Genetic Diversity And Phylogenetic Relationships Among Some Horse Breeds Reared In Egypt

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ABSTRACT

Genetic diversity among some indigenous Arabian horses and other horse breeds in Egypt were evaluated using inter simple sequence repeats (ISSR) markers which might be useful in distinguishing between the breeds. Five breeds of horses were included in this study. This technology has great potential for use in horse breeding situations where levels of genetic variation could be monitored and crossbreeding could be controlled in a commercial breeding program. Out of fifteen primers screened, five primers generated reproducible, scoreable and polymorphic bands. The results showed that a total number ISSR bands produced were 85, out of these 26 bands were polymorphic. Amplified products ranged from 368 bp to 2334 bp in size. The genetic distance had the highest value (4.900) between Thoroughbred and Oldenburg breeds and the lowest value (2.450) between Obian Arabian horse and Kuhaylan Arabian horse breeds. The dendrogram of genetic relationship based on overall ISSR primers revealed that Obian Arabian horse breed, Kuhaylan Arabian horse breed and Anglo-Arabian horse breed were clustered in one cluster, while Thoroughbred and Oldenburg were grouped in separate clusters. The present study provides information about genetic diversity among some Arabian horse and other horse breeds reared in Egypt to highlight their polymorphisms.

INTRODUCTION

Horses have shared an intimate association with human civilization as they have fulfilled key agricultural, economical and cultural roles in both historic and current societies. However, several breeds thought to exist today, many are now under threat of extinction as a result of modern industrial practices *(1)*.

Arabian horse is an important breed resource for local horses in our country. It has a lot of advantages such as powerful endurance, rough feeding resistance, beautiful appearance, suitability for riding and strong disease resistance *(2)*. These advantages have become a driving force for in-depth study on Arabian horse. Several societies have been organized world wide during the past century to determine the characteristic features and precise origin of various breeds of horses in order to preserve their unique traits. Such a society was organized in Egypt and its goal is to study the populations of Arabian horse breeds.

Genetics has an important role in horse breeding and young horses may command high purchase prices based on their pedigrees. Horse breeders pay close attention to pedigrees, avoid close inbreeding and apply selection to develop a wide range of athletic type. At the same time breeders are aware about many equine health problems that have strong genetic components including infectious disease, allergic conditions and musculoskeletal diseases. Genomic studies may be used to identify the genetic basis underlining more complex hereditary traits associated with performance, health and even suggest methods to improve the quality of horses available to horsemen *(3)*.

ISSR (Inter simple sequence repeats) markers are more likely than other methods to detect small differences between populations due to their high levels of allelic variation, being able to discriminate in both overall heterozygosity and mean number of alleles. In horse breeding this technology has the potential to be of great use in monitoring levels of genetic variation within stocks as well as for relatedness purposes *(4)*.

The joint effort of breeders is to use the quality sources of the pure Arabian horse for the regeneration of the breed and its registration as a

genetic source of horses in the Arab Republic of Egypt. To achieve this target it is necessary to characterize the differences of the Arabian horse types in relation to other horse breeds.

The main objective of the study is to evaluate the level of genetic diversity among the indigenous Arabian horse breeds and other breeds using a set of fifteen ISSR primers and deduce the phylogenetic relationship between them.

MATERIALS AND METHODS

The present work was carried out on five different breeds of horses reared at El-Sharkia and Cairo governorates. The age of animals ranged from 3-8 years old. The molecular techniques were performed in the Central lab of Genetics in Genetics Department, Faculty of Agriculture, Ain Shams University, Cairo.

I. Animals studied

A total of 50 horses representing five horse breeds (Thoroughbred, Obian, Oldenburg, Anglo-Arabian and Kuhaylan), each breed represented by ten animals.

II. ISSR- PCR:

A. Preparation of blood sample

Blood samples for DNA extraction were collected on EDTA as anticoagulant and then stored at –20°C.

B. Genomic DNA Extraction and Purification

Extraction of genomic DNA was performed from whole blood using AxyPrep Blood DNA Miniprep Kit (Cat. no. AP-MN-GDNA-50, Axygen Bioscience, CA, USA) following the manufacturer protocol. The estimation of the DNA concentration in different samples was done by U.V spectrophotometer.

C. Polymerase Chain Reaction (PCR) conditions

Genomic DNA from each ten blood samples of each population were mixed to obtain bulked sample. Fifteen ISSR primers were used in the study (Table 1). Primers were initially screened to identify well-amplified polymorphic bands among populations. PCR reaction was performed in 25 µl final volume using Perkin Elmer 2400 (Germany) thermal cycler. Samples were preheated for 4 min at 94°C, subjected to 40 cycles (94°C for 60 sec, 40°C for 120 sec and 72°C for 120 sec) and a final volume for 7 min at 72°C.

Table 1. Names and sequences of the ISSR primers used.

No.	ISSR primers	Sequences	PCR product (bands)
1	814	$(CT)_8TG$	+
2	844A	$(CT)_8AC$	-
3	844B	$(CT)_8GC$	-
4	17898A	$(CA)_6AC$	-
5	17898b	$(CA)_6GT$	+
6	17899A	$(CA)_6AG$	-
7	17899B	$(CA)_6GG$	-
8	HB8	$(GA)_6GG$	-
9	HB9	$(GT)_6GG$	-
10	HB10	$(GA)_6CC$	-
11	HB11	$(GT)_6CC$	+
12	HB12	$(CAC)_3GC$	+
13	HB13	$(GAG)_3GC$	-
14	HB14	$(CTC)_3GC$	+
15	HB15	$(GTG)_3GC$	-

The resulting PCR products of ISSR-based PCR analyses were detected using agarose gel electrophoresis (1.2% in 1X TBE buffer), stained with ethidium bromide (0.3 ug/ml), visually examined with UV transilluminator and photographed using a CCD camera (UVP, UK).

D. Data Analysis

Clear, unambiguous and reproducible bands were considered for scoring. Each band was considered a single locus. Data were scored as (1) for the presence and (0) for the absence of a given band. Band size was

estimated by using Totallab, TL120 1D v2009 (nonlinear Dynamics Ltd, USA). Data were analyzed using qualitative routine to generate similarity coefficient and used to construct a dendrogram using unweighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering routine. The cluster analysis and dendrogram construction were performed with *STATISTICA (Stat Soft, Inc., 2007) (5)* and *NTSYSpc (Ver. 2.1) (6)*.

RESULTS AND DISCUSSION

Genetic diversity and relatedness between the five horse breeds was evaluated using fifteen ISSR primers, out of these, five primers used in the study were successful in generating reproducible and reliable amplicons for different horse breeds. These primers yield informative and identifiable ISSR DNA markers and revealing differences between breeds. All Polymorphic ISSR products were confirmed by repeating the PCR reaction. A fragment was considered polymorphic when absent in at least one breed. These primers detected scoreable polymorphisms in banding patterns among the five horse breeds. The banding pattern obtained from ISSR primers (814, 17898b, HB11, HB12 and HB14) are shown in figures 1, 2, 3, 4 and 5.

Genetic relationships among the horse breeds based on ISSR primers

In this study, initially fifteen ISSR primers were tested to amplify the pooled DNA samples. Reproducible and distinct ISSR profiles in one or more breeds were generated through five primers out of the fiifteen primers, whereas the other primers failed to produce polymorphic patterns. From these primers, a total of 85 ISSR bands (Table 2) were obtained from the five horse breeds. Amplified products ranged from 368 bp to 2334 bp in size.

Table 2. Number of amplified ISSR fragments from the five breeds of horses.

Primer	Breed					Total
	Thoroughbred	Obian	Oldenburg	Anglo-Arabian	Kuhaylan	
814	6	4	4	4	4	22
17898b	4	3	3	3	3	16
HB11	2	2	3	3	3	13
HB12	3	3	6	6	3	21
HB14	3	3	2	3	2	13
Total	18	15	18	19	15	85

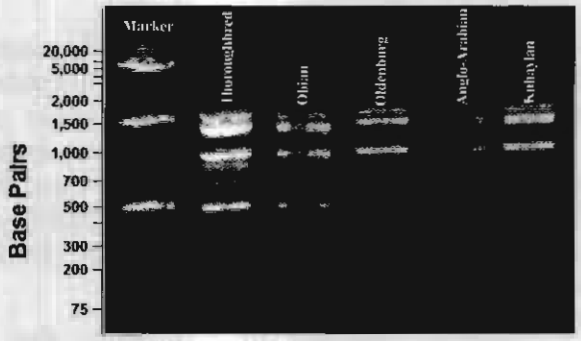

Figure 1. Electrophoretic banding pattern of ISSR fragments produced with primer 814.

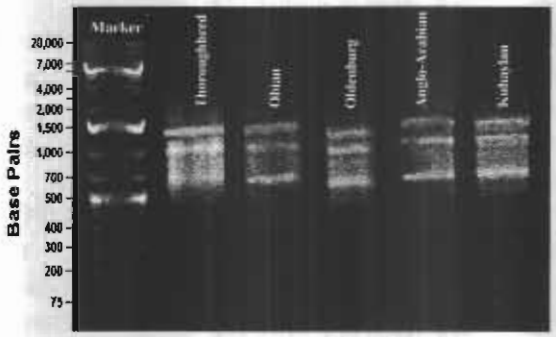

Figure 2. Electrophoretic banding pattern of ISSR fragments produced with primer 17898b.

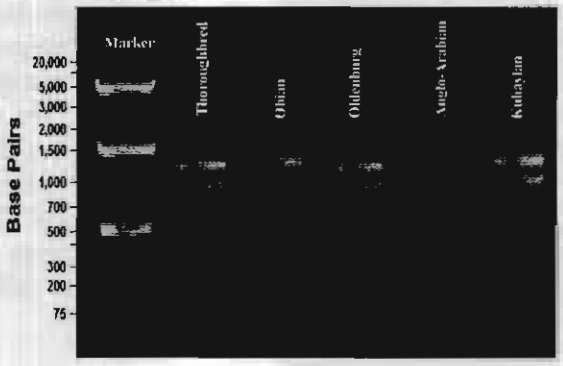

Figure 3. Electrophoretic banding pattern of ISSR fragments produced with primer HB11.

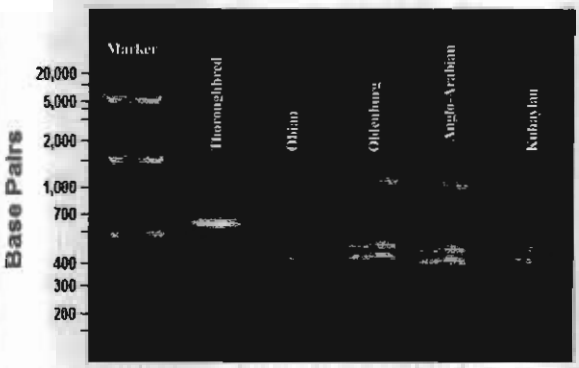

Figure 4. Electrophoretic banding pattern of ISSR fragments produced with primer HB12.

Figure 5. Electrophoretic banding pattern of ISSR fragments produced with primer HB14.

The number of amplified ISSR fragments from the five breeds of horses was illustrated in Table (2). Primer 814 showed the highest total number of amplified bands (22) for all breeds (Thoroughbred, Obian, Oldenburg, Anglo-Arabian and Kuhaylan) collectively while primers HB11 and HB14 showed the lowest number (13). Anglo-Arabian breed showed the highest number of amplified ISSR fragments (19) after using all the five primers while Obian and Kuhaylan breed showed the lowest number of amplified ISSR fragments (15) after using all the five primers.

The number of polymorphic bands per primer ranged from 1 to 10 with an average 5.2 bands/ primer (Table 3). Moreover, the total number of ISSR bands was 32, out of these 26 were polymorphic. Primer HB12 recorded the highest number of polymorphic bands (10) with a percent of 100% while primers HB14 recorded the lowest number (1) with a percent of 33%. On the same line, the number of bands per each primer ranged from 4 (HTG7) to 9 (VHL20, HTG10, ASB2) in a study on genetic diversity of Hucul horse breeds *(7)*. Moreover, genetic relationships between the Haflinger, Maremmano and Arabian breeds were studied and a total of 94 bands were detected across the breeds, with a mean of 8.5 bands per primer and the number of observed bands was highly variable, ranging from 5 (HTG7) to 14 (ASB2) *(8)*.

Table 3. Total No. of bands, % of polymorphic loci from the ISSR primers.

Primer	Total No. of bands	No. of polymorphic bands	% of polymorphic loci
814	6	2	33
17898b	8	8	100
HB11	5	5	100
HB12	10	10	100
HB14	3	1	33
Total	32	26	---

The high allele (band) frequency observed for some of the horse breeds studied indicates the possibility that they might be considered as a marker bands for each breed.

The Similarity coefficients among the studied horse breeds based on amplified ISSR-PCR fragments using all ISSR primers was estimated in table (4). The highest value of similarity (0.833) was recorded between Obian and Kuhaylan breeds, while the lowest similarity (0.455) was recorded between Thoroughbred and Oldenburg breeds.

Table 4. Similarity coefficients (Dice Similarity Measure) among the studied horse breeds.

Breed	Thorough-bred	Obian	Oldenburg	Anglo- Arabian	Kuhaylan
Thoroughbred	1.000				
Obian	0.615	1.000			
Oldenburg	0.455	0.564	1.000		
Anglo Arabian	0.636	0.718	0.636	1.000	
Kuhaylan	0.683	0.833	0.683	0.780	1.000

Genetic distances among the studied five horse breeds are shown in Table (5). The genetic distances ranged from 2.450 (most related) between Obian and Kuhaylan breeds to 4.900 (distantly related) between Thoroughbred and Oldenburg breeds. The lowest genetic distances between Obian and Kuhaylan breeds may be due to that the functional genetic pool of these breeds is similar and they might have common breeding origins.

Table 5. Genetic distances, calculated as the total number of ISSR band differences, among the studied horse breeds.

Breed	Thorough-bred	Obian	Oldenburg	Anglo- Arabian	Kuhaylan
Thoroughbred	0.000				
Obian	3.870	0.000			
Oldenburg	4.900	4.120	0.000		
Anglo Arabian	4.000	3.320	4.000	0.000	
Kuhaylan	3.610	2.450	3.610	3.000	0.000

In the present study, high genetic diversity level reported between the Thoroughbred horse breed when compared to the Obian and Kuhaylan Arabian horse breeds. In another study, genetic diversity between Thoroughbred and Arabian horse populations were analyzed using 12 microsatellite markers, ten were highly polymorphic and two were low polymorphism. Concerning AHT4, HTG10 and VHL20 loci, Thoroughbred population exhibited different alleles compared with Arabian population. The highest levels of heterozygosity exist on ASB2 locus, in both populations. Moreover, no significant differences were observed between the two populations in expected heterozygosity suggesting that more intensive breeding practices may have resulted in a further erosion of genetic variability ***(9)***.

According to Food and Agriculture Organization recommendations, highly polymorphic microsatellite markers are the method for investigating genetic relationships and breed differentiation ***(10)***.

The recent developments in molecular genetics allowing the detection of genes responsible for economic traits have opened a new area in farm animal selection, including horses. Recently, breeders have turned to molecular biology and use PCR (Polymerase Chain Reaction) for detection of inter simple sequence repeats (ISSR) and short sequence repeats (STR), which are also referred to as microsatellites. These markers are evenly distributed across genome and highly polymorphic. The use of microsatellites as a marker provides a sensitive method for individual identification. It can also be used to screen for markers linked to performance traits or genetic disorders ***(11)***.

Genetic diversity in three samples from three breeds of horses (Arabian, Thoroughbred and Anglo-Arabian horses) were studied using 12 microsatellites, based on average heterozygosity ratio. Variability was relatively lower in Thoroughbred horse (0.7036), while it was almost the same in Arabian and Anglo-Arabian horses (0.7217 and 0.7232 respectively) ***(12)***. The author indicated that the test using DNA molecular markers analysis constitute a highly efficient and reliable alternative for the identification of individual horses and is a useful tool for horse breeders and horse registries.

Furthermore, in another study, Thoroughbred has the high genetic diversity level when compared with Arabian and Pantaneiro horses ***(13)***.

The breed relationships based on genetic distances are presented in dendogram format (Fig. 6). Table 6 simplifies the visualization of clusters to compare the effects of distance measure on reconstruction of breed relationships. Cluster II was the largest having three breeds (Obian, Kuhaylan and Anglo-Arabian), while clusters I (Thoroughbred), III (Oldenburg) each had single breed.

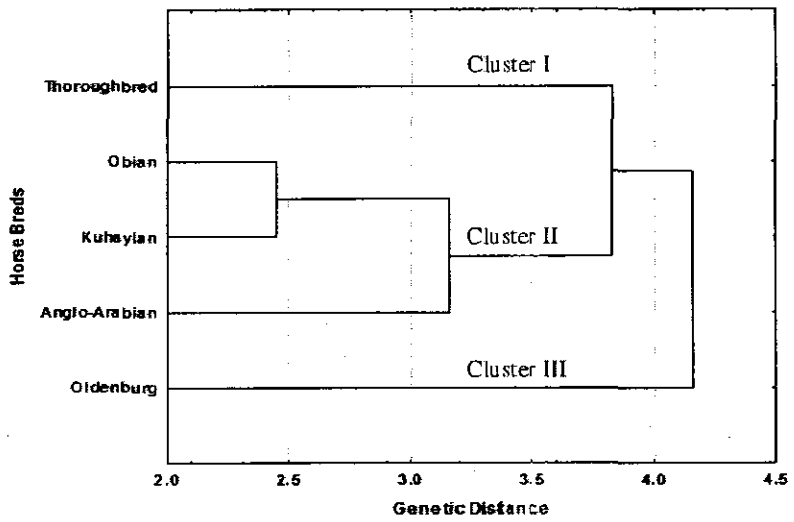

Figure 6. Phylogenetic tree (Linkage dendrogram) of studied horse breeds based on amplified ISSR-PCR fragments using all ISSR primers.

Table 6. Grouping pattern of the studied five horse breeds based on analysis of their ISSR band differences.

Cluster	Number of genotypes	Horse breeds falling in cluster
I	1	Thoroughbred
II	3	Obian, Kuhaylan and Anglo-Arabian
III	1	Oldenburg

The Anglo-Arabian horse breed was clustered away from Thoroughbred. Conversely, another similar study on seven Japanese, four mainland-Asian horse populations and two European horse populations clustered Anglo-Arab and Throughbred horse breeds in one European cluster *(14)*.

Despite the variability that was found between breeds in this study, ISSR markers failed to discriminate between Obian and Kuhaylan Arabian horse breeds except for primers HB11 and HB14. The Obian and Kuhaylan Arabian horse breeds showed limited genetic variability across several genetic markers, most likely due to the extensive cross-breeding that has taken place over the years, resulting in a mixing of breeds.

The ISSR marker analysis of the different horse breeds under the study revealed that the Kuhylan, Obian and Anglo-Arabian horse breed stocks was genetically rather heterogeneous and had considerable genetic resources. This especially applied to the Kuhylan and Obian Arabian horse breeds.

The use of ISSR markers were able to consistently separate Arabian breeds from Thoroughbred breed for various distance measures. This separation is consistent with theories that the Arabian breeds evolved from one ancestral type of horse, while Thoroughbred

breed evolved from a different ancestral type *(15)*.

The placement of the Anglo-Arabian horse breed between the two Arabian breeds is consistent with the documented origin of the Anglo-Arabian horse breed, from cross between Arabian lines and Thoroughbred. Historical accounts suggested that some influence of a breed into another breed has occurred as a result of crossbreeding between the breeds. Close relative crossing might cause a decrease in heterozygosity. Furthermore, a decrease in the heterozygosity level could be due to the selective advantages of different alleles in different loci or different selection criteria for different alleles *(16)*.

In conclusion, the present work contributes to the knowledge of population structure and assessment of existing genetic diversity among five indigenous horse breeds. Further genetic analysis of these horse breeds and their comparisons need to be carried out to determine the phylogenic evolutionary relationships and genetic distances among the indigenous equine breeds.

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الملخص العربي

التنوع الوراثي وعلاقات القرابة الوراثية بين بعض سلالات الخيول المرباة في مصر

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تم تقييم التنوع الوراثي بين بعض سلالات الخيول العربية المحلية وبعض سلالات الخيول الأخرى الموجودة في مصر باستخدام (ISSR) والتي قد تكون مفيدة في التمييز بين السلالات. حيث تم إجراء هذه الدراسة على خمس سلالات من الخيول. أوضحت هذه التكنولوجيا (ISSR) أن لها دور قوي كبير إذا ما استعملت في تربية الخيول حيث تمكن من رصد مستويات التنوع الوراثي كما تمكن من التحكم في برامج التهجين المستخدمة عند تربية الخيول والخلط بين السلالات. خمسة بادءات من أصل خمسة عشر مستخدمة هم فقط من أنتجوا حزم متعددة الاشكال. أوضحت النتائج أنه تم الحصول على عدد 85 حزمة من ISSR منها 26 حزمة متعددة الأشكال. تراوحت أحجام الحزم الناتجة من 368 إلى 2334 زوج من القواعد وتراوحت المسافة الجينية في أعلى قيمة لها (4.900) بين سلالة الحصان الإنجليزي وسلالة الأولدنبورغ وأدنى قيمة لها (2.450) بين سلالات الحصان العربي العبيان والكحيلان. أوضح الدندوجرام الناتج من استعمال كل بادءات ISSR مجتمعة أن سلالات الحصان العبيان والكحيلان و الأنجلو العربي كلهم جمعوا في مجموعة واحدة ، في حين أن سلالات الخيول الإنجليزية و الأولدنبورغ يقع كل منهما في مجموعة منفصلة. هذه الدراسة تقدم المعلومات حول التقدم المحرز في مجال التنوع الوراثي بين الخيول العربية المصرية وغيرها من سلالات الخيول ، وبالتالي تبرز تعدد الأشكال الوراثي فيما بينهم.