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Phylogeny of gazelles in some islands of Iran based on mtDNA sequences: Species identification and implications for conservation

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ABSTRACT

Different species of gazelles are among the most endangered mammals on the Asian steppes and occur in the central, southern and northwestern regions of Iran. The previous conservation efforts in this region have been incomplete due to confusion about the phylogenetic relationship among various populations. So that, different conservation programs such as *ex-situ* breeding and transfer of captive stocks to potential conservation units encountered the fundamental difficulties. This paper provides a phylogenetic study based on the analysis of Cytochrome *b* gene of mitochondrial DNA sequences in a number of individuals collected from the four wild island gazelles population (Kish, Kharg, Hormoz and Hengam) in the south of Iran which are applicable in phylogenetic studies and species identification for conservation. After comparing the obtained and retrieved sequences of Genbank in MEGA5 Software and then tracing a related phylogenetic tree, we found two different species in four islands. Therefore, from phylogenetic point of view, Kharg and Kish Islands' populations belong to Iranian gazelle (*Gazella subgutturosa*) and Hengam and Hormoz Islands' populations are Jebeer or Indian gazelle (*Gazella bennettii*). Thus, for these two different species, separate conservation programs should be taken to manage populations of these regions and to prevent the exchange gazelles between these Islands.

INTRODUCTION

It is expected that half of the earth's plant and animal species will become extinct in less than a century. This sharp decline of biodiversity has been termed as extinction spasm and it is largely caused by human activities (Proenca & Pereira, 2013). Different species of gazelles are among the most endangered mammals on the Asian steppes (Wacher *et al.*, 2010). These species are distributed more in the central and southern regions of Iran. All gazelle species populations have declined because of poaching, habitat degradation, and alternation of natural habitats to agricultural land, construction, mining, and military activities (Hemami &

Groves, 2001). Also, gazelles are amongst the most taxonomically complex mammal groups (Groves, 1967, 1996, 1997; Vassart *et al.*, 1994, 1995; Lorenzen *et al.*, 2008). Taxonomic status of most gazelle species, particularly under the species level is not clearly understood (Wronski *et al.*, 2010). Thus, different scientific and management programs such as ex-situ and captive breeding which were used to restock their populations have not been so prosperous. Conservation efforts for gazelles in the world include the prevention of hunting and establishment of protected areas (Lerp *et al.*, 2011). Furthermore, most natural habitats of species have not

been degraded through over-exploitation, so reintroduction efforts remain a feasible option if poaching is prevented (Hemami & Groves, 2001; Lerp *et al.*, 2011). Some previous conservation efforts for these species have been incomplete due to confusion about the phylogenetic relationships among various populations. For example, in Moghan plains in Northwestern Iran and in Ghazvin plains in the central part of the country, the attempts to reintroduce gazelles were not successful (Zachos *et al.*, 2010). Clarifying taxonomic relationships is essential for conservation of biodiversity, conservation genetics, and determining populations or individuals for future reintroductions (Wronski *et al.*, 2010). Describing the genetic variation found in organisms is one of the most important objectives of conservation geneticists. Genetic variation is fundamental to retaining the biodiversity found in species, populations and ecosystems. It is defined by allelic diversity and by the percent of heterozygosity found in a population (Conway, 2001). A population with high genetic diversity may be more capable in dealing with changes in its surroundings such as outbreak of a new disease. Also, endangered organisms with small populations (especially in closed Islands without any relationship with other populations) may experience a reduction in the level of genetic variation in their populations (Conway, 2001; Aeini *et al.*, 2007). Descriptions of morphometric characters such as pelage coloration, skull and other skeletal morphometrics are commonly used as a basis for classification (Groves, 1969; Rebholz & Harley, 1999). However, these methods lead to the different classification schemes in gazelles. The genetic researchers have the potential to assess the taxonomic and phylogenetic status of species which have been difficult to classify (Furley *et al.*, 1988; Rebholz & Harley, 1999). As there is much misunderstanding and misinformation in the literature about several species of gazelles living in Iran, and considerable fresh gathered information over the past few years, it is appropriate to review their status

and distribution, and to provide some genetic data. The lack of detailed information about phylogenetic relationships among gazelles as a very diverse group of mammals made conservation efforts complicated. This is especially because, no thorough phylogenetic or phylogeographic analysis focusing on island gazelles have been conducted. Kharg Island (also called Khark), is administered by the adjacent coastal Bushehr Province. The existence of gazelles is biologically important characteristics of this Island. Kharg and Kharku were designated as protected areas in 1960 with a total area of 2438 ha. This reserve was upgraded to wildlife refuge in the early 1970s, but Kharg was separated a few years later leaving only Kharku protected in the Kharku wildlife refuge (312 ha). The area of Kharg Island is 2100 ha (Behrouzi Rad, 2013). During the Iran-Iraq War (1985), 30 gazelles from Kharg Island were transferred to Kish Island in an attempt to preserve them. This was done in cooperation with the Iranian Department of Environment that now can be seen in the southern and eastern parts of this island. Based on the last census, their number has grown to over 600 gazelles (Iranian Department of Environment, 2013, unpublished data). Hormoz and Hengam Islands are located in the Persian Gulf in south of Iran. However, no genetic or ecological studies have been conducted on gazelles of these Islands, but based on some morphological characters and according to the Iranian Department of Environment these gazelles are Jebeer. In the present study, the taxonomic status of gazelles in four southern islands of Iran, namely Kish, Kharg, Hengam and Hormoz were investigated by analyzing 410 base pairs (bp) of cytochrome b gene (Cyt-b) of mitochondrial DNA (mtDNA). The sequences derived from collected samples from the wild in the present study as well as eight sequences of different Gazella species retrieved from GenBank were used to estimate the phylogenetic relationships. The obtained results can help to fill a remarkable gap in scientific knowledge about the gazelles in Iran and it

is expected to provide a reliable insight for better management of this species.

MATERIALS AND METHODS
Sample collection and DNA extraction

Samples were collected from free-living populations of gazelles in Kharg, Kish, Hor-moz and Hengam Islands in the Persian Gulf, south of Iran. Fresh feces were collected in the field (Table 1). By this non-invasive sampling method, capturing the animals was avoided and thus the risk of injures and disturbing these endangered animals were reduced.

Table 1. The characteristics of collected samples in the four islands.

Sample location	Sample ID	Accession number	Sample type
Kharg Island Lat. 29° 22' , Long. 50° 30'	Kharg 1 Kharg 2 Kharg 3 Kharg 4	KM387303 KM387304 KM387305 KM387306	feces
Kish Island Lat. 26° 36' , Long. 54° 04'	Kish 1 Kish 2 Kish 3 Kish 4	KM387291 KM387292 KM387293 KM387294	feces
Hormoz Island Lat. 27° 04' , Long. 56° 28'	Hormoz 1 Hormoz 2 Hormoz 3 Hormoz 4 Hormoz 5	KM387295 KM387296 KM387297 KM387298 KM387299	feces
Hengam Island Lat. 26° 39' , Long. 55° 54'	Hengam 1 Hengam 2 Hengam 3 Hengam 4 Hengam 5	KM387300 KM387301 KM387302 KP729618 KP729619	feces

For each individual, one sample was collected and preserved by using ethanol 96% and then transferred to the laboratory. DNAs were extracted using the AccuPrep® Stool DNA Extraction Kit following the manufacturer's instructions.

Complementary data retrieved from Gen-Bank

The data set was completed with the Cyt-b sequences present in GenBank for the genus Gazella. After discarding the partial sequences with a high number of unknown bases and also sequences that have been shown to be chimeric, what remained are: G. subgutturosa, G. bennettii, G. gazella, *G. cuvieri, G. leptoceros, G. spekei, G. erlageri* and *G. dorcas* (Table 2). Also, one sequence of *Procapra gutturosa* was retrieved as outgroup.

Polymerase chain reaction (PCR), amplification, sequencing and alignments

After DNA extraction, a 410 base pairs fragment of the mitochondrial cytochrome b (cyt-b) gene was amplified using the polymerase chain reaction technique, then they were sequenced in two directions. The primers used for PCR and sequencing (Rebholz & Harley, 1999) were: cyt-b: L14724, 5'_TGATATGAAAAACCATCGTTG_3';H15 149,5'_CCTCAGAAAGATATTTGTCCTC_3' . The PCR reactions were performed in a volume of 25 µl, containing 2 µl of DNA (50 ng), 1 µM of each primer, 1 × PCR buffer, 200 µM of each dNTP, 1.5 mM MgCl2, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems). PCR was performed for these primers according to the protocol shown in Table 3. Then, sequencing was conducted using the BigDye Terminator Kit® (Applied Bio-

systems, Foster City, CA, USA) and the sequences were run on a capillary ABI 3730 DNA Analyzer sequencer® (Applied Biosystems, Foster City, CA, USA). The chromatograms were edited for correction with Seqscape v2.7 software (Applied Biosystems) and then the sequences were aligned with the ClustalW algorithm implemented in the Mega 5 software (Tamura *et al.,* 2011) with a final correction by eye. All sequences were

submitted to NCBI Genbank and deposited under accession numbers KM387291 to KM387306, KP729618 and KP729619.

Phylogenetic analysis of mitochondrial data and other genetic analysis

Phylogenetic analyses were performed on mitochondrial data using Neighbor-Joining and Maximum Likelihood methods. Based on the data from Mega 5 software, the model

Table2. mtDNA sequences retrieved from Genbank.

Species name	Accession number
Procapra gutturosa	DQ269151
Gazella erlangeri	JN632639
Gazella subgutturosa	JN632644, AF187715, DQ269164
Gazella spekei	JN632642
Gazella bennettii	JN632635, AF187698
Gazella cuvieri	JN632636
Gazella leptoceros	JN632641, JN410259
Gazella gazella	JN632640, JN410260, JN410261, AJ222682
Gazella dorcas	JN410222, JN632638, JN410258, JN410242

Table 3. PCR Conditions using primers L14724 and H15149 (Hammond et al., 2001).

Number of cycles	Step	Duration	Temperature
1 cycle	initial denaturation	5 min	95 °C
35 cycle	denaturation	30s	95 °C
	primer annealing	30s	50 °C
	extension	30s	72 °C

with the lowest value of AICc is the best. Therefore Neighbor-joining analyses and Maximum Likelihood values were calculated using the Tamura-Nei (TN93+G) model. Bootstrap analyses were performed in the neighbor-joining and Maximum Likelihood options of MEGA 5 with1000 replicates. Also, Arlequin V 3.5 software (Excoffier and Lischer, 2010) was used for exact determination of haplotypes, estimation of polymorphism sites, percentage of organic bases and the other genetic analysis with 100.000 Markov chain steps and a burnin of 10.000 steps.

RESULTS AND DISCUSSION

The analyses were carried out using thealignment sequences of 410 bp of the mito

chondrial cytochrome b (cyt-b) gene, comprising 36 sequences of gazelles with 1 sequence of other antelopes as outgroup. The resulting phylogenetic tree from the Maximum Likelihood and Neighbor-Joining analysis in MEGA5 software is shown in Figs. 1 - 2, respectively. Both obtained trees similarly show that Kharg and Kish Islands gazelles are *Gazella subgutturosa*, while Hengam and Hormoz Islands gazelles are *Gazella bennettii*.The results of haplotype number using Arlequin V 3.5 Software are shown in Table 4. The molecular diversity indices and percentage of organic bases for comparison between populations are shown in Tables 5 and 6, respectively.
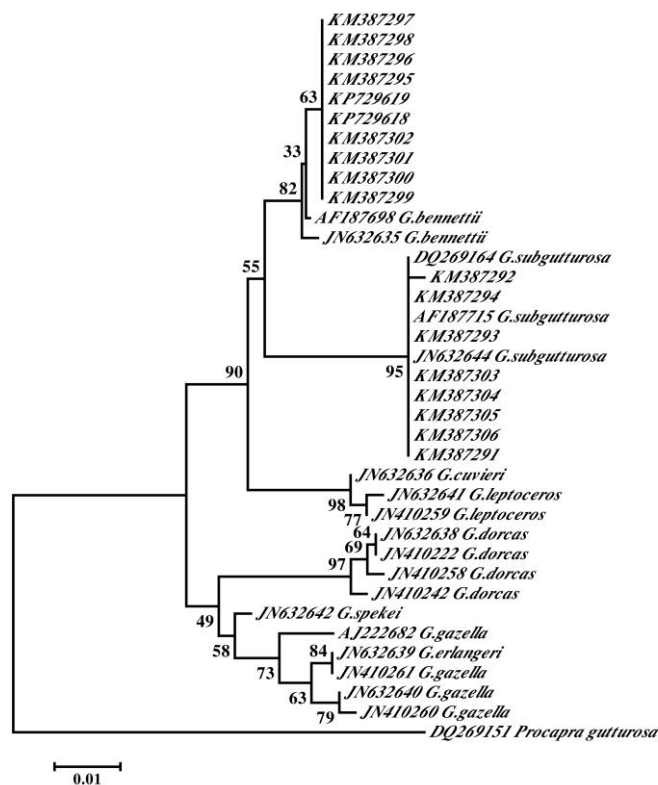
Fig. 1. Maximum Likelihood tree based on the analysis of 410 bp of Cyt-b sequences. The resulting phylogenetic tree shows Kharg and Kish Islands gazelles are *Gazella subgutturosa* and Hengam and Hormoz Islands gazelles are *Gazella bennettii.*
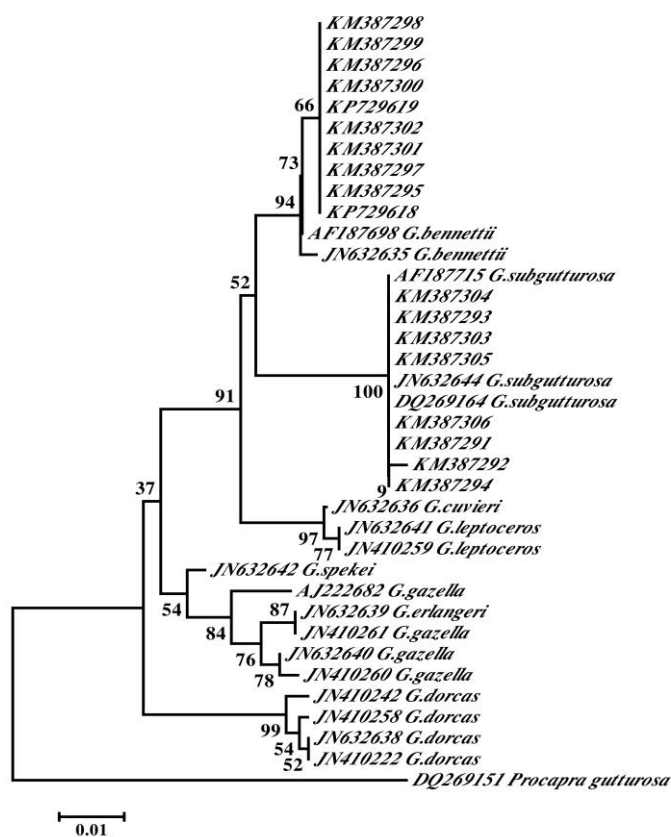

Fig. 2. Neighbor-Joining tree based on the analysis of 410 bp of Cyt-b sequences. The resulting phylogenetic tree shows that Kharg and Kish Islands gazelles are *Gazella subgutturosa*, while Hengam and Hormoz Islands gazelles are *Gazella bennettii.*

Table 4. Haplotype distribution in study areas.

Haplotype	frequency	Kish Island	Kharg Island	Hormoz Island	Hengam Island
H1	3	Kish 1 Kish 3 Kish 4			
H2	1	Kish 2			
H3	4		Kharg 1 Kharg 2 Kharg 3 Kharg 4		
H4	5			Hormoz 1 Hormoz 2 Hormoz 3 Hormoz 4 Hormoz 5	
H5	5				Hengam 1 Hengam 2 Hengam 3 Hengam 4 Hengam 5

Table 5. Molecular diversity indices in four populations.

Study area	Number of samples	Number of haplotype	Level of missing data	Number of nucleotide sites	Number of polymorphic sites	Number of observed transitions	Number of observed transversions
Kish Island	4	2	5%	410	1	0	1
Kharg Island	4	1	5%	410	0	0	0
Hormoz Island	5	1	5%	410	0	0	0
Hengam Island	5	1	5%	410	0	0	0

Table 6. Percentage of organic bases for 410 bp of Cyt-b gene.

Study area	A	G	T	C
Kish Island	31/28	14/39	26/28	28/05
Kharg Island	31/22	14/39	26/34	28/05
Hormoz Island	32/68	13/90	27/07	26/34
Hengam Island	32/68	13/90	27/07	26/34

DISCUSSION

Phylogeny of gazelles

Based on the obtained ML tree (Fig. 1), the genus Gazella are well supported by two different lineages: one comprising *G. dorcas* plus *G. gazella*, *G. spekie*, *G. erlangeri*; and the other including *G. bennettii*, *G. subgutturosa*, *G. leptoceros* and *G. cuvieri*.

According to Lerp *et al.,* (2013), the genus Gazella has two major clades: (1) predominantly Asian clades (*G. bennettii, G. subgutturosa, G. marica, G. leptocerus* and *G. cuvieri*)

and (2) a predominantly African clade (*G. dorcas, G. saudiya, G. spekei, G. gazella* and *G. arabica*). Based on their results the sister species pairs, a desert adapted form and a humid mountain adapted form can be inferred. Two desert species; *G. dorcas* in Africa and *G. saudiya* in Arabia have a sister group relationship with mountainous *G. gazella* in the Levant and *G. arabica* in Arabia. Also, this relationship has been observed within Africa between the desert slender-horned gazelle (*G. leptoceros*) and the mountainous Cuvier's

gazelle (*G. cuvieri*) of the Atlas Mountains. A third species pair occurs in Asia; Desert species goitered gazelle (*Gazella subgutturosa*) and mountainous Chinkara (*Gazella bennettii*) is one of these situations.

In the ML tree shown above, all samples of Dorcas gazelles formed a reciprocally monophyletic clade with a sister group relationship to *G. gazella, G. spekie* and *G. erlangeri*. In the second lineage, *G. cuvieri* and *G. leptoceros* clade formed a reciprocally monophyletic clade with a sister group relationship to *G. subgutturosa* and *G. bennettii*. Finally, *G. subgutturosa* is the sister taxon of *G. bennettii*. Therefore, our findings are congruent with the results from a recent phylogenetic investigation of Hammond *et al.,* (2001), Wacher *et al.,* (2010), Lerp *et al.,* (2011), Lerp *et al.,* (2013) and Fadakar *et al.,* (2013). They analyzed the mitochondrial DNA Cyt-b gene and inferred Asian clade consists of *G. subgutturosa* and *G. bennettii* both forming a reciprocally monophyletic clade in our present phylogeny. Members of the African clade include *G. cuvieri* and *G. leptocerus*, together form a highly supported monophyletic.

Also, within the NJ tree, two different lineages were well supported: one comprising *G. dorcas* and the other including *G. bennettii, G. subgutturosa, G. leptoceros, G. spekei, G. erlangeri, G. gazelle* and *G. cuvieri*. In the second lineage, all samples of *G. gazella* and *G. erlangeri* formed a reciprocally monophyletic clade with a sister group relationship to *G. spekei* and *G. cuvieri*; and *G. leptoceros* clade formed a reciprocally monophyletic clade with a sister group relationship to *G. subgutturosa* and *G. bennettii*. Finally, based on the ML tree, *G. subgutturosa* is the sister taxon of *G. bennettii*. As a result, ML and NJ trees are more congruent with other researchers' results. So, Kharg and Kish Islands gazelles are Iranian gazelle (*Gazella subgutturosa*), while Hengam and Hormoz Islands gazelles are Jebeer or Indian gazelle (*Gazella bennettii*). According to the phylogenetic trees (Figs. 1 - 2), two populations from Hormoz and Hengam Islands share the same branch, while the two other populations from Kharg

and Kish place in the other branch. Therefore, this region of Cyt-b gene is appropriate for the species level recognition of gazelles and it is not capable of distinguishing between populations of the same species. This finding is already confirmed by Fadakar *et al.,* (2013) about Iranian gazelles.

According to the results of haplotype diversity (Table 3), all the Hengam individuals belong to one haplotype. Also, four individuals of Kharg are one haplotype, while five individuals of Hormoz are another haplotype. Therefore, there was no nucleotide diversity within these populations. Indeed, analysis of 14 sequences yielded only three haplotypes, one for each population, exhibiting few number of haplotypes. As well, Kish Island individuals exhibit two haplotypes. It seems due to lack of relationship between different Islands' populations, the fewest haplotype diversity and probably inbreeding is expected in these Islands. However, the sampling process is hard and a large scale study requires for more detailed knowledge about these populations. In Kish Island, we found two different haplotypes which are distinct from all other samples, but this is probably the result of two mutational steps. In another study in Zanjan Province (Aeini *et al.,* 2007) the low level of haplotype and genetic diversity was observed in Persian gazelles of Sohrein, predicting that the population could be endangered.

Also, the high similarity between Hormoz and Hengam Island populations indicates the possible gene flow in these populations in the past. They probably were transferred to these Islands from the same initial population. Also, Kish Island gazelles were derived from Kharg Island; hence, their populations are close to each other. Base composition of cytochorome-b sequences of five samples in Hengam and Hormoz Islands (A= 32.68, C= 26.34, T=27.07, G=13.90) show a little difference to that of the four Kharg Island ones (A= 31.22, C=28.05, T=26.34, G= 14.39) and also Kish Island samples (A= 31.28, C=28.05, T= 26.28, G= 14.39) (Table 6). The overall genetic distance between Kish, Hengam and

Hormoz populations was around 0.98, while between Kharg, Hormoz and Hengam was 1.0, which according to Kankilic *et al.,* (2012), is sufficient for separation of these species from each other. Hormoz and Hengam populations do not have any genetic distance to each other, as well as Kish and Kharg ones.

Gazelles are as endangered species and exact genetic data is essential for different conservation programs such as ex-situ breeding and reintroduction or transfer of captive stocks to the potential conservation units. This situation is more complicated in small and isolated island populations confronted by different problems such as inbreeding depression and subsequent extinction. The present study is a preliminary pace of this kind of studies in these islands. Results of genetic analyses of these islands' gazelles accompanied with morphological distinctiveness, suggest that it is an evolutionary significant unit. Thus, it should take separate conservation programs with more detailed data. To provide more reliable results on phylogenetic relationship among gazelles, sequencing on the complete Cyt-b gene and also other loci such as nuclear DNA with more samples is proposed.

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تبارشناسی آهوها در برخی جزایر ایران بر اساس توالی DNA میتوکندریایی: شناسایی گونه‌ها و کاربردهای آن در حفاظت

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چکیده

گونه های مختلف آهو از جمله درمعرض خطرترین پستانداران مناطق استپی قاره آسیا هستند و در مناطق مرکزی و جنوبی ایران دیده می‌شوند. تلاش‌های حفاظتی در این نواحی در گذشته، به دلیل ابهام در درک روابط فیلوژنتیک در میان جمعیت‌های مختلف ناقص بوده است. بطوریکه برنامه‌های حفاظتی مختلف نظیر تکثیر در خارج از محل طبیعی (*ex situ*) و انتقال ذخایر توسعه یافته در اسارت به واحدهای حفاظتی بالقوه، با مشکلات اساسی روبرو بوده است. در مطالعه حاضر، روابط فیلوژنتیک آهوها بر اساس آنالیز توالی‌های ژن سیتوکروم b میتوکندری تعدادی از افراد جمع‌آوری شده از جمعیت‌های آهو در چهار جزیره کیش، خارک، هرمز و هنگام در جنوب ایران مورد بررسی قرار گرفته است که می‌تواند در یافتن روابط تبارشناختی و تشخیص گونه برای امور حفاظتی، کاربرد داشته باشد. بعد از مقایسه توالی‌های به دست آمده در این مطالعه و نیز توالی‌های اخذ شده از ژن بانک در نرم افزار مگا ۵ و تحلیل بعدی درخت فیلوژنتیک مربوط به آن‌ها، وجود دو گونه مختلف در این ۴ جزیره مشخص شد. بنابراین، به لحاظ نگاه فیلوژنتیک، آهوهای جزایر خارک و کیش، آهوی ایرانی (*Gazella subgutturosa*) و آهوهای جزایر هنگام و هرمز، جبیر یا آهوی هندی (*Gazella bennettii*) هستند. بنابراین، برای این دو گونه متفاوت بایستی برنامه های حفاظتی مجزا جهت مدیریت جمعیت‌های این مناطق اتخاذ نمود و از تبادل آهوهای این جزایر، ممانعت نمود.

*مولف مسئول