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RESEARCH ARTICLE

Characterization of Genetic Diversity in Dhofari Wild Gazelles

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توصيف التنوع الجيني للغزال البري بمحافظة ظفار

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Abstract. Wild gazelles are scattered in most arid and semi-arid areas in the Sultanate of Oman particularly in valleys, mountains and sandy zones of Rub' al Khali desert. Recently, however, gazelles' numbers have been declining in Oman mainly due to the loss of habitat. Consequently, a gradual loss of their genetic diversity is inevitable. However, little is known about the status of the genetic diversity of the Omani wild gazelles. This study aimed to determine the extent of inbreeding, population structure and genetic diversity in the Omani gazelles' populations in Dhofar region. Samples from four different locations namely Gara, Stom, Solot and Ayon were collected. DNA belonging to 74 gazelles' fecal samples was extracted using the human stool DNA extraction protocol. Following extraction, four microsatellite nuclear markers were used to calculate the levels of inbreeding, population differentiation and genetic diversity. PCR inhibitors were significantly removed using Bovine Serum Albumin (BSA) and dimethyl sulfoxide (DMSO). The mean inbreeding for the population was 0.228 for all loci with a standard error of 0.09. It is therefore postulated that Dhofari gazelles are generally undergoing gradual inbreeding, which may lead to lack of fitness in future generations. The genetic differentiation (Fst) ranged between 0.071 (between Gara and Stom) and 0.231 (between Gara and Ayon). On the other hand, the Fst estimate between Solot (most distant) versus other Dhofari gazelles populations (pooled together) was 3.7%. Principle Components Analysis (PCA) clustered Ayon and Gara populations apart from one another and closer to Stom while placing Solot further than all other populations, which is in agreement with the Fst results and the geographical distribution. In conclusion, the results of this preliminary study provides an insight towards the conservation of wild gazelles in Dhofar in Oman. It provides an initial report on the status of the genetic diversity of Dhofari wild Gazelles and serves as a reference point for future studies assessing their genetic diversity and variability.

Keywords: noninvasive samples, microsatellite, inbreeding, genetic diversity, population structure.

الملخص: تنتشر الغزلان البرية في معظم المناطق القاحلة وشبه القاحلة من سلطنة عمان وخاصة في الوديان والجبال والمناطق الرملية من صحراء الربع الخالي، وفي الآونة الأخيرة ، تواجه الغزلان إنخفاضًا في أعدادها، الأمر الذي قد يؤدي الى فقدان التنوع الجيني تدريجيا مما قد يتسبب في انقراضها من البرية، ويعتبر الفقدان التدريجي للتنوع الجيني خسارة لأحد المقومات الرئيسية التي قد تحمي من الأنقراض، وحتى الان لا يُعرف سوى القليل عن حالة التنوع الوراثي للغزلان العربية البرية في محافظة ظفار. هدف هذا المشروع إلى تحديد أفضل الطرق لأخذ عينات روث الغزال وتحليلها والتعرف على أفضل بروتوكول لإستخلاص الحمض النووي الريبوزي (DNA) والتحسين من أداء تفاعلات سلسلة البوليميرز (PCR). ويعتبر إستخلاص الحمض النووي من الروث من أفضل البدائل للحيوانات البرية وهو بديل عن إستخلاصه من الدم والذي قد يتطلب تخدير الحيوان مما يعد عملية مضنية وخطرة على الحيوانات البرية، أما الأهداف الثانوية فهي تطوير فهم مستويات زواج الأقارب و نوعية التركيبة الجينية والتنوع الوراثي لدى مجموعات الغزلان البرية، وقد إستنتج أن أفضل بروتوكول لإستخراج الحمض النووي من عينات الروث هو البروتوكول الذي يستخدم في استخراج الحمض النووي للبراز البشري، وبعد استخراج الحمض النووي من هذه العينات تم استخدام أربعة علامات وراثية من نوى الخلايا الجسمية في تحديد مستوى زواج الأقارب والتنوع الجيني في الغزلان، ولقد تحسنت هذه القياسات بعد أن تمت إزالة مثبطات تفاعل سلسلة البوليميريز PCR وذلك عن طريق إضافة مادة المصل البقري الزالالي (BSA) وثنائي ميثيل سلفوكسيد (DMSO) وأظهرت النتائج بأن متوسط زواج الأقارب هو ٠,٢٢٨ لجميع عينات محافظة ظفار وبنسبة خطأ قياسي (SE) قدره ٠,٠٩، حيث يشير ذلك بأن الغزلان تمر بمرحلة مبكرة من التزاوج الداخلي التدريجي، أما فيما يتعلق بالتنوع الوراثي الجيني فقد إعتمد على حسابات Fst المتبعة بين مجموعتين من الغزلان مثل نتائج Fst التي تصل الى ٣,٧٪ بين مجموعة غزلان منطقة صولوت Solot و باقي الغزلان المضمنه في الدراسة، وأما قياسات ال Fst لباقي الغزلان البرية فهي تتراوح ما بين ٠,٠٧١ (بين وادي غارة ووادي ستوم) و ٠,٢٣١ (بين وادي غارة ووادي عيون)، ولقد حللت الخلفية الجينية بطريقة تحليل المكونات الرئيسية للمجموعات الأربع من الغزلان البرية الموجودة في أربع وديان رئيسية وأشارت النتائج عن بعد غزلان عيون عن غارة جينيا وتبعد عنها بقليل غزلان وادي ستوم وأما غزلان منطقة صولوت فقد كانت الأبعد عن غزلان المناطق الأخرى، وتتفق هذه النتائج الFst وواقع التوزيع الجغرافي لمواقع جمع العينات، وفي الختام ، تعد نتائج هذه الدراسة هي الأولى من نوعها في دراسة إمكانية إستخلاص الحمض النووي من عينات الروث في الغزال في السلطنه وهي ذات أهمية في معرفة التركيبة الجينية للغزلان البرية في سلطنة عمان ويمكن استخدام نتائجها كمرجع للدراسات المستقبلية المعنية بالتنوع الجيني في الغزال العربي في السلطنة.

Introduction

The genus gazelle belongs to family Bovidae. According to the International Union of Conservation of Nature, IUCN, there are eleven different gazelle species (Mallon and Kingswood, 2001). In Oman, two known Gazelle species have been documented. Namely, *Gazella arabica* and *Gazella subgutturosa marica*. *Gazella arabica* is a vulnerable gazelles species found in the Arabian peninsula (IUCN, 2017; Massolo et al., 2008). In Oman, gazelles are found in protected areas and are scattered in various the wild areas around the country. The numbers of gazelles in the wild are estimated to be 1737 animals in Jabal Samhan and Nejed areas of Dhofar governorate (Al Hikmani et al., 2015).

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The first action taken by the Omani government to protect wild gazelles was to set up several sanctuaries that encompassed gazelles. These sanctuaries include Ras Al Shagar protected Area, Al Wusta Wildlife Reserve, and Jabal Samhan protected area, established in 1982, 1994 and 1997 respectively. Together, these sanctuaries have greatly attributed to the protection of gazelles' population in Oman although several populations are still struggling to survive due to habitat destruction (as a result of highways construction and urban sprawl), pouching and reduction of pastures due to lack of rainfall (Ministry of Environment and Climate Affairs, Sultanate of Oman, personal communication). Other areas where wild gazelles have been reported including Al Saleel Natural Park, Al Hajar Mountain, and southern coastal plain of Mirbat and Sadah. Additionally, few separated individuals have been reported throughout Dhofar Nejd areas (Ministry of Environment and Climate Affairs, Sultanate of Oman, personal communication).

Unfortunately, gazelles population are continuously declining due to illegal hunting and animal capturing (Al Hikmani et al., 2015; Massolo et al., 2008). Gazelles are a main dietary component for some wild species, such as the Arabian leopard *Panthera pardus nimr* (commonly found in Dhofar mountains) which (Judas et al., 2006). In addition, gazelle's juveniles are considered one of the opportunistic mammalian preys for the scavenger white vulture's (considered an endangered species by the IUCN, 2017) which relies on gazelles juveniles as feed for its hatchlings (Margalida et al., 2012). In addition, habitat degradation and population fragmentation also threaten Arabian gazelles. The presence of various kinds of flora in many valleys in Oman is a primary source of feed for many wildlife species. However, plants destruction along valleys due draught or weather conditions is common. An example of conditions is the floods caused by the tropical cyclone Gonu in northern Oman in 2007, and more recently, the destructive cyclone (Mekunu) and the cyclone (Luban), which impacted Dhofar Governorate in 2008. These cyclones could diminish the numbers of wild gazelles and the types of plants that they feed on in addition to threatening their livelihood as gazelles become unable to adapt to sudden habitat and environmental catastrophes (Ministry of Environment and Climate Affairs, Sultanate of Oman, personal communication). Another challenge facing the gazelle populations is the construction of road networks across gazelles habitats separating gazelles herds smaller herds, which could increase inbreeding and reduce fitness. For instance, the road between Qurayyat and Sur Wilatats splits the wild gazelles population into two herds (groups) of gazelles with little interbreeding between both populations. The pouching of adult gazelles in various parts of Oman remains a continuous threat the gazelles' population.

Non-invasive sampling is advantageous and it is easier and cheaper than invasive sampling (Taberlet et al., 1999) and is also in line with ethics and conservation strategies for wild animals. Nevertheless, there are some drawbacks associated with in non-invasive sampling. For instance, in fecal samples, there is a chance of cross contamination of feces belonging to different individuals. This can be avoided by properly selecting the pellets exactly from the top of the fecal colony to get fecal pellets belonging to only one individual. The challenge associated with fecal DNA is its degradation because of the sun's UV light (King et al., 2018). The fecal moisture is yet another concern as it enhances the attachment of the soil to the feces and increase the inhibitors from the soil, which in turn prevents proper amplification of DNA. Thus, gathering fresh fecal samples and preserving them in very dry and low temperature is essential for successful DNA amplification (Murphy et al., 2007). The feces of both Nubian ibex and domestic goats is occasionally confused with the gazelles scats and this could affect the accuracy of the results of genetic diversity studies. In such cross-species contamination of fecal samples, the alleles obtained for the analysis could give false genotyping results resulting in allelic dropout or multiple alleles. In order to avoid such complications, developing basic knowledge of differentiation between species fecal samples becomes essential. The separation between individual gazelles fecal samples is also important in assessing the level of genetic diversity of the species. Collecting scat samples from a spot scat of different gazelles are found and it is assumed that it belongs to one individual could result in higher estimates of heterozygosity.

Although the threats of gazelle populations in Oman are continuous, the impacts of these threats on the fitness and genetic variation of these populations had not been assessed. It is therefore a matter of importance to assess the status of the genetic variation in the Omani Gazelle population. Such assessment is not only important to guide policy makers to take appropriate actions today, but can be a reference point helping future researchers compare today's genetic variation with their future findings. Monitoring the genetic diversity of Omani gazelles is crucial as it helps us to predict their future fitness, disease susceptibility and the levels of inbreeding (Hedrick and Garcia-Dorado, 2016; Szulkin et al., 2013). Therefore, genetic diversity assessment is required for shaping policies in gazelles' protection. The aim of this project was to determine population structure and genetic diversity of the Arabian gazelles in Dhofar using microsatellite DNA markers. We utilized a non-invasive sampling approach in which we extracted DNA from gazelles' scat samples. Our approach is the initial of its kind in assessment of genetic diversity in gazelles' populations in Oman.

Materials and Methods

Collection and Grading of Faecal Samples for DNA Extraction

In this project, 110 gazelles' feces specimens were collected from 69 locations from four different valleys in Dhofar Governorate Stom, Gara, Ayon and Solot (Table 1). Gazelles scat samples were located by tracing gazelles toe prints. Gazelle toes prints are small footprints shaped like a longitudinal symmetrical cross section of an apple with clear med-line as shown in Figure 1.

Table 1. Collection of scat samples in gazelles habitats for Dhofar governorate.

Population	Valleys	Locations	Samples
1	Solot	25	38
2	Wadi Ayon	11	14
3	Wadi Gara	18	29
4	Wadi Stom	15	29
	Total	69	110

Figure 1. An image showing gazelles toe print on the soil.

The diameter of a scat pellet's area is roughly 30 cm. In collection zones, different pellets from different gazelles usually exist in the same spot. Therefore, in a cluster of feces, old or fresh samples can be found. However, only fresh scat pellets were sampled carefully from the top of any scat colony in order to best ensure they belong to the same individual. Although we took this measure, it is still imperative to indicate that the samples number is not always reflective of the individuals' number in a certain site since an individual can defecate at more than one spots within a location. We graded the gazelle's faecal sample based on their color on a scale of A (fresh gazelle's feces) to D (at least 5 days old feces). The colors of grades A to D samples ranged from dark black to white respectively as shown in Figure 2. The difference in color is attributed to evaporation of moisture and physicochemical changes the difference of plants species that gazelles graze on in different locations. The physical characteristics and grading of scat samples of various colors are given in Table 2.

Samples collection was conducted in the same day and in any given location to limit the collection of scat from the same herd twice. This mitigated the chance of double sampling of the same individual as gazelles could migrate from one location to another especially during lack of water resources and competitions for pasture. The fecal samples were collected in plastic bags, labeled and preserved in a cool box and later frozen at -80°C until DNA extraction.

DNA Extraction

Pre-DNA extraction, crusting of fecal samples was performed using sterile and disposable blades into 4 mL Eppendorf tubes. For the extraction protocols in this project, we crusted the outer layer of 11 pellets from each sample for a total weight of 0.18 g to 0.22 g. In total, 913 pellets belonging to 83 fecal samples were crusted for DNA extractions. DNA extraction was performed using the QIAamp® human stool DNA extraction protocol following the manufacturer's procedure. DNA concentration and purity were assessed using a nano-drop.

Polymerase Chain Reaction (PCR)

Fecal samples carry some compounds that inhibit PCR reactions. These compounds came from the soil, bile salts, complex polysaccharides, collagen, heme, humic acid, and urea. To overcome PCR inhibitors, 50% DMSO and 10% BSA were used in an amount 2.5µL of total PCR volume. The composition of the master mix was used for amplifying various gazelle molecular markers. These were performed in 25 µL of total volume (1X) containing on average 25 ng/µL of DNA, 200 µM of each dNTP, 2 µM $MgCl_2$, 5 pmol of each primer, 1-unit hot start polymerase, 2.5 µL of the same amount of both 50% DMSO and 10% BSA. Finally, 7.8 µL nuclease free water were added to complete the total PCR volume. The phases of PCR cycles started with 94°C for 7 min and ended with 72°C for 7 min and in-between cycles were as follows: (i) DNA denaturation at 94°C for 30 s, (ii) primers annealing phase for 30 s (at a temperature chosen to be the lowest annealing temperature amongst both primers), and (iii) DNA extension took place at 72°C for 30 s in presence of (Taq) polymerase enzyme.

Fragment Analysis

A size standard "ROX 400" (ABI) (Internal Lean Standard) was run concurrently in each capillary to create the standard curve. Three markers (FAM, HEX and TAMARA) labeled with different dyes, were used to label the product of the 9 Microsatallite markers shown in Table 4. The markers were run in groups of three (A<B and C) according to the melting point, to reduces the required consumables and duration of analysis. The used composition of the Master Mix for each run is given in Table 3. 10.1µL of the total volume were loaded in 96

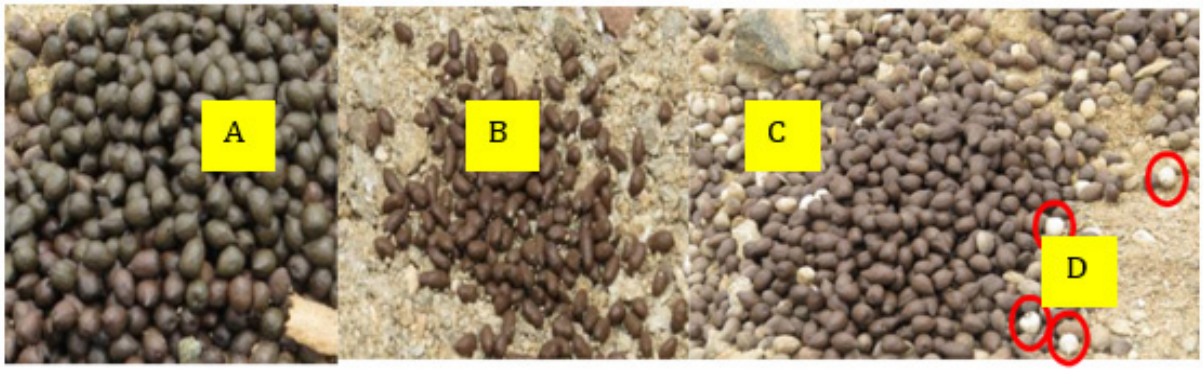

Figure 2. Gazelle fecal samples grades A (fresh) to D (oldest).

Table 2. Physical characteristics of fecal samples as graded in this study.

Grade	A	B	C	D
Color	Black/ green	Brown/black	Black/Brown	White
Moisture	Moist	Dry	Dry	Dry
Mucus presence	Yes	No	No	No
Soil presence	Soil (crystals)	Sometimes	No	No
Scattered	Combined	Somewhat	Scattered	Very Scattered

plate and centrifuged 500 rpm for 1 min. Then, the plate was incubated at 95°C for 5 min and kept on ice for 1min before being centrifuged at 500 rpm for 1 min before genotyping using the ABI Genetic Analyzer (model 3130xl).

Measures of Genetic Diversity and Population Structure

The fixation index (F), inter-population differentiation (FST), Hardy Weinberg Equilibrium (HWE) and Principle Component Analysis (PCA) were calculated using GenALEx V.6.51 (Peakall & Smouse, 2012).

Table 3. Composition of the Master Mix used.

Master mix	1X(µL)
Formamide	9.2
Internal Lean Standard, Rox dye	0.3
DNA form three dilution mixes of labeled dyes of PCR results (2:2:3 or 2:2:4)	0.6
Total volume	10.1

Results

Fragment analysis

Out of the 110 DNA samples extracted in this study, 74 samples yielded enough DNA for successful genotyping and only four polymorphic microsatellite markers (BM4505, TEXAN19, INRA40, and BM415) were successfully amplified. For these markers, four population Gara, Solot (area between Merbat and Sadah), Stom, and Ayon were investigated. Stutter peaks were carefully evaluated to avoid mistakes in allele sizing.

Genetic Diversity

A chi-square test of the HWE was performed at a α=0.05 with the null hypothesis (Ho) that the distribution of sample markers followed HWE. The results indicated that there was no significant deviation from HWE in markers frequencies in two populations, Gara and Ayon. However, three markers in Solot, and two in Stom showed a significant deviation from HWE at α=0.05 as illustrated in Table 5. Although not all the loci were in HWE, we decided to include which are not at HWE due to the limitation of small sample size and the few markers used in this study. Therefore, all four loci were used to evaluate the genetic diversity of the four population. The fixation index (F), mean and standard error (SE) of sample size (N), no. alleles (Na), effective alleles (Ne),

Table 4. Multiplexes A, B, C: primer names, primers sequences, dye name and melting temperatures of various primers used in the study.

#	Loci	Primer sequence (´5-> 3´) Forward Reverse	Dye	Tm C°
A	BM302	F-GAATTCCCATCACTCTCTCAGC R-GTTCTCCATTGAACCAACTTCA	5´ HEX	58.4
	SR-CRSP6	F-CATAGTTCATTCACAATATGGCA R-CATGGAGTCACAAAGAGTTGAA	5´ FAM	57.5
	ETH10	F-GTTCAGGACTGGCCCTGCTAACA R-CCTCCAGCCCACTTTCTCTTCTC	5´ TAMRA	66.4
B	TEXAN6	F-AGGCAGTTACCATGAACCTACC R-ATCCTGGTGGGCTACAGTCTAC	5´ FAM	62.1
	BM4505	F-TTATCTTGGCTTCTGGGTGC R-ATCTTCACTTGGGATGCAGG	5´ HEX	58.4
	TEXAN19	F-CTGAAACCCTCTTATTCAAATTGTG R-TGCAGAGTCAGATAAAAATCCC	5´ TAMRA	58.4
C	OarFCB304	F-CCCTAGGAGCTTTCAATAAAGAATCGG R-CGCTGCTGTCAACTGGGTCAGGG	5´ FAM	66.8
	INRA40	F-TCAGTCTCCAGGAGAGAAAAC R-CTCTGCCCTGGGGATGATTG	5´ TAMRA	59.4
D	BM415	F-GCTACAGCCCTTCTGGTTTG R-GAGCTAATCACCAACAGCAAG	5´ TAMRA	59.4

observed heterozygosity (Ho), and expected heterozygosity (He) and the genetic differentiation values (Fst) for all the populations are presented in the Tables 6 and 7, whereas the PCA is shown in Figure 3.

Table 5. Summary of Chi-Square Tests for Hardy-Weinberg Equilibrium. The markers names, degree of freedom (DF), Chi-Square value (ChiSq) and its probability are shown.

Population	Locus	DF	ChiSq	**Prob.**
Gara	BM4505	15	20.160	0.166
Gara	TEXAN19	28	39.020	0.081
Gara	INRA40	1	0.194	0.659
Gara	BM415	1	0.000	1.000
Solot	BM4505	3	6.000	0.112
Solot	TEXAN19	36	56.525	0.016
Solot	INRA40	21	67.089	0.000
Solot	BM415	3	20.989	0.000
Solot	BM4505	15	30.000	0.012
Solot	TEXAN19	36	73.229	0.000
Solot	INRA40	21	30.238	0.087
Solot	BM415	6	11.194	0.083
Ayon	BM4505	3	4.160	0.245
Ayon	TEXAN19	28	48.333	0.010
Ayon	INRA40	15	22.440	0.097
Ayon	BM415	1	0.141	0.708

Table 6. Mean and standard error (SE) of sample size (N), no. alleles (Na), no. effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index (F) for the populations.

Pop	Mean/ SE	N	Na	**Ne**	**Ho**	**He**	**F**
Gara	Mean	7.250	4.500	2.863	0.519	0.563	0.030
	SE	0.479	1.500	0.708	0.086	0.122	0.075
Solot	Mean	21.250	5.500	3.127	0.248	0.617	0.595
	SE	6.223	1.500	0.651	0.118	0.106	0.152
Stom	Mean	13.000	6.500	4.172	0.663	0.740	0.067
	SE	2.415	1.041	0.611	0.111	0.048	0.222
Ayon	Mean	8.500	4.750	2.494	0.357	0.505	0.219
	SE	1.555	1.377	0.713	0.070	0.119	0.143

Table 7. Gazelle populations pairwise population Fst values.

Gara	Solot	Stom	Ayon
Gara			
Solot	0.000		
Stom	0.073	0.000	
Ayon	0.104	0.133	0.000

Fst Values below diagonal. Fst=0 (panmixis), Fst=0.01 (moderate diversity), Fst<0.2 (High diversity).

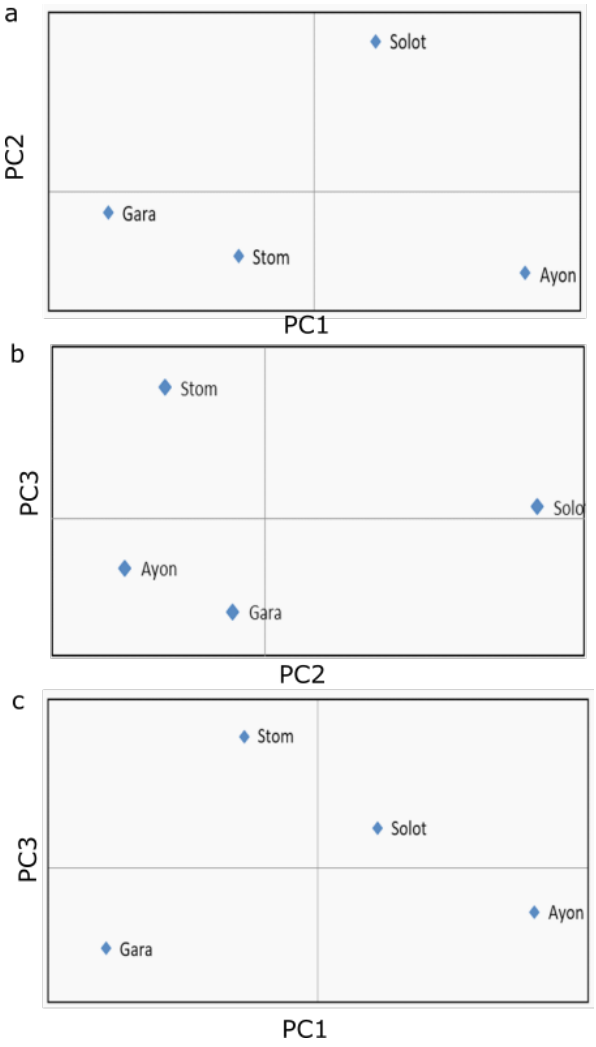

Figure 3. Clustering of gazelle populations using principal coordinates analysis a (PC1 vs PC2), b (PC2 vs PC3) and c (PC1 vs PC3).

Discussion

The largest allele number obtained was in Solot (21.25) followed by Stom (13) and lowest sample size observed was in Gara (7.25). However, the number of alleles only increased slightly as the sample size increased. The observed heterozygsity did not depart largely from the observed heterzygosity except for Solot and Ayon. The results of the fixation index (Table 6) showed some degree of inbreeding in all the locations. However, it was the highest in Solot (0.595) and lowest in Gara (0.030). The Fst values showed the presence of a clear population differentiation in our data with no panmixic populations (lower that 1%). The Fst value between Solot and Stom populations was 7.3%, which indicates that the two populations are genetically close. This is also an indication that the two populations are near panmixia and might be undergoing random mating. The Fst value between Gara and Ayon (23.1%) is a relatively higher genetic differentiation compared to that between other populations. This suggests that there was little interbreeding or migration between the two populations. The Fst value between Gara and Stom was 7.1%, which was moderate and similar to the value between Solot and Stom (7.3%), while that between Ayon and Solot was 10.4%. However, the genetic diversity between Gara and Solot, was 14.5%, which was higher than Ayon and Solot.

Altogether, our results indicated a moderate to high genetic diversity in wild Dhofar gazelle's populations. In contrast, computing Fst pairwise between two gazelles population (Solot versus remaining populations) gives 3.7% of genetic diversity. Taken together, our results show that all populations had a moderate genetic differentiation from one another. However, the levels of genetics differentiation found in this study are considered within the range reported for gazelle populations. In a previous study on wild gazelles of the southern Levant, the pairwise Fst between Dorcas gazelles (Arava) and Acacia gazelles was found to be 30.9 % which is a relatively high genetic differentiation (Hadas et al., 2015). Principle components analysis of PC 1 vs PC 2 shown in Figure 3a agreed with Fst results and showed that Gara and Stom (lowest pairwise Fst) were closer to each other compared to the rest of the populations. It also placed Gara and Ayon distantly from one another (Highest pairwise Fst).

Conclusion

There was a low to moderate inbreeding levels and moderate to high genetic diversity in wild gazelles populations included in this study, which indicated higher within population mating and lower between populations mating. Our genetic differentiation (Fst) analysis showed that the highest differentiation was between Gara and Ayon (23.1%). The PCA was in agreement with the genetic differentiation analysis and clustered Gara and Ayon further away from one another. In addition to that, the PCA analysis clustered the populations according to their geographical distribution in the map with Solot being further away from the other sampling locations. Our study illustrates the successful utilization of noninvasive sampling in assessment of genetic diversity in wild gazelle populations in Oman. Nevertheless, additional markers and a larger sample size are required in order to get accurate estimation of population genetics parameters in future studies. Alternatively, utilization of modern genotyping techniques such as genotyping by SNPs would ultimately yield more markers and increase the confidence and reliability of the results compared to microsatellites.

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