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Mitochondrial DNA sequencing of Kehilan and Hamdani horses from Saudi Arabia

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ABSTRACT

The Arabian horse breed is well known for its purity and played a key role in the genetic improvement of other horses worldwide. The mitochondrial genome plays a vital role in maternal inheritance and it's helpful to evaluate its genetic diversity and conservation. It has higher mutation rates than nuclear DNA in vertebrates and therefore reveals phylogenetic relationships and haplotypes. In this study, the mitochondrial genome mutations in two Saudi horse strains, Kehilan and Hamdani demonstrated various changes in the gene and amino acid levels and included two other Saudi horses (Hadban and Seglawi) from the previous study for phylogenetic comparison. The whole mitochondrial genome sequencing resulted in intra and inter mtDNA variations between the studied horses. Interestingly, the Hamdani horse has nucleotide substitutions similar to those of the Hadban horse, which is reflected in the phylogenetic tree as a significantly close relationship. This type of study provides a better understanding of mitogenome structure and conservation of livestock species genetic data.

1. Introduction

Mitochondrial DNA (mtDNA) has become an essential tool for studies on maternal inheritance, population genetics, evolution and phylogenetic relationships. Its haploid, heteroplasmic and unlike nuclear genomes, has recombinations and higher mutation rates, making it reliable for biodiversity studies (Myćka et al., 2022). The control region of mtDNA is known for its maternal inheritance and higher mutation rate (Hutchison et al., 1974; Ishida et al., 1995, 1994). Some of the studies focused particularly on the control region, which is the hypervariable and small region to explore the variations and useful in population and phylogenetic analysis of large samples (Kavar and Dovč, 2008; Luís et al., 2006; Vilà et al., 2001). There is an region of particular interest in mtDNA related to heredity, known as the Displacement loop (D-Loop) which is about 1100 bp in length (Ishida et al., 1995) with a mutation rate of 2.8 to 5 times greater than the rest of mtDNA (Aquadro and Greenberg, 1983; Cann et al., 1984). Mammalian species have been recorded with intraspecific differences in their mtDNA (Achilli et al., 2012; Lippold et al., 2011).

The first horse mitochondrial genome sequencing (Xiufeng and Árnason, 1994) paved the way for the diversity and evolutionary studies in zebra and donkey (Ann Oakenfull et al., 2000) and ancient Equus mtDNA analysis for phylogeny and evolution (Vilstrup et al., 2013). Horses were domesticated 5000 years ago and since then they formed inseparable connection with people (Anthony, 2010; Librado et al., 2021). They are famous due to their inclusion in the cultural traditions and specific features apart from their economic significance (Bower et al., 2012). During domestication, wild horses vastly contributed to the domestic horse diversity and there were 18 haplotypes found in modern horse mtDNA sequences (Achilli et al., 2012; Lippold et al., 2011). A significant mtDNA diversity was observed among the domestic horse populations which were geographically bounded and maternally inherited (Achilli et al., 2012; Cieslak et al., 2010; Jansen et al., 2002; Lippold et al., 2011; Vilà et al., 2001). The domestication process was procured by the gene flow of maternal inheritance which was familiar to the early breeders (Petersen et al., 2013; Wallner et al., 2013). Maternally inherited mtDNA analysis revealed remarkable diversity compared to paternal or nuclear genome (Lindgren et al., 2004; Ling et al., 2010). However, one of the recent studies used Y chromosome markers to trace the Arabian sire and successfully record the breeding data for the past 200 years in west (Remer et al., 2022).

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The genetic ancestral information of horse breeds origin or ancestry are usually stored in studbook records but can also be revealed using genomic investigation studies or mtDNA analysis which can verify or negate studbook information (Raudsepp et al., 2019). mtDNA exploration in horses deduced the place of origin, phylogeny and evolutionary analyses to characterize the different founder mare breeds (Aberle et al., 2007; Bowling et al., 2000; Głazewska, 2010; Hill et al., 2002; Royo et al., 2005). The Eurasian steppe was believed to be an important place of domestication with its widely shared overland routes (Anthony and Brown, 2003; Benecke and von den Driesch, 2003; Kuzmina, 2003; Levine, 1999; Olsen, 2003). One of the studies reported that the Iberian Peninsula was also possibly the site of horse domestication (Lopes et al., 2005). One of the recent studies reported that there are 18 haplogroups based on horse mtDNA (A-R) and majority of them were domesticated in Asia (Gáspárdy et al., 2023). They were not well differentiated geographically due to the mixing and modern breeding methods. Due to the widespread biogeographical patterns of large, domesticated horses, their genetic diversity is more complex (McGahern et al., 2006). They have been used as carriers during prehistorical times and have undergone considerable breed mixing leading to a significant alteration in their genetic structure. However, mtDNA plays a key role in determining the origin of breeds (Gáspárdy et al., 2023).

The Arabian horses were mentioned as the oldest breeds dispersed globally and they contributed to the improvement of other horse breeds (Bowling et al., 2000; Głazewska, 2010; Khanshour et al., 2013; Remer et al., 2022; Zechner et al., 2002). However, there are certain places where the Arabian bloodlines have not been introduced (Głazewska, 2010; Hendricks, 1995). During the 7th to 9th centuries, the Persian horse significantly contributed to the Central Asian and European lines validated by the ancient DNA (Fages et al., 2019). The Arabian horses got attention in the 18th century among Western world and the breeders from Europe started importing them from Middle East. The 19th century and early 20th century in Europe marked the introduction of Arbian horses in for breeding. The practice was widely adopted by a number of countries and established various horse breeds based on Arabian horse such as British, Egyptian, French, Polish, Russian and US Arabian. There are 63 registered Arabian pedigree records globally according to the World Arabian Horse Organization (WAHO) (https://www.waho.org). Generally, outside the Middle East, selective breeding is implemented whereas in the Arabian horse population majorly followed maternal breeding which resulted in a few Arabian male lines known as tail male lines (Remer et al., 2022).. The Arabian stallions are spread over worldwide and they were restored in their places of origin (WAHO). Bedouins bred the Arabian horses known as Kehilan, Seglawi, Hamdani, Hadban and Abeyan and historically they were known for their purity and strict breeding lines (Głazewska, 2010; Pruski, 1983). Glazewska, (2010) stated the pedigree records of American Arabian and Western Arabian (Shagya and Polish) horses as no older than 100 and 200 years respectively. Syrian, Saudi Arabian and Iranian horse populations have different registered maternal pure lines as 'Arabians' and are referred to as the Middle Eastern horse populations (Khanshour et al., 2013). There is a popular belief that the Arabian horses were derived from five founder mares, however the modern haplogroups suggest that they have mixed clustering (Wang et al., 2016).

There are little or few studies on partial mitochondrial gene analysis of Saudi Arabian horses, to this end, this study aims to sequence the whole mitochondrial genome of two horse strains, Kehilan and Hamdani to find the variations and analyze them phylogenetically.

2. Materials and methods

2.1. Sample information and ethical approval

Acquired the blood samples of two horses Kehilan and Hamdani from the jugular vein at private farms in Jeddah, Saudi Arabia. Animal ethics approval was obtained and the experiments were carried out as per the guidelines of the Animal Ethics Research Committee (Reference No. 298–14).

2.2. Genomic DNA extraction, integrity and quantification

The blood samples were drawn into the labeled EDTA tubes under aseptic conditions and then carried on an icebox to the lab and stored at −20 °C. Genomic DNA was isolated using 0.2 ml of blood through a Qiagen DNA extraction kit as per the manufacturers instructions (Cat. No. 51104; Hilden, Germany). In the final step the extracted genomic DNA was eluted into Tris Ethylenediaminetetraacetic acid buffer (TE) to avoid degradation. The DNA concentration was quantified by agarose gel electrophoresis and spectrophotometer which was stored at −20 °C until further usage.

2.3. Polymerase chain reaction (PCR) and sequencing

The primers were obtained from the previous study (Xu et al., 2007) and synthesized from Macrogen Inc, South Korea (Table 1). The extracted DNA was used in PCR amplification. The 25 μL of PCR reaction mixture containing; 5 μL Jena Bioscience Taq PCR Master Mix (Taq DNA Polymerase, PCR Buffer, MgCl2, and dNTPs), 2 μL of template DNA (100 ng), 1 μL of each forward and reverse primers (10 picomoles) and distilled water to make up the 25 μL volume. The following conditions were set on the PCR machine (MULTIGENE, Labnet International Inc., NJ, US); Initial denaturation at 95 °C for 5 min, 34 cycles of denaturation at 95 °C for 30 sec, annealing at 56–58 °C for 30 sec, initial extension at 72 °C for 90 sec (primers 9 and 10 for 150 sec) and a final extension at 72 °C for 10 min. The amplicons were stained on 1% agarose gel with ethidium bromide and the amplified band size was confirmed with a 1 kb DNA marker. Multiple sets of overlapping primers resulted in accumulation of amplicons with varying size. These amplicons were purified and sequenced (Sanger Sequencing) from Macrogen Inc., Korea.

Table 1
MtDNA primers used to amplify the whole mitochondrial genome.

S. No	Sequences	Amplified length in bp	Amplified region on mtDNA
1	F: TAACATGAATCGGCGGACA	2000	15,191
	R: TTGCTGAAGATGGCGGTAT		706
2	F: ATTTCCATAGACAGGCATCC	1500	16,599
	R: TCACCTCTACCTACGAATCTTCT		1,439
3	F: CGTAAGGGAACGATGAAAGAT	1400	1,232
	R: AATAGATAGAAACCGACCTGGAT		2,565
4	F: ACGAGAAGACCCTATGGAGC	2000	2,169
	R: GTGGAATAGGTTAGTCGTATGTAG		4,835
5	F: TACCTCTAACCACTACACTAATC	1500	4,646
	R: CATAATGGAAATGTGCTACTA		6,499
6	F: CGAGCATACTTCACATCAGC	2000	6,265
	R: TAGCGAAAGAGGCGAATAGA		7,995
7	F: GACTTTACTACGGTCAATGCTC	1500	7,616
	R: TTTCCTTCTATTAGGCTATGGT		9,108
8	F: CCACCCACAGGTATCCAC	2100	8,993
	R: GATGAGGACGGCTACGAT		11,113
9	F: GAGGCTACGGAATACTACGAA	1500	10,992
	R: TAAAGGATTGCTTGAAGGG		12,303
10	F: GCTAACAACCTTTTCCAACTG	2500	12,182
	R: GACCAGGGTAATGTGCGATA		15,359

2.4. Sequencing analysis

The codon code aligner V.5.0 program (https://www.codoncode.com/aligner/) was used to analyze the sequencing data such as trimming, editing, confirming the SNP's on both sequences (forward and reverse) and nucleotide base quality was assessed on chromatogram peaks and quality Phred score 20 or above. The reference sequence also used to crosscheck errors and missing data in sequenced files. Then, the purified nucleotide sequences were aligned to the reference sequence and edited each sequence either manually or automatically. Additionally, they were translated using the codon code software and analyzed at the amino acids level to observe the variations and compare them with reference sequences (JN398377 and X97337.1).

2.5. Phylogenetic relationship

The Molecular Evolutionary Genetics Analysis (MEGA6) program (https://megasoftware.net/) was used to build a Neighbor-joining phylogenetic tree (Tamura et al., 2013) and included two mitochondrial sequences of Genbank accessions MK100122.1 (Hadban horse) and MK100123.1 (Seglawi horse) from the previous study. Some other horse mitochondrial Genbank sequences were included in the analysis and *Equus asinus* (X97337.1) was considered as an outgroup.

2.6. Bioinformatic tools

The sequence data from NCBI were aligned in MEGA and imported to DnaSP v5 (https://www.ub.edu/dnasp/DnaSP32Inf.html) program. The data file was converted to Roehl Data File (rdf file) format which provided the general information of haplotype data. We opened the rdf file in Network 10.2 (fluxus-engineering.com) to create the median joining network with default settings and draw the haplotype network. The NCBI sequences of cytb gene used were (OQ320039.1, OQ331231.1, JQ340098.1, FJ765122.1, MG001421.1, MG001420.1, MG001414.1, KT368732.1, JQ340168.1, JQ340167.1, Q340153.1, JQ340152.1, JQ340135.1, JQ340124.1, JQ340123.1, GU734783.1, KF038159.1, AP013093.1, AP013089.1, MK449357.1, MK467454.1, HQ439448.1, JN398454.1, JN398453.1, JN398450.1, JN398449.1, JN398448.1, JN398447.1, JN398446.1, HQ439500.1, HQ439477.1, HQ439465.1, HQ439457.1, HQ439450.1, HQ439448.1, HQ439447.1, HQ439444.1, DQ223536.1, DQ297656.1, FJ765139.1, FJ765112.1, MN187576.1, MN503280.1, MK100122.1, MG001436.1, MG001435.1, MG001432.1, MG001430.1, MG001429.1, MG001427.1, MG001426.1, KT221837.1, KT757754.1, KT757739.1, KT368738.1, KT368731.1, KT368726.1, JQ340161.1, JQ340149.1, JQ340125.1, JQ340116.1, JQ340102.1, JQ340099.1, JQ340096.1, KT368740.1, ON168403.1, KJ917295.1, KJ917268.1, AP013102.1, AP013101.1, AP013084.1, AP013080.1, KC202959.1, KC202957.1, KC202956.1, MK449358.1, MK467453.1, JN398377.2, JN398397.1, JN398394.1, JN398393.1, JN398392.1, JN398391.1, JN398388.1, JN398387.1, JN398386.1, JN398385.1, JN398384.1, JN398383.1, JN398382.1, JN398381.1, JN398380.1, JN398378.1, HQ439491.1, HQ439488.1, HQ439487.1, HQ439475.1, HQ439471.1, HQ439469.1 and HQ439458.1.

3. Results

3.1. Mitochondrial genome compositions and variations of two Saudi Arabian horses

The sequenced mitogenomes of Kehilan and Hamdani horses ranged from 16649 bp to 16655 bp respectively and presented similar characteristics. The mitochondrial genome structure and organization was similar to other horses and composed of 13 protein coding genes, 22 transfer RNA (tRNA) and 2 ribosomal RNA (rRNA) along with the control region. The nucleotide base composition of both the mitochondrial genomes were similar; A (32.1%), G (13.4%), T (25.8%), C (28.6%) except Kehilan which differed in A (32.2%) base composition by 0.1%. The estimated GC content was 42%. The estimated haplotype diversity (Hd) was 1.00 and nucleotide diversity, Pi = 0.008. The genomic DNA was extracted, amplified (through overlapping mtDNA primers) and all the sequences were assessed critically to analyze the differences with respect to the reference sequence. Both forward and reverse sequence analysis verified the particular SNP's chromatogram peaks. This was further assessed by reference sequence and quality Phred score to refine the variable sites. This study revealed 109 genetic variations including transitions, transversions, insertions and deletions in the studied horse strains (Table 2). Hamdani horse represented the highest number (109) of substitutions compared to the Kehilan (66). The edited sequences were submitted to Genbank of Kehilan (OQ320039) and Hamdani (OQ331231).

3.2. Differences in coding region of the mitochondria

The protein coding genes exhibited 53 amino acids changes in Kehilan (9) and Hamdani (44) strains as shown in Table 3. The horse reference sequence (HRS) JN398377 and X79547 were used as reference sequences. The HRS differs from X79547 at nucleotide positions 357 and 358, where two C's were removed, and the nucleotide positions are highlighted in bold font according to the X79547 representation in Table 3. Hamdani showed higher variations at DNA and protein levels than Kehilan.

In gene ND1 there was one amino acid change from Lys-Arg at 3387 nucleotide position in Kehilan horse whereas in Hamdani there were two changes at 3306 and 3494 from Arg-Met and Pro-Thr respectively. There were no changes observed in the ND2 gene of any studied strains. In COX1 and COX2 genes, there was each amino acid modification (6785 Ile-Val and 7628 Pro-Ser) in Kehilan and in Hamdani 4 (5888 Ser-Pro, 6312 Pro-Leu, 6507 Pro-Leu and 6714 Thr-Ile) and 3 (7245 Gln-Arg, 7614 Leu-Gln and 7668 Cys-Tyr) amino acid changes respectively. The genes ATP8, ATP6, COX3, ND3, and ND4L didn't result in any mutations in Kehilan, while in Hamdani, there were 1 (7902 Thr-Ile), 6 (8010 Asn-Ser, 8243 His-Tyr, 8363 Asn-Asp, 8366 Thr-Ala, 8561 Ser-Thr, and 8570 Ser-Pro), 0, 2 (9705 Tyr-Cys and 9777 Leu-Pro), and 0 amino acid modifications detected, respectively. The ND4 gene from both the strains has two amino acid changes i.e kehilan-10829 Leu-Val and 11,555 Ser-Pro; Hamdani-10829 Leu-Val and 11,269 Met-Ile, observed when compared to JN398377. However, according to the reference X79547 there was an amino acid change Leu-Val at 10,829 nucleotide position in both strains which is highlighted in bold letters in Table 3. In gene ND5 there were two amino acid changes (12863 Trp-Arg and 13,052 His-Tyr) whereas in Hamdani 20 amino acid varia-tions (11792 Ile-Val, 11,862 Pro-Leu, 11,882 Thr-Ala, 11,888 Arg-Cys, 11,945 His-Tyr, 11,954 Arg-Trp, 11,969 His-Tyr, 11,988 Ser-Asn, 12,086 Arg-Gly, 12,128 Pro-Ser, 12,144 Pro-Leu,12149 His-Tyr, 12,164 His-Tyr, 12,191 Ser-Pro, 12,334 Ile-Met, 13,335 Ser-Asn, 13,517 Pro-Thr, 13,542 Phe-Tyr, 13,545

Table 2
Nucleotide base substitutions in Hamdani and Kehilan horses.

Substitutions/Change	Hamdani	Kehilan
Transversions	16	18
Transitions	86	28
Deletions	5	15
Insertions	2	5
Total	109	66

Table 3
Amino acid changes in Kehilan and Hamdani horse strains with respect to the reference JN398377 and X79547.

Gene	Position	Kehilan	JN398377	Position	Hamdani	JN398377
ND1	3387	Lys	Arg	3306	Arg	Met
				3494	Pro	Thr
ND2						
COX1	6785	Ile	Val	5888	Ser	Pro
				6312	Pro	Leu
				6507	Pro	Leu
				6714	Thr	Ile
COX2	7628	Pro	Ser	7245	Gln	Arg
				7614	Leu	Gln
				7668	Cys	Tyr
ATP8				7902	Thr	Ile
ATP6				8010	Asn	Ser
				8243	His	Tyr
				8363	Asn	Asp
				8366	Thr	Ala
				8561	Ser	Thr
				8570	Ser	Pro
COX3						
ND3				9705	Tyr	Cys
				9777	Leu	Pro
ND4L						
ND4	**10,829**	**Leu**	**Val**	**10,829**	**Leu**	**Val**
	11,555	**Ser**	**Pro**	**11,269**	**Met**	**Ile**
ND5	12,863	Trp	Arg	11,792	Ile	Val
	13,052	His	Tyr	11,862	Pro	Leu
				11,882	Thr	Ala
				11,888	Arg	Cys
				11,945	His	Tyr
				11,954	Arg	Trp
				11,969	His	Tyr
				11,988	Ser	Asn
				12,086	Arg	Gly
				12,128	Pro	Ser
				12,144	Pro	Leu
				12,149	His	Tyr
				12,164	His	Tyr
				12,191	Ser	Pro
				12,334	Ile	Met
				13,335	Ser	Asn
				13,517	Pro	Thr
				13,542	Phe	Tyr
				13,545	Ser	Phe
				13,557	Pro	Leu
ND6						
CYTB	15,291	Arg	His	14,554	His	Gln
	15296,7	Ser	Gln	14,619	Pro	Gln
				14,805	Thr	Ile
				15,204	Val	Ala

Nucleotide changes in bold are according to X79547.

Ser-Phe and 13,557 Pro-Leu) observed as the highest variations. The gene ND6 does not show any mutations in the studied horses. Finally, the CYTB gene ends up with two amino acid changes in Kehilan horse (15291 Arg-His and 15,296 Ser-Gln) and four alterations with Hamdani (14554 His-Gln, 14,619 Pro-Gln, 14805Thr-Ile and 15,204 Val-Ala) at dissimilar positions.

There were 56 nucleotide changes in the non-coding region including insertions or deletions in rRNA, tRNA, and misc feature of which 34 were in Hamdani and 20 in Kehilan as shown in Table 4 and Table 5.

3.3. Phylogenetic relationship

A Maximum-likelihood phylogenetic tree has been constructed with two Saudi horse strains (Kehilan and Hamdani) mtDNA sequenced data sets. The previous study of Saudi horse strains Hadban and Seglawi mtDNA along with some Genbank sequences were included in the phylogenetic tree to obtain the complete relatedness among the submitted mtDNA sequences. The MEGA6 program was used to construct phylogeny with a distance matrix of 1,000 bootstrap replicate values. In total, the phylogenetic tree included 26 horse mitochondrial sequences from NCBI along with *Equus asinus* (GenBank: X97337.1) as an outgroup (Fig. 1).

3.4. Network analysis of sequence data

The median joining network was constructed based on the cytb gene of studied strains along with the NCBI sequenced data set (n = 98) from different regions (Fig. 2). There were 7 haplotypes observed based on their similarity, and the Hamdani and Kehilan formed individual haplogroups H_1 and H_2 respectively. The haplogroups H_4 and H_7 witnessed higher sample sizes of 35 and 59 respectively. Hadban horse from the previous study segregated in haplogroup H_7.

4. Discussion

The Mitochondrial genome provides a vital source for maternal diversity, phylogeny, evolutionary and place of origin analysis to correlate among animals evidenced by plenty of studies (Dhorne-

Table 4
Variations in non-coding region of Kehilan.

Position	Place	Kehilan	JN398377
158	rRNA	G	A
356	rRNA	C	T
358	**rRNA**	**C**	**–**
597	rRNA	T	G
599	rRNA	A	–
604	rRNA	A	G
605	rRNA	A	C
608	rRNA	G	T
611	rRNA	G	T
612	rRNA	G	T
613	rRNA	–	T
620	rRNA	–	C
621	rRNA	–	T
622	rRNA	–	C
623	rRNA	–	T
630	rRNA	A	G
633	rRNA	C	T
634	rRNA	–	G
635	rRNA	–	T
636	rRNA	–	T
642	rRNA	C	A
643	rRNA	G	T
644	rRNA	T	C
653	rRNA	G	–
859	rRNA	C	T
2232	rRNA	–	T
5098	**tRNA**	**A**	**T**
5240	**tRNA**	**–**	**A**
5279	**tRNA**	**–**	**A**
15,391	**tRNA**	**–**	**T**
15,498	MF	C	T
15,723	MF	A	G
15,773	MF	T	C
15,829	MF	G	A

Nucleotide changes in bold are according to X79547, Deletion/Insertion -, MF- Misc Feature.

Table 5
Variations in non-coding region of Hamdani breed.

Position	Place	Hamdani	JN398377
158	rRNA	G	A
356	rRNA	C	T
358	**rRNA**	**C**	**–**
235	rRNA	C	A
258	rRNA	A	T
513	rRNA	C	A
669	rRNA	C	T
739	rRNA	C	T
860	rRNA	G	A
961	rRNA	C	T
1386	rRNA	–	T
1683	rRNA	C	A
2227	**rRNA**	**–**	**T**
5099	**tRNA**	**A**	**T**
5241	**tRNA**	**–**	**A**
5280	**tRNA**	**–**	**A**
7003	tRNA	A	G
11,685	tRNA	T	C
11,693	tRNA	T	C
11,727	tRNA	G	A

Nucleotide changes in bold are according to X79547, Deletion/Insertion -, MF- Misc Feature.

Pollet et al., 2020; Hill et al., 2002; Khanshour and Cothran, 2013; Moridi et al., 2013). The mitochondria provide a great source as the molecular biology markers whether it could be of short gene sequences (D-Loop, control region and cytb) or complete genome which has been deployed since a long time ago. This study sequenced two mitochondrial genomes of the Saudi horse strains namely Kehilan and Seglawi. Their mitochondrial genomes were approximately 16.6 kb in length and consisted of 13 coding genes, 2 rRNAs and 22 tRNAs. Both horses showed more than 99% similarity of the nucleotide bases throughout the mitochondrial genome. Hamdani horse (showed 109 nucleotide variations and Kehilan with 66 variations which are comparable to the previous study of two Saudi horses (Hadban (89) and Seglawi (60) (Sheikh et al., 2019). The mitochondrial protein coding genes of Hamdani (44) differed with Kehilan (9) horse however, they were relatively similar to the Saudi horses of Hadban (34) and Seglawi (11). The transitional changes were prevalent in both the horses mtDNA which was also reported in a study with advanced nanopore sequencing method of horse mitochondrial genome sequencing (Dhorne-Pollet et al., 2020). On the other hand, the nuclear DNA also reported to have the transitional bases substitution effect (Lanave et al., 1986; Pereira et al., 2009).

The ND5 gene recorded the highest mutations (20) in the Hamdani horse whereas in Kehilan horse (2) they were comparatively less and the ATP6 gene of the Hamdani horse portrayed 6 variations. Interestingly, the two strains found identical polymorphism at the 10,829 nucleotide position of the ND5 gene which was reported in the Hadban horse (Sheikh et al., 2019). This ND5 gene have been reported with the relation to the positive selections and adaptation to the extreme environments (Wang et al., 2016).

Of note, three genes ND2, COX3 and ND6 have not been found with any variants in Hamdani and Kehilan horses as well as Hadban and Seglawi from the previous study. Moreover, the ND4L gene in Hamdani and Kehilan horses and cytb gene in Hadban and Seglawi horses has not evidenced any polymorphic sites. However, the cytb gene of Hamdani and Kehilan represented 4 and 2 amino acid alterations respectively and this could be due to the heteroplasmy of the mitochondrial cytb gene (Zhao et al., 2015).

The Hamdani (current study) and Hadban (Previous study) horses formed a sister relationship with each other and fall in-between closely related branches of Arabian and American paint horse branches above them and Deqin, Akhal Teke, Arabian horses branches lie below them. This supports that the exported founder mare (Arabian horse) influence contribution to the American horse (Khanshour and Cothran, 2013).

The horses from this study were compared to the haplotypes of Achilli et al, (2013) study and found that Kehilan horse falls under haplogroups A1 and A'B with the corresponding mutations at the nucleotide positions 10,829 and 158, 356 respectively. Hamdani horse showed diverse similarity of the mutational positions 6507, 7245, 7614, 7668, 7902, 8363, 9777, 12334, 13335, 14,805 and 15,204 resembling the haplogroups P, M'N'O'P, O'P'Q, A-K, A-L, O'P'Q, O'P'Q, J'K, A'B'C'D, A-K, and O'P'Q respectively.

The Maximum-likelihood phylogenetic tree represented that the Arabian and Syrian horses were scattered in multiple nodes (Fig. 1) showing high genetic diversity among them which was similar to the study by Khanshour et al, (2013). One of the studies based on mitochondrial D-Loop revealed comparable result to this current study of Kehilan diversity than Seglawi and Hamdani (Almarzook et al., 2017). The variations in the mitochondrial genome of protein-coding genes revealed that Hamdani and Hadban are more closely related than Seglawi and Kehilan horses. Seglawi horse stands out as a single branch and is far to the Hamdani and Hadban group leaving a bunch of horses (American paint, Arabian, Naqu Syrian and Przewalskii horses) in between. However, Kehilan horse observed further away from the previous Saudi horses and relatively diverse from its three neighbors. It branched in between the Syrian, Arabian and Italian horses towards to the Seglawi and Akhal Teke, Chincoteague pony and Arabian horses on another side. The mtDNA data demonstrated that the Hamdani and Hadban horses were closely allied. However, Kehilan horse showed significantly fewer and dissimilar variations than the other three Saudi horses, as represented by the genetic data and phylogenetic tree.

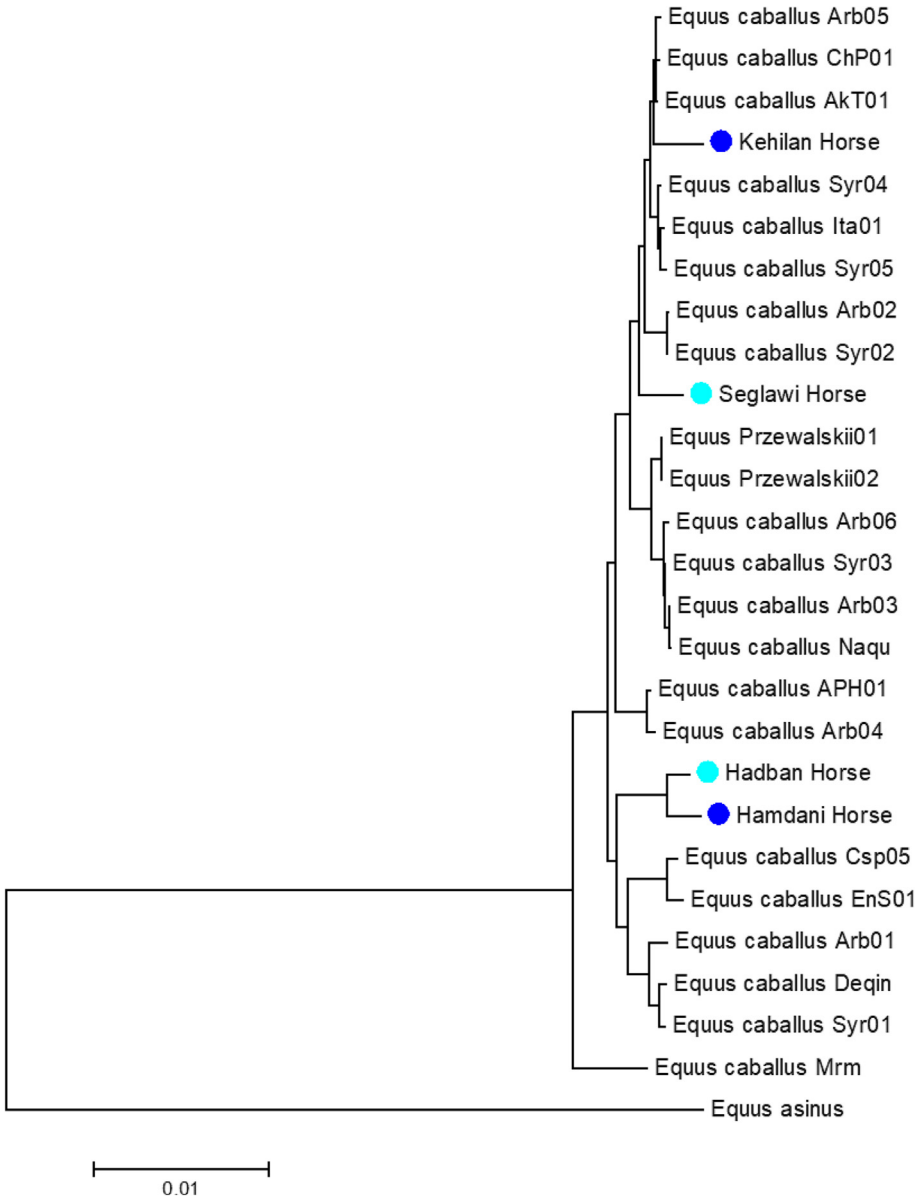

Fig. 1. Maximum likelihood phylogenetic tree of four Saudi horse strains along with other Genbank sequences constructed based on whole mtDNA. Hadban and Seglawi were from our previous study. Hamdani and Hadban showed a close relationship.

The Kehilan horse showed unique variability in the current study however, Kahlila or Kahlawi horses of Syrian Arabian origins showed high variability in haplotypes with D-Loop region of mtDNA (Almarzook et al., 2017; Khanshour and Cothran, 2013). One of the studies on mtDNA revealed that there was haplogroup sharing between Arabian and Italian horses due to Arabian horse contribution and maternal gene flow (Cardinali et al., 2016).

The Studied Saudi horses showed the separate nodes and did not show any admixture revealing the precise selection or breeding results of Bedouins, which plays a key role in Arabian horse strain uniqueness (Hendricks, 1995). This type of genetic diversity analysis is useful as a tool in the conservation of livestock species (Bruford et al., 2003; Freeman et al., 2006). Two haplotypes were produced from the studied samples, and the median joining network revealed a closer relationship between the Saudi horse haplotypes H_1 (Kehilan) and H_7 (Hadban) than the other ones. The statistical analysis was non-significant due to small population size.

Although the studbook may have errors, mtDNA assessment effectively solves it and illustrates the maternal genetic data. The mtDNA facilitated maternal phylogenetic relationship however, on the other hand, Y chromosomal marker analysis provides paternal lineage of haplotypes, beneficial for further resolution of the Arabian horse characterization. This could be extended furthermore paleogenetic studies to answer the breed origin, admixtures and address the lost lineages (Orlando, 2020; Remer et al., 2022). Therefore, it is recommended to imply both the mtDNA and Y chromosome markers to better understanding of various factors such as maternal inheritance, diversity, breeding, conservation, place of origin, classifications and paternal ancestry.

5. Conclusion

mtDNA serves as an important molecular marker for selecting Saudi Arabian horse strains and preserving their genealogy data. This study evaluated the mitochondrial genomes of two Saudi Ara-

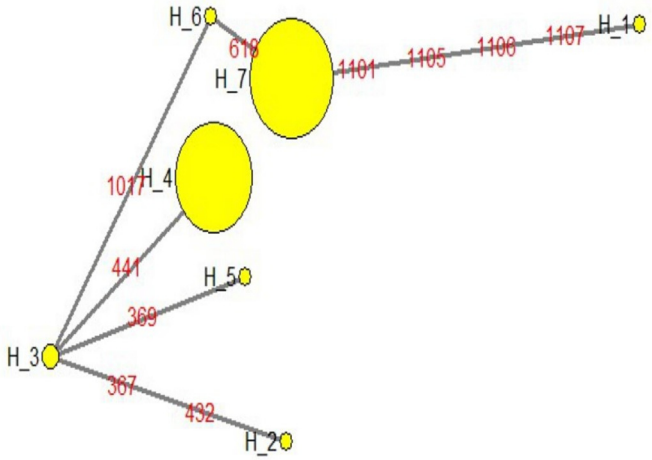

Fig. 2. Median joining network of cytb gene from 100 NCBI sequences. Each node represented the mutation sites from one haplogroup to another. H_1 and H_2 is Hamdani and Kehilan horse strains respectively. H- Haplotype.

bian horses, Hamdani and Kehilan for variations at DNA and amino acid levels and derived the phylogenetic relationship between them along with some NCBI published horse mtDNA sequences. Hamdani horse formed a sister relation with Hadban Saudi horse in terms of mtDNA variations and phylogeny, whereas Seglawi and Kehilan showed distantly related than their neighboring Saudi horses. The Arabian horse strains harbored broad diversity; however, there are no clear classifications. They contributed significantly to other horse breeds during earlier migrations from the Middle East. Breed conservation is important for the native horse population of the Arabian Peninsula, as it influences maternal genetic diversity. To explore further, a broad-scale perusal, pedigree analysis, and nuclear genome sequencing should be combined.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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