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Fertility Reduction in Male Persian Fallow Deer (*Dama dama mesopotamica*): Inbreeding Detection and Morphometric Parameters Evaluation of Semen

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Abstract

Persian fallow deer (*Dama dama mesopotamica*) is only found in a few protected and refuges areas in the northwest, north, and southwest of Iran. The aims of this study were analysis of inbreeding and morphometric parameters of semen in male Persian fallow deer to investigate the cause of reduced fertility of this endangered species in Dasht-e-Naz National Refuge, Sari, Iran. The Persian fallow deer semen was collected by an electroejaculator from four adult bucks randomly during the breeding season and from five dehorned and horned deer's in non-breeding season. Twelve blood samples were taken and mitochondrial DNA was extracted, a non-coding region called d-loop was amplified, sequenced and then were considered for genetic analysis. The Persian fallow deer's normal and abnormal spermatozoa were similar to that of domestic ruminants but very smaller and difficult to observe at the primary observation. The post-mating season collected ejaculates contained abnormal spermatozoa, debris and secretion of accessory glands in horned bucks and accessory glands secretion free of any spermatozoa in dehorned or early velvet budding bucks. Microscopic evaluation in all four bucks during the mating season showed the mean concentration of 9 × 10^6 spermatozoa/ml. The mean ± standard deviation of age, testes length and testes width was 4.60 ± 1.52 years, 3.58 ± 0.32 and 1.86 ± 0.09 cm, respectively. The results identified 1120 loci in which 377 were polymorphic. In conclusion, reduced fertility of male Persian fallow deer may be caused by inbreeding of the protected herd in a limited area of Dasht-e-Naz National Refuge.

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Keywords

Persian Fallow Deer, Genetic Analysis, Semen Quality

1. Introduction

The Persian fallow deer (*Dama dama mesopotamica*) belongs to the family Cervidae and were formerly found in Iran, Iraq, Palestine, Jordan, Lebanon, Syria, and eastern Turkey. Today, the only surviving indigenous populations are in the Dez and Karkheh Wildlife Refuge in southwestern of Iran. This species was previously abundant throughout western Asia, but, is now one of the rarest deer in the world [1]. Its taxonomic status is disputed, with some maintaining it as a subspecies of the fallow deer while others treat it as a separate species, *Dama mesopotamica* [2]. This is only found in a few protected areas in the northwest, north, and southwest of Iran [3] (**Figure 1**). Due to this condition, six animals were captured (3 males, 3 females) in order to begin the species' conservation at the Dasht-e-Naz (near the southern shore of the Caspian Sea) and the Karkheh Wildlife Refuges. One of the males was sent to Germany in 1964 as part of the European captivity breeding program [3]-[5].

The seasonality of reproduction examined in many species of the deer family. The researchers used various approaches like direct behavioral observations. The changes in pituitary hormone levels [6]-[10] and androgenic activity of the testes [11]-[15] have also been described. Deer reproduction seasonality studies are very often based on quantitative and qualitative evaluations of semen. Today, three different methods of semen collection are using: electroejaculation, artificial vagina (AV), and post-mortem epididymis recovery [16] [17]. The period of sexual activity (occurrence of libido) of Persian fallow deer buck amounted to 90 days and lasted from early August to middle April of each year with few days' variations [18]. The macroscopic appearance is the swelling of the neck, perpetual and abdominal blackish region and the harem control and defensive trait of dominant stag but the phenotypic appearance described before is the best land mark of physiological activity of stags and finally the hardness of horns [18]. Therefore, the aims of this study were analysis of inbreeding and morphometric parameters of semen in male Persian fallow deer to investigate the cause of reduced fertility of this endangered species.

2. Materials and Methods

2.1. Animals

All procedures are in accordance with animal guideline care of Ethical Committee of Islamic Azad University of Chalus, College of Agriculture and Livestock Sciences, Chalus, Iran. All applicable institutional and national guidelines for the care and use of animals were followed.

Figure 1. Male Persian fallow deer in rut season.

During two reproductive seasons 2010-2011 at Dasht-e-Naz National Refuge of Persian fallow deer which is a 55 ha woodland enclosure located at 53˚12'18"E and 36˚41'50"N, 25 km of northeast of Sari, Mazandaran province-Iran. This area was formally classified as a Wildlife Refuge in 1964 in order to conserve Persian fallow deer. It contains suitable habitat for the deer within its long established fenced area.

2.2. Semen Sampling

The Persian fallow deer stags were randomly captured during the mating season. Four adult stags in August and September, and five dehorned and horned deer's in the non-breeding season, January and February by anesthetizing using ketamine (4 mg/kg, Alfasan, Woerden, Netherlands) and xylazine (4 mg/kg, Rompun, Bayer, Suffolk, UK) which injected by darting (Dan-Inject® darting system, Dan-Inject International, Denmark). The animal's heads were covered immediately on capture. Testicular measurements were taken with a caliper and a tape after restraining. Their semen was collected. Ejaculates of each stag were collected by an electroejaculator. Using artificial vagina for collection of semen was reported previously [19].

2.3. Semen Analysis

The ejaculates were evaluated using standard procedures of semen evaluation of domestic animals. The pH of separated fractions was measured by pH-meter. Sperm concentration was determined with a light microscope. For sperm morphology analysis semen samples were prepared either as air-dried smears or fixed in formal saline. The preliminary morphological evaluation of semen was performed on 200 spermatozoa by previously reported method [20]. Morphometric measurement was done using an Image Analyzer Software (Digimizer, version 4.2.0.0) on smear stained with methylene blue and epifluorescence microscope (Nikon eclipse 50i; Nikon, Tokyo, Japan). The sperm progressive motion and duration of motility were evaluated using semen diluted in PBS at the ratio of 1:50 and assessed using a light microscope (×250) equipped with a heated block (38˚C). Color of fraction was estimated visually. The volume of the fraction at non-breeding season was measured using calibrated Eppendorf tube to the nearest 0.05 ml and at the breeding season fraction was measured using graded semen plastic collector to the nearest 0.1 ml. In addition, sperm viability was evaluated by eosin-nigrosin staining. Briefly, a fraction of each sperm suspensions were mixed with an equal volume of 0.5% eosin-nigrosin solution and smears on a glass microscope slide then they were evaluated with light microscopy in randomly 10 selected fields microscope for the percentage of vital (unstained) and dead (stained) spermatozoa.

2.4. Blood Sampling and Polymerase Chain Reactions

For genetic polymorphism analysis, 12 blood samples were taken from Persian fallow deer and mitochondrial DNA was extracted from each sample using AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea). A mitochondrial control region (d-loop) was amplified using the ProL and PheHb primers [21]. Polymerase chain reaction (PCR) reactions were conducted in a reaction volume of 20 microliters. Successful PCR amplification was verified on a 1% agarose gel containing ethidium bromide. After confirming the presence of the target product with approximately 1 kbp size, PCR reactions were repeated to produce about 150 microliters for each sample. These products were purified from agarose gel using AccuPrep® Gel Purification Kit (Bioneer, South Korea), sent to Bioneer Company (South Korea) for sequencing and then were considered for genetic analysis.

2.5. Polymorphism and Phylogenetic Analysis

As matter of fact that the length of extracted sequences was different, it was difficult to measure the inbreeding amount. Therefore, we run multiple alignments analysis adopting some gap penalties to come up with equal length sequences. For doing so, we called a function (clustal) provided in phanorn, an R add-in software. Then, Arlequin software was run to analysis these sequences. In estimating inbreeding without pedigree, heterozygosity of individual is surrogate. Inbreeding reduces heterozygosity on genome-wide scale. In this study, much emphasis was put on measures in which heterozygosity play pivotal role. As matter of fact that decephering inbreeding requires nucleous DNA seqeunces, we couldn't be able to infere the amount of inbreeding in this population. Therefore, we resort on some genetic evolutionay sudies using these sequences. In this way, we used different pacakges e.g R, Mega, DNAsnp. Since the length of DNA sequense were not equal, we first alinged

them using ClustalW program and save the result. This result underwent different analysis such as phylogentic and DNA model evolution analysis. Statistical evaluation was performed using SPSS statistical package (SPSS 11.5, SPSS Ltd., UK) for Windows.

3. Results

Analysis of Persian fallow deer semen parameters in breeding and non-breeding seasons are presented in **Table 1**. The Persian fallow deer semen, both with normal and abnormal spermatozoa, is similar to which of domestic ruminants but very smaller and difficult to observe at the primary observation. The first ejaculate that collected at the beginning of January had a watery or milky consistency. Ejaculates collected during non-breeding season contained both spermatozoa and secretion of accessory glands. Actually the period of sexual performance consist of two of sexual activity phase in Persian fallow deer, post mating season and mating season. The post-mating season collected ejaculates contained abnormal spermatozoa, debris and secretion of accessory glands in horned bucks and accessory glands secretion free of any spermatozoa in dehorned or early velvet budding bucks. From early January and mid February the ejaculates of the mating period (rut season) collected. From early August contained two typical fractions (spermatozoa and accessory glands secretion). Macroscopic appearance of ejaculates was closely related to sexual behavior of ejaculates collected in rut period and it had the consistency of cream to milky. Microscopic evaluation in all four bucks showed the mean concentration of 9 × 10^6 spermatozoa per ml. The rate of abnormality among the spermatozoa in all five bucks consists of dag defect and head less ordinary. Morphometric evaluation of Persian fallow deer spermatozoa was done on head (longitudinal and diametric) mid piece and tail as shown in **Table 2**. Also, **Figure 2** presents the normal and defected sperms in semen of Persian fallow deer.

Reproductive characteristics of male Persian fallow deer during non-breeding season for five different deer are presented in **Table 3**. The mean ± SD of age, testes length and testes width was 4.60 ± 1.52 years, 3.58 ± 0.32 cm and 1.86 ± 0.09 cm, respectively.

Table 1. Persian fallow deer semen parameters in breeding (rut) and non-breeding seasons in Dasht-e-Naz National Refuge, Sari, Iran.

Variables	Rut season	Non-breeding season
Sperm		
Concentration (×10^6 per ml)	90	ND
Motility rate (%)	25	ND
Disorders rate (%)	75	ND
Viability (%)	90	ND
Color	Milky to cream	White and clear
pH	6.8	6.5
Volume (ml)	1.8	0.75 ml

ND, not determined.

Table 2. Mean ± SD of morphometric analysis of Persian fallow deer spermatozoa using Digimizer image analysis software during rut season in Dasht-e-Naz National Refuge, Sari, Iran.

Variables	Value (μm)
Length	258.00 ± 2.00
Head	
Length	33.47 ± 4.00
Diameter	16.24 ± 1.00
Width	2.21 ± 3.00
Mid piece	49.50 ± 1.00

Twelve mitochondrial DNA sequences with different lengths were statistically analyzed to get some insight into possibility of finding inbreeding measure across these sequences. The results identified 1120 loci (assuming each nucleotide as locus) in which 377 were polymorphic. Nucleotide composition (relative values) were C (22.41%), T (31.56%), A (30.89%) and G (15.14%). Mutation analysis of mitochondrial DNA is presented in **Table 4**. Expected heterozygosity across these sequences was different with mean 0.40 and SD of 0.18. There were many nucleotides for which the degree of expected heterozygosity estimated showed more than 80%. However, we have to cross our finger in being such rigid in our argument. Phylogenetic analysis showed similar results using different algorithms. Base upon these resutls we can to some extent build up the pedigree file (**Figure 3**).

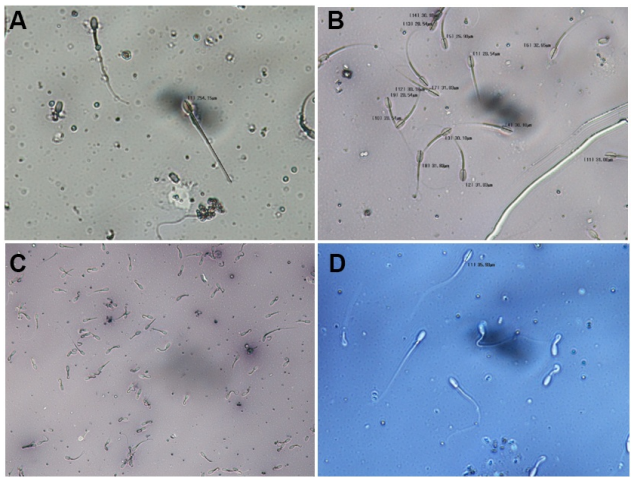

Figure 2. Morphometric evaluation (A) and (B) and disorders (C) and (D) of spermatozoa in Persian fallow deer.

Table 3. Reproductive characteristics of male Persian fallow deer during non-breeding season in Dasht-e-Naz National Refuge, Sari, Iran.

No.	Age (year)	Testes length × width (cm)	Semen volume (ml)	Sperm concentration ($\times 10^6$/ml)
1	4	3.4 × 1.9	0.25	0
2	4	3.5 × 1.8	0.25	0
3	3	3.2 × 1.8	0.2	0
4	7	4.0 × 2.0	0.2	10*
5	5	3.8 × 1.8	0.2	41**

*All of spermatozoa were dead in staining. **5% of spermatozoa were alive with individual motility.

Table 4. Mutation analysis of mitochondrial DNA of 12 blood samples was taken from Persian fallow deer in Dasht-e-Naz National Refuge, Sari, Iran.

Variables	Number	Variables	Number
Observed polymorphism		Transversions	147
Transitions	147	Substitutions	232
Transversion	147	Ins/del	164
Substitutions	294	Number of usable nucleotide sites	1120
Ins/del	164	Mean number of pair-wise differences	149.61 ± 69.08
Observed sites		Nucleotide diversity (average over loci)	0.13 ± 0.07
Polymorphic	377	Estimated mean of Theta(s)	76.82 ± 29.71
Transitions	128	Theta (Pi)	149.61 ± 77.78

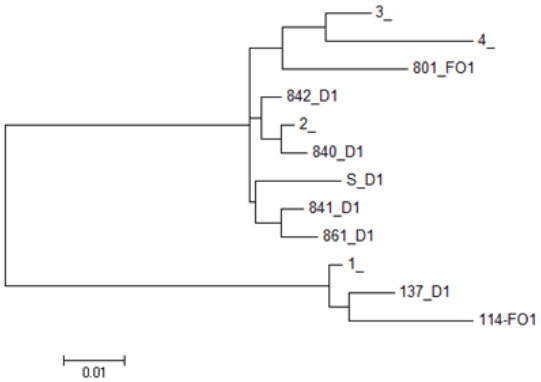

Figure 3. Maximum likelihood phylogenetic tree obtained from 12 blood samples were taken from Persian fallow deer in Dasht-e-Naz National Refuge, Sari, Iran.

4. Discussion

In the present study morphologic and sexual characteristics in addition to non-coding DNA seqeunces of 12 Iran's native deer populations in Dasht-e-Naz National Refuge were evaluated. The main purpose in this study was to get some insight into the amount of genetic proximity in this population. Because of low population of Persian fallow deer there is rare information about it. However, approximately similar studies had been performed on the other deer species. Semen characteristics of *Mazama americana*, a species of brocket deer from forests in South America, were evaluated in previous reports. In that study, five males of the species were used, and three semen samples per buck using electroejaculator were collected at intervals of 2 weeks [22]. Also, semen characteristics of pampas deer (*Ozotoceros bezoarticus*) were studied [23]. Our results demonstrated that Persian fallow deer semen had higher volume (especially in rut season) but lower pH and concentration against both deer species. However, the length, width and diameter of head and mid piece of Persian fallow deer spermatozoa were obviously higher than those of other two mentioned deer species.

The high mutation rates of mitochondrial DNA can produce intraspecific polymorphism and deep interspecific divergence in relatively short evolutionary times [24]. By using of this technique, we identified 1120 loci (assuming each nucleotide as locus) in which 377 were polymorphic. Again, although there are no reports about polymorphism in Persian fallow deer, some studies are conducted in other deer species. In an earlier study by Randi *et al.* [25], polymorphism of mitochondrial DNA in the populations of Siberian (*Capreolous pygargus*) and European roe deer (*Capreolous capreolous*) was studied. They reported that average interspecific sequence divergence was 4.9%. Also, genetic diversity in European red deer (*Cervus elaphus*) was evaluated and reported. Our results in this area are new and there are no previous reports. On the other hand, phylogenetic analysis demonstrated that our male Persian fallow deer had some inbreeding that can negatively influence their fertility. It has been demonstrated previously that Persian fallow deer from different sampling sites of Iran were genetically similar but distinct to European fallow deer, deserving to be considered as one independent management unit [26]. However, the Iranian stock should be considered of special conservation interest because it has the highest allelic richness [27]. Pureblood Persian fallow deer must be avoided from genetic contamination and one must be aware that at least 30 polymorphic markers should be used to accurately detect hybridization [28].

In conclusion, it must be remembered that genetic analysis should be stressed in breeding plans aimed at reducing the mating between relatives for preventing the inbreeding effect [29]. Reduced fertility of male Persian fallow deer which is confirmed in mating season by observation of abnormalities of sperm morphology and subnormal quality of semen using sperm analysis and evaluation of their reproductive indices may be caused by inbreeding of the protected herd of Persian follow deer in a limited area of Dasht-e-Naz National Refuge. Moreover, morphological evaluation of semen and sperm plus to sexual characteristics of male Persian fallow deer can be helpful to prevent reducing fertility.

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