capacity of the UMA El Pochote does not support more than 100 deer. Therefore, future studies on the population of the reproductive nucleus of *O. v. veraecrucis* at UMA El Pochote, should focus on determining kinship relationships between specimens, to select those with different genetic diversity and include them in a planned crossbreeding program.

Conclusions

The genetic diversity characteristics of *O. v. veraecrucis* at UMA El Pochote show similar parameters compared to studies conducted in wild populations and other captive management systems. This is possibly due to the fact that the individuals that make up the population under study have their geographical origin in different areas of the known geographical distribution for this subspecies in the state of

Veracruz, Mexico, which may explain the genetic heterogeneity and the low genetic structure, since there is an independent mixture of alleles. This population showed a medium allelic diversity and conservation is required to guarantee population viability; but it is necessary to establish a N_e of 625 copies to retain 100% of the existing genetic diversity. However, it is important to consider the genetic characterization for the selection of specimens destined for mating with which the existing genetic variability is maintained, without the risk of drift losses and inbreeding.

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