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Genetic Diversity and Population Structure among Arabian Horse Genealogical Lineages in Bulgaria

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Abstract: The present research aimed to characterize the genetic diversity and relationships among extant Arabian horse sire lines in Bulgaria, using 15 equine microsatellite markers. The evaluation included 537 Arabian horses representing nine sire lines (SAKLAWI I, LATIF, SEANDERICH, IBRAHIM, SHABAB, DJEBEL MOUSA, KUHAILAN AFAS, BAIRACTAR, and SARHAN). The obtained results indicated that within these lines, the mean number of alleles ranged from 4.15 in SARHAN to 5.54 in SAKLAWI I and LATIF. The mean expected heterozygosity (He) ranged from 0.54 in the SEANDERICH line to 0.67 in SAKLAWI I. The inbreeding coefficient for the entire Arabian populations was rather low: F_{IS} = −0.109, fluctuating from −0.204 in SHABAB to −0.041 in SAKLAWI I. The mean genetic differentiation, F_{ST}, was 0.096, demonstrating that nearly 90% of the total genetic variation was due to genetic differentiation within each population. STRUCTURE analysis indicated a genetic similarity between SHABAB and LATIF, between IBRAHIM and KUHAILAN AFAS, as well as between SAKLAWI I, SEANDERICH, and BAIRACTAR. This study of the genetic diversity of Arabian sire lines in Bulgaria can assist in developing a national strategy for the exclusion of non-purebred animals from breeding programs in order to preserve the genetic profile of the original Arabian lines.

Keywords: Arabian sire lines; microsatellites; genetic variability; genetic differentiation; conservation

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1. Introduction

The history of mankind changed forever with the domestication of the horse, and from that moment onward, horses and horsemanship have played an essential role in human civilization. The horse was domesticated around the 3rd millennium BC during the Early Bronze Age. Unlike other ungulates, not only were horses used as a source of meat and milk, but their stamina and speed also revolutionized warfare and transportation [1]. The horse became even more important to human civilization during the Middle Ages, with the introduction of the horse collar and horseshoes, which enabled its use in agricultural activities [2,3]. Thus, horse domestication contributed to irreversible changes in economic and sociopolitical systems, ideologies, human gene pools, and the spread of languages [4,5], giving rise to an exceptional horse–human interaction that also includes emotional attachments [6,7].

Among over 500 famous horse breeds in the world [8,9], the Arabian horse is one of the oldest light, warm-blooded horse breeds [10–12]. The origin of Arabian horse is well

documented [13–15]. According to archaeological evidence, the first horse bones appeared in burials on Syrian sites during the Akkad period (c. 2350–2150 BC) in the Bactria–Margiana Archaeological Complex or Oxus Civilization (c. 2100–1800) in southern Central Asia [16,17]. Further confirming these findings, there are also horse images appearing in the art during the period of the Dynasty of Akkad from late 2400 BC to 2200 BCE [18,19]. It is generally accepted that horses appeared in Egypt during the Second Intermediate Period (c. 1650–1550), when competition for power in Egypt and Nubia led to the formation of two new dynasties: the 15th, called the Hyksos (c. 1630–c. 1523 BCE), and the 17th (c. 1630–1540 BCE), ruled from Thebes [20]. Since horses were primarily used as draft animals immediately after domestication, their absence from the Arabian Peninsula during this period is easily explained [21,22].

There are various hypotheses regarding the creation of the breed, the most plausible being related to the Bedouin tribes inhabiting the Arabian Peninsula approximately 4000–5000 years ago [23,24]. For the last century, the Bedouins have shaped the Arabian horse, depending on the climatic and natural conditions of the environment. As a result of these specific conditions, the breed's typical exterior was formed, i.e., with a thin skin and a tail held high when the horse is in motion, both useful properties in cooling the body [22,25]. The most popular horses were the blond chestnuts, providing protection against the harsh desert sun, with flat and round hooves, enabling them to traverse the sandy and stone-strewn ground of their homelands [25]. Through wars and trade, Arabian horses spread to many parts of the world, where they adapted in a variety of ways, giving rise to the modern Arabian horse. Considering the fact that it is one of the most famous breeds worldwide, some countries have tried to create their own strain of the Arabian horse. Thus, the six types of Arabian horses—Egyptian, Russian, Polish, Crabbet, Spanish, and Shagya—have appeared [26].

It is well known that for over 100 years, the Arabian horse has been widely used for the "improvement" of many other horse breeds [11,27–29]. The cumulative influence of a stallion leads to the formation of a "sire line". These were usually genetically strong fathers who passed on their traits, so they were frequently used for breeding, and their offspring could then be selected accordingly. Therefore, the sire line can only appear over several generations and cannot be "established" as such from the beginning. However, sire lines can be used as a tool to consolidate types, to manage inbreeding or line breeding, and to monitor genetic diversity [10,29,30]. Currently, more than 25 globally active Arabian sire lines exist [31].

In recent years, the application of DNA microsatellite markers has proven extremely useful in elucidating the population structure and genetic diversity in many animals and plant species [32–34]. One of the weakness when using microsatellite markers in livestock species appears to be the different size of the alleles of the same microsatellite marker under different PCR conditions [35]. As a result, new microsatellite markers were additionally designed in the animal species to facilitate comparison between studies. In order to ensure comparability and to enable merging the results regarding microsatellite genotyping from different labs, only recommended STR loci should be applied. A list of recommended microsatellites to be used in livestock, as well as for genealogical control of domestic species, has been issued by the Food and Agriculture Organization of the United Nations and the International Society of Animal Genetics (ISAG) [36,37].

Arabian horses have been genetically evaluated based on microsatellite markers [11,38–41] and the Y chromosome [10,30,42–44]. In contrast to Y chromosome sequence analysis, there was no investigation of Arabian sire lineages on the basis of microsatellite markers.

Therefore, in the present study we aimed to investigate the current status of the genetic diversity and inbreeding of the population of Arabian horses in Bulgaria by means of microsatellite genotyping, using samples from nine Arabian lineages.

2. Materials and Methods

2.1. Animal Welfare and Ethical Statement

All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Bulgarian Food Safety Agency (BFSA) (Art. 154 of the Law on Veterinary Activity), in accordance with European Union Directive 86/609.

2.2. Sample Collection

A total of 537 hair samples were obtained from the manes and/or tails of Arabian horses (male and female) in 2000–2023. The samples were preserved under dark storage in filter paper packages, with silica gel to dehydrate the sample, so as to prevent degradation. Samples from both private and state studs were included. All samples represented nine Arabian sire lineages: Foundation sire SAKLAWI I (SAK)—Egyptian Arabian (n = 144); Foundation sire LATIF (LAT)—Egyptian Arabian (n = 44); Foundation sire SEANDERICH (SEA)—Spanish Arabian (n = 37); Foundation sire IBRAHIM (IBR)—Polish Arabian (n = 91); Foundation sire SHABAB (SHA)—Qatar Arabian (n = 36); Foundation sire DJEBEL MOUSA (DJE)—French Arabian (n = 16); Foundation sire KUHAILAN AFAS (KUH)—Polish Arabian (n = 131); Foundation sire BAIRACTAR (BAI)—German Arabian (n = 27); Foundation sire SARHAN (SAR)—French Arabian (n = 11) (Figure 1). The pedigree information for each animal was obtained from the Executive Agency for Selection and Reproduction in Animal Husbandry (Sofia, Bulgaria), the Purebred Arabian Breeding Association (Plovdiv, Bulgaria), and the stud farm "Kabiuk" (Shumen district, Bulgaria). Pedigree information was required regarding the identification of unrelated purebred individuals. We eliminated all samples with a doubtful or excluded pedigree. A detailed description of all studied lineages is presented in the Supplementary Material S1.

Figure 1. Genealogical lineages of the studied Arabian founder stallions breeding in Bulgaria.

2.3. DNA Extraction and Microsatellite Genotyping

Genomic DNA was isolated from the hair follicles of about 20 hairs, using a Tissue DNA purification kit (Cat. No. E3550, EURx Ltd., Gdansk, Poland), according to the manufacturer's instruction. The quantity and quality of the extracted DNA was evaluated spectrophotometrically and according to 1% agarose gel electrophoresis staining with SimpliSafe™ (Cat. No. E4600; EURx Ltd., Gdansk, Poland) under UV light. Then, the DNA was stored at −20 °C prior to analysis.

A total panel of 15 microsatellite markers (AHT4, AHT5, ASB2, ASB17, ASB23, HMS1, HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, HTG10, and VHL20) specific to the autosomal DNA of *Equus caballus* was used. The microsatellite loci are included in the panel recommended by the International Society for Animal Genetics (ISAG) for genetic diversity studies and parentage verification. The 15 microsatellites were amplified in one multiplex reaction, using a Stockmarks horse genotyping kit (Cat. No.: PN4336407—Applied Biosystem—USA) according to the method described by Sargious et al. [45]. The primers used for multiplexed PCR were labeled using 6FAM, VIC, NED, and PET standards. The fragment sizes of the microsatellite alleles were determined using an ABI3130XL genetic analyzer (Applied Biosystem, New York, NY, USA), with LIZ600 labeled size standard. The data obtained were further analyzed using GeneMapper 5.0 software (Applied Biosystem, New York, NY, USA).

2.4. Statistical Analyses

The number of alleles per locus (Na), the effective number of alleles (Ne), the mean number of alleles (Nm), the observed heterozygosity (Ho), the expected heterozygosity (He) for microsatellite markers, the unbiased expected heterozygosity (uHe) for the studied sire lines, and the Shannon's information index (I), the gene flow (Nm), and the Hardy-Weinberg equilibrium per locus across microsatellite markers and Arabian horse lineages were calculated using GenAlEx 6.5 (New Brunswick, NJ, USA) [46]. GenAlEx 6.5 also implemented the principal coordinate analysis (PCoA) on the basis of the genetic distance matrix, based on the Nei genetic distance [47] among all nine Arabian horse lineages. The HP-Rare program was used to perform rarefaction on the measures of allelic richness (AR) [48]. Wright's F-statistics (F_{IT}, F_{IS}, F_{ST}) [49] were calculated using POPGENE software [50]. The confidence intervals at 95% (95% CI) of the *F*-statistics were assessed through jackknifing the horse samples and 5000 bootstraps over the loci. Estimations and randomization tests were performed with Fstat 2.9.4 [51]. The polymorphic information content (PIC) of each locus was calculated using the equation of Botstein et al. [52], utilizing Cervus v3.0.7 software [53]. Cervus v3.0.7 software was also used to estimate the null allele frequencies—i.e., alleles that consistently fail to amplify during PCR due to priming site mutations, differential amplification of size variants, or inconsistent DNA quality.

STRUCTURE 2.3.4 software was used to analyze the population structure of the investigated goat breeds [54]. The number of presumptive clusters (K) was run, from 2 to 12, where K was the number of the tested clusters. Ten interactions were performed for each K value. All runs were performed with a length of 50,000, followed by 150,000 Markov chain Monte Carlo (MCMC) repeats after burn-in, with 20 replicate runs for each K, using an admixture model and independent allele frequencies. The software package Clumpak (http://clumpak.tau.ac.il/, accessed on 23 January 2020) was utilized to identify the most probable values of K [55].

The MEGA v.11 [56] was used to construct neighbor-joining trees for the studied phylogenetic relationships among all Arabian sire lines.

3. Results

3.1. Polymorphism of Microsatellite Markers

After the fragment analysis, many animals failed to be genotyped for the ASB17 or ASB23 loci. For example, 23 animals of the SAK sire line (30/144 20.8%) failed to genotype for the ASB17 and ASB23 markers, by observing a match for samples with no results for either loci. The genotyping results were also similar in the other horse lineages. We do not have an exact explanation for the failed genotyping at these particular microsatellite markers; however, for the microsatellite analysis to be correct, these markers were removed from further analysis. A total of 608 alleles were identified in the entire Arabian horse population, consisting of 537 animals, across 13 microsatellite loci, with a mean of 5.19 $\pm$ 0.13 per locus (Supplementary Table S1). The highest Na of the alleles (6.78) was found in locus ASB2, while the lowest allele number (3.11) was observed in

HTG7. The mean value of the effective number of alleles (Ne) showed the highest rate in the HSM3 locus (3.97), while the lowest (1.80) was observed in the HTG7 marker. Only two microsatellite markers (HTG7 and HMS6) exhibited a PIC content of less than 0.5, while in all others, the PIC showed values higher than 0.5, indicating that these loci were highly polymorphic. The expected heterozygosity (He) varied from 0.39 in the locus HTG7 to 0.75 in the locus HMS3, with an average value of 0.62 ± 0.01 across the Arabian horse population. The observed heterozygosity (Ho) showed the highest mean value in the locus HMS3 (0.85) and the lowest in the locus HTG7 (0.43), indicating a high level of genetic variability among the three breeds. The Shannon's information (diversity) index (I), which is an indicator of the genetic diversity of the population, ranged from 0.69 in the loci HTG7 to 1.54 in the HMS3 marker (Supplementary Table S1). The null allele frequency was not high, ranging from −0.0332 for the HTG4 marker to 0.0287 for the HTG7 locus (Supplementary Table S1). Hence, no marker was excluded from the subsequent analysis of genetic differentiation because of the low estimated frequency of the null allele.

The global deficit of heterozygotes across the Arabian horse population (F_{IT}) was −0.003, i.e., there was no heterozygote deficit in the population as a whole (Supplementary Table S2). The value of the inbreeding coefficient (F_{IS}) across the analyzed population was very low (−0.110), which indicated that the individuals in the population were less related under a model of random mating, i.e., there was an absence of inbreeding in each Arabian horse lineage. The mean F_{ST} index, which measures the population differentiation due to genetic structure, showed a value of 0.096, indicating a difference of around 10% between Arabian horse lineages. This value showed the very low level of genetic differentiation between the studied sire lines. The gene flow (Nm) varied from 1.49 for the locus HMS2 to 3.59 for the marker HMS3, with a mean value of 2.53 ± 0.20 for the entire Arabian horse population. This value indicated a moderate gene flow among the studied lineages.

The coefficient of genetic differentiation G_{ST} exhibited a value of 0.084, indicating a very low genetic differentiation between sire lines (Supplementary Table S2). The mean total genetic diversity (H_T) estimated from the pooled allele frequencies was 0.689 ± 0.03, which revealed a high level of total genetic diversity of the overall population.

In all 116 HWE tests (13 loci in nine Arabian horse lineages), only the SEA and SAR sire lines did not show a significant deviation in the Hardy–Weinberg equilibrium tests in each marker (Supplementary Table S3). The remaining lines showed, to a lesser or greater extent, a significant deviation at a particular locus.

3.2. Genetic Variability between and within the Arabian Horse Lineages

Table 1 summarizes the statistics of the molecular genetic polymorphism in the Arabian horse lineages. A total of 84 distinct alleles were detected at the 13 microsatellite markers in 537 studied animals. The SAK I and LAT sire lines exhibited the highest number of alleles, 72, while the SARHAN lineage showed 54 alleles revealed by microsatellite markers. The private allele list was observed at very low values among all Arabian lineages. The LAT had the largest number of private alleles at 2 (2/84, 2.3%), while in some lineages, such as IBR, BAI, DJE, etc., no private allele was detected. Across the nine Arabian horse lineages, the average number of alleles per locus was 5.19 ± 0.37. The average Ho frequency (0.78) was the highest in the SHA sire line and the lowest in the SEA (0.63) line (Table 1). The average uHe value varied from 0.55 in the SEA line to 0.67 in the SAK and LAT lineages. The heterozygote deficit within populations (F_{IS}) for the two goat breeds showed very low values (KLH, 0.037; BSHL, 0.011), which indicated the absence of inbreeding processes. Also, the mean F_{IS} = −0.109 was highly variable among the studied horse lineages. The confidence intervals (95%) varied from 3.3 to 8.3 for the LAT and IBR lineages, respectively, according to the LD method.

Table 1. Genetic diversity estimates by Arabian horse lineages. Sample size (N), number of alleles, mean number of alleles per population (Na), effective number of alleles per locus (Ne), observed heterozygosity (Ho), unbiased expected heterozygosity (uHe), allelic richness (AR), inbreeding coefficient (F_{IS}), and effective population size (95% confidence interval), estimated with the linkage disequilibrium (LDNe).

Breed	Acronym	N	Number of Alleles	Na	Ne	Ho	uHe	AR	F_{IS}	LDF_{IS}
SAKLAWI I	SAK I	144	72	5.54	3.31	0.69	0.67	3.51	−0.041	4.3 (3.8–4.7)
LATIF	LAT	44	72	5.54	3.18	0.73	0.67	3.02	−0.109	3.3 (3.2–3.4)
SEANDERICH	SEA	37	69	5.31	2.42	0.63	0.55	2.62	−0.159	4.4 (4.0–4.7)
IBRAHIM	IBR	91	70	5.38	2.86	0.64	0.61	2.79	−0.060	8.3 (7.3–9.1)
SHABAB	SHA	36	68	5.23	3.07	0.78	0.66	2.97	−0.204	3.4 (3.2–3.5)
DJEBEL MOUSA	DJE	16	67	5.15	2.93	0.67	0.64	2.94	−0.074	6.5 (5.9–6.8)
KUHAILAN AFAS	KUH	131	70	5.38	3.00	0.66	0.62	2.85	−0.075	7.0 (6.4–7.5)
BAIRACTAR	BAI	27	66	5.08	2.87	0.70	0.64	2.87	−0.112	5.1 (4.8–5.3)
SARHAN	SAR	11	54	4.15	2.79	0.71	0.64	2.88	−0.143	3.8 (3.5–3.9)
Mean			67.5	5.19	2.94	0.69	0.63	2.94	−0.109	5.1
SE			5.48	0.37	0.44	0.01	0.04	0.24	0.011	1.7

Abbreviations: SAK I—SAKLAWI I; LAT—LATIF; SEA—SEANDERICH; IBR—IBRAHIM; SHA—SHABAB; DJE—DJEBEL MOUSA; KUH—KUHAILAN AFAS; BAI—BAIRACTAR; SAR—SARHAN.

The AMOVA analysis showed substantial subdivision between the studied sire lines (F_{ST} = 0.008, $p < 0.001$), but with a large fraction of variation found within the individuals (F_{IT} = 0.014, $p < 0.001$) (Supplementary Table S4). Partitioning levels of genetic diversity within and among the populations revealed that only 1% of the total genetic variance existed within the populations.

3.3. Admixture Analysis and Genetic Differentiation

The admixture and genetic differentiation among the Arabian horse lineages were evaluated by STRUCTURE analysis and principal coordinate analysis (PCoA).

The results from the STRUCTURE analysis, implemented by Kopelman et al. [55], showed a clear optimum at K = 3 (Figure 2A). This means that all individuals were assigned into three different clusters. Supplementary Table S5 shows the proportion of individuals assigned to each of the three clusters, depending on the Q value that resulted from the STRUCTURE analysis. The first cluster mainly consisted of KUH, where 98.2% of the KUH individuals were assigned into this cluster. This cluster also included many individuals of the IBR and SAR sire lines (98.0% and 94.3%, respectively). The second cluster consisted of 98.2% of SAK I samples and 97.9% of SEA samples, in addition to 96.8% of the BAI lineage. Three different sire lines, 98.0% of SHA, 97.7% of LAT, and 97.2% of DJE, formed the third cluster.

At K = 3, which was the best value of the number of clusters that represent the structure of the data, the KUH sire line mixed together with IBR and SAR and formed a distinct cluster, with very few individuals from the sire lines from the second and third clusters (Figure 2B). Also, the SAK I, together with most of the IBR and SAR sire lines, formed a second cluster. Among the second cluster, the SAK I lineage seemed to form the most mixed population, as many individuals from the second cluster were included in this line. SHA, LAT, and DJE formed a separate cluster, with a few individuals (most visible at the DJE sire line) from the lineages of the second cluster.

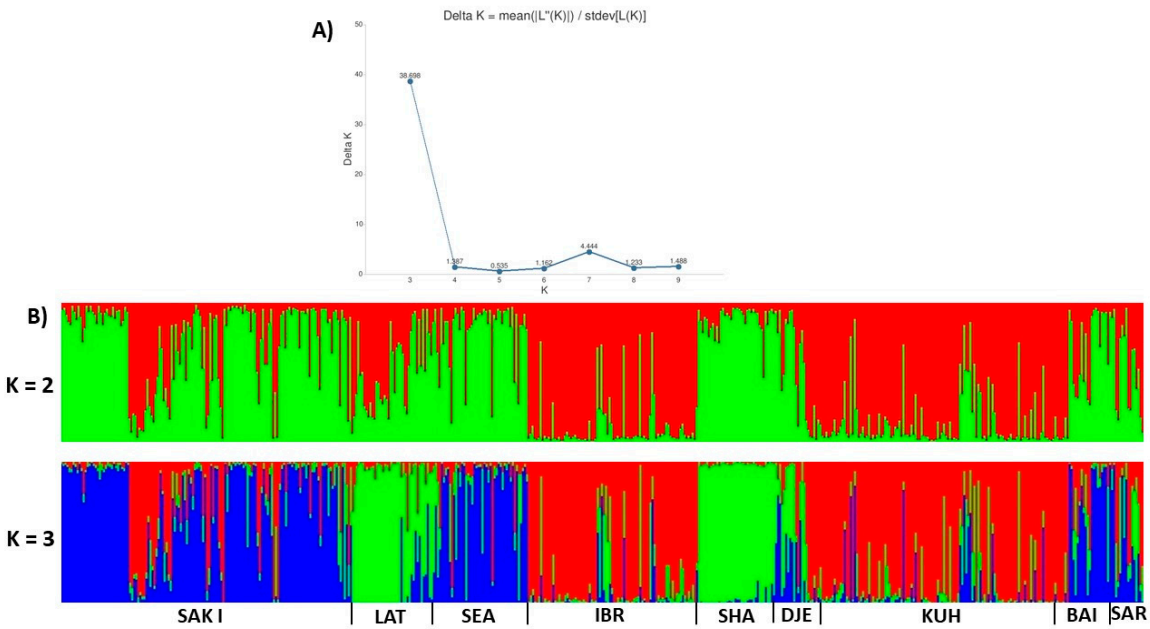

Figure 2. (**A**) The optimal ΔK was found at K = 3, with clustering assignment dependent upon to the Bayesian method under an admixture model obtained by STRUCTURE software for K = 2 and K = 3. Each individual is represented by a single column that is divided into segments whose size and color correspond to the relative proportion of the Arabian sire lineages corresponding to a particular cluster. Black lines separate the populations. Arabian horse lineages abbreviations can be seen in Table 1. (**B**) Each individual is represented by a single column that is divided into segments whose size and color correspond to the relative proportion of the Arabian sire lineages corresponding to a partic-ular cluster. Black lines separate the populations. Arabian horse lineages abbreviations could can be seen in Table 1.

PCoA analysis was applied to examine the relationships among the Arabian horse lineages and between individuals (Figure 3). For better understanding of the distribution of the studied horse samples, we generated two PCoA plots (1 vs. 2 and 1 vs. 3 axes). In general, the PCoA plot supported the data obtained from the STRUCTURE analysis. Because of the low genetic differentiation among all lineages, we did not observe a separation of different sire lines in the PCoA plot (1 vs. 2 axes), although some individuals from different sire lines (LAT and SEA) separated from the overall gene pool. In the PCoA plot (1 vs. 3 axes), we again observed a common genetic pool formed by the studied samples. Unlike in the first option, here, some individuals were separated from the IBR and SAK sire lines.

Figure 4 shows the neighbor-joining tree, comprising all 537 samples from the nine Arabian lineages. The SHA and SEA populations, SAK I and DJE, as well as IBR and BAI, clustered together and seemed to be the closest lines among all populations. The LAT sire line showed some differentiation from the SAK I—DJE branch, while the KUH lineage grouped closely to the IBR—BAI branch. The most unexpected was the phylogeny of the SAR sire line. It is logical to assume that the small number of animals from some of the sire lines did not provide a precise phylogenetic resolution.

The pairwise F_{ST} value and Nei's genetic distance among all Arabian lineages is shown in Supplementary Table S6. The calculated F_{ST} value was very low among all sire lines, with the lowest value of 0.011 (IBR and KUH) and the highest of 0.101 (DJE and SAR). The Nei's genetic distances showed genetic similarity between the IBR and KUH lines, with values of 0.035, while the genetic distance was the most clearly pronounced between DJE and SAR (0.451).

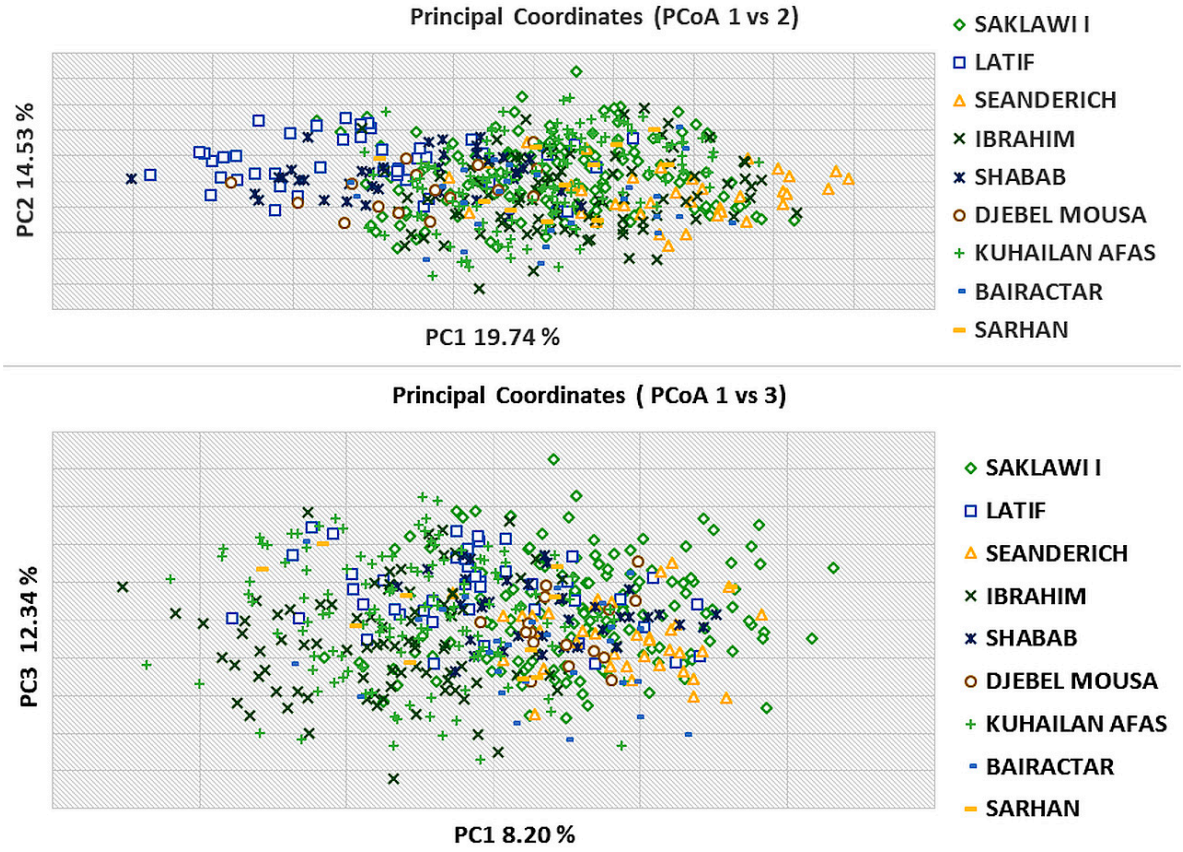

Figure 3. Principal coordinate analysis (PCoA 1 vs. 2; 1 vs. 3 axes) of the nine Arabian horse lineages created by GenAlEx. A two-dimensional plot of the PCoA analysis shows the clustering of the 537 Arabians. Arabian horse lineages abbreviations can be seen in Table 1.

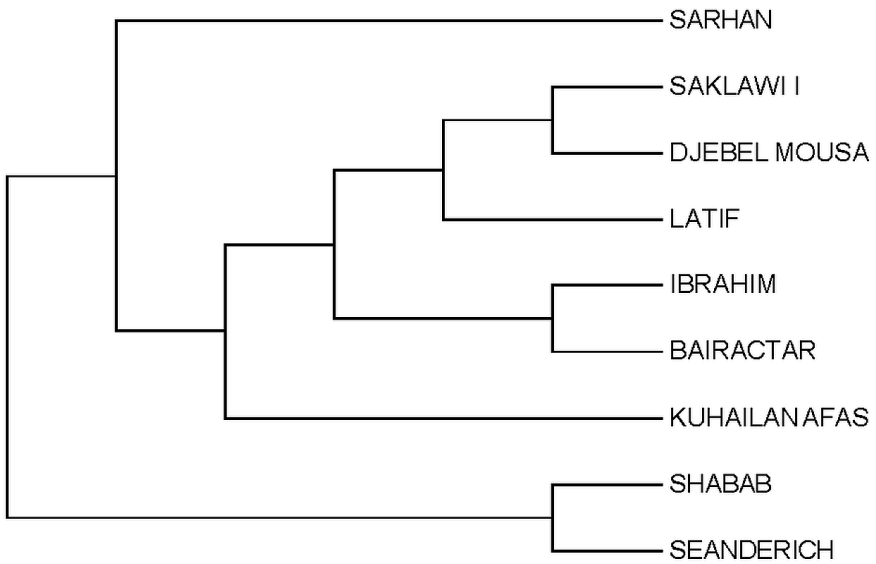

Figure 4. Neighbor-joining phylogenetic tree showing the genetic relationship among nine Arabian horse lineages conducted in MEGA11 [56], based on the Tamura–Nei model [57]. All positions containing gaps and missing data were eliminated. There were a total of 195 positions in the final dataset. Arabian horse lineages abbreviations can be seen in Table 1.

4. Discussion

To our knowledge, this work is the first study to apply microsatellite markers to highlight the genetic variability and population structure of the investigated nine Arabian horse lineages.

4.1. Genetic Diversity within and among the Arabian Horse Sire Lines

All Arabian lines showed high heterozygosity, but the LAT and SAK I sire lines both presented the highest value (0.66). The latter is among the highest heterozygosity values reported for other Arabian horse populations using the same or similar loci. For example, The He in Saudi Arabian, Syrian registered, Syrian nonregistered, and Iranian Arabian lines has been reported as 0.68, 0.69, 0.75, and 0.71, respectively [38], and as 0.69 in the Egyptian Arabian line [39], varying from 0.693 to 0.707 in Polish Arabian line [41], as 0.67 in the Turkish Arabian line [58], etc. The high genetic diversity observed in the LAT and SAK I sire lines was supported by the high Ne (3.18 and 3.31, respectively) (Table 1). In comparison, the Ne value in Saudi Arabian, Syrian registered, Syrian nonregistered, and Iranian Arabian lines has been reported as 3.30, 3.51, 4.23, and 3.61, respectively [38], as 2.99 in the Egyptian Arabian line [39], and the Polish Arabian line has shown a mean value ranging from 3.50 to 3.86 [36], while a mean value in the Turkish Arabian line is 3.34 [58]. The highest genetic diversity of the SAK I line may be associated with introgression from the other lineages (mainly IBR and KUH). The latter consideration is supported by the mixed genetic profile in this population compared with other populations in this study (Figure 2). Most likely the presence of other lineages in the SAK I line population is managed by breeders due to the danger of inbreeding and the reduction of the genetic diversity [59,60]. The SEA sire line revealed the lowest value of genetic diversity compared with the other populations (Table 1). In general, the founder effect, genetic drift, selection, and/or inbreeding result in low genetic variability in farmed stocks, including horses [61,62]. The level of genetic diversity found in a population largely depends on the mating system, the evolutionary history of a horse breed, and the population history, as well as the geographical ranges of the populations [63]. In addition, the presence of null alleles produces an excess of homozygotes in the population dataset, which in turn may affect the genetic diversity of the population [64,65].

One of the key indicators regarding heterozygosity in different horse populations (Finnish horse breed, Italian horse breed, and thoroughbred horses in Russia) is Na [66–68]. The preference of this parameter over heterozygosity results from the fact that in some cases, heterozygosity provides an overly optimistic view when there are many alleles at a locus or when the population goes through a small or recent bottleneck [41,69]. The Na ranged from 4.15 in the SAR to 5.54 in the SAK I and LAT populations, which may be indicative of a recent bottleneck or a founder effect in the latter sire lines. According to some authors, one disadvantage of Na is that the values are strongly influenced by the sample size [41,70]. Considering that, we also calculated the AR. The levels of AR showed the same pattern as that of the Na among all studied populations, which means that the sample sizes for all sire lines had no noticeable effect on Na. The value of the inbreeding coefficient F_{IS}, which also reveals the degree of deviation from random mating, ranged between −0.041 in SAK I to −0.204 in the SHA sire line, i.e., all lineages revealed a negative value of F_{IS} (Table 1). This negative F_{IS} rate may be associated with an excess of heterozygosity, which is most often observed following a decrease in the number of individuals in a population [71]. The negative F_{IS} value seen in all nine Arabian lines represents an excess of heterozygosity, which may be a result of outbreeding, i.e., a certain level of genetic introgression of all lineages before and/or after their introduction in Bulgaria. Otherwise, the excess of heterozygosity in the Bulgarian Arabian may be due to errors in random sampling prior to the pedigree record of this population.

Alternatively, rapid advances in sequencing technologies are making genotyping and genome sequencing more affordable and readily available [72,73]. Novel techniques such as next-generation sequencing (NGS) represent a powerful tool to generate large-

scale genome-wide datasets for investigating the genomic signatures of genetic admixture between different animal breeds [74,75].

4.2. Relationships and Genetic Differentiation among the Arabian Horse Sire Lines

The AMOVA results showed a very low percentage of variation among the sire lines (1%) (Supplementary Table S4), which was supported by the low level of F_{ST}. The overall F_{ST} value (0.096) showed a low level of sub-population stratification in the nine Arabian sire lines. A similar level was reported in Saudi Arabians, Iranian Arabians, Polish Arabians, and American Arabians [38], as well as in Tunisian Arabians [42]. All these results show that there is very low genetic differentiation among the individual populations (strains) of the Arabian horse. This finding was supported by the F_{ST} values (Supplementary Table S6). The F_{ST} value could be low (<0.05), medium (0.05–0.15), or high (>0.15) [76–78]. In our case, the SAK I sire line expressed the lowest values of F_{ST} compared with all other lines. Interestingly, the lowest genetic distance was observed between the SAK and the KUH sire lines (0.025), which suggested that individuals of the two lines may share closely related paternal ancestors. However, the results of STRUCTURE and phylogenetic analyses did not confirm this suggestion. According to the pedigree analysis of studbooks data, it can be seen that the founder Arabian sire lines with different blood lines originated from different countries, which might also explain the lower differentiation between SAK I and the other lineages. Furthermore, the pedigree analysis based on historical records only seems insufficient to explain this relatedness within Arabian sire lines. The general outcomes from the PCoA matched the results explained by both the genetic relatedness and genetic distances among studied sire lines (Figure 3). The PCoA plot clearly demonstrated that none of the individuals (except for a few animals) formed separate clusters due to the low genetic differentiation among all sire lines. In addition, the plot revealed that the LAT and SEA sire lines (PCoA 1 vs. 2 axes), as well as the IBR and SAK (PCoA 1 vs. 3 axes) lines, seemed to be without genetic introgression from other lines, as a few potential outlier individuals separated from the other gene pool to the left and the right side of the plot, respectively. This suggestion was supported when the neighbor-joining phylogenetic tree was constructed (Figure 4). The LAT and SEA lineages formed different branches, but it was very strange that the SHA line clearly separated from all other lineages. The SHA clustered close to the LAT line, which was visible from the PCoA analysis.

4.3. Population Structure and Assignment of the Arabian Sire Lines

The Bayesian clustering analysis at the optimal value of K = 3 confirmed the close relationship between the LAT and SHA sire lines (Figure 2). In all other lineages, to a greater or lesser extent, an admixed structure was observed. These data were confirmed by the results from PCoA and the pairwise F_{ST} tests. The STRUCTURE analysis identified the SHA sire line as the purest among all the lines. The extreme homogeneity of this lineage is probably due to the conservative breeding in this population, which descended from a limited number of founders. The STRUCTURE analysis showed similarity between the SHA and LAT sire lines. In fact, only these lineages were imported to Bulgaria from Arabian countries (Qatar and Egypt, respectively), which may suggest that their populations share a similar origin. The genetic similarity of the IBR and KUH lineages can be easily explained, considering that both lines are of Polish origin. Genetic similarity was found between SAK I (Egypt Arabian), SEA (Spanish Arabian), and BAI (German Arabian). The pedigree information shows that both the sire and the dam of SEA are determined to have been from the SAK I lineages [79]. Concerning the BAI sire line, it is the oldest active sire line in the world [30,38]. Similarly to SEA, the BAI sire line originated from the SAK I line and became the foundation sire of the Weil royal stud [80]. These observations confirmed that the SAK I, SEA, and BAI lineages share a common ancestor.

Knowledge of genetic characterization and genetic structure is the first step in breed conservation and may have implications for future breeding strategies and management plans. The analysis of the genetic structure of a population can be carried out using ge-

nealogical or molecular information [81,82]. In the case of missing or incomplete pedigrees, it would be better to use molecular information to characterize a population; moreover, molecular information indicates the additional relatedness between animals appearing as founders in the pedigree. Particularly, microsatellite genotyping provides information about the genetic profile of each animal, hence determining whether there is any genetic admixture with other breeds. This is crucial regarding the selection of purebred individuals for inclusion in selection programs in order to protect the purity of the breed.

5. Conclusions

Overall, this study enriches the understanding of the Arabian population structure. The current status of genetic diversity of the investigated populations of Arabian horse lineages breeding in Bulgaria showed high genetic diversity in all of them. Based on history, pedigree analysis, and STRUCTURE analysis, the investigated Bulgarian Arabians formed three distinct groups, with significant genetic relationships between them. Genetic similarity was observed between LAT and SHA, as well as between the IBR and KUH sire lines. The SAR population was more distant. The assessment of the inbreeding coefficient showed that the excess in homozygosity was common to all these lineages. In addition, the genetic differentiation indicated very low values among all sire lines. These results can facilitate conservation programs for this important breed and enhance the efforts to implement appropriate and strategic breeding programs in order to improve the management of Arabians. Furthermore, this study may encourage the Arabian horse breeders of the SAK I and SAR lineages, which were the most mixed lines, to eliminate impure animals from their stud lines, which would ensure the preservation of the original Arab lineage. In addition to the conventional selection method, the use and application of molecular studies for analysis of the population structure and the genetic diversity of each lineage is imperative. Finally, future studies using whole genome sequencing data should be pinpointed, since the latter will be more sensitive in detecting introgression or possible genetic admixtures among the horse lines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16050281/s1, Table S1: Number of identified alleles (Na), number of effective alleles (Ne), Polymorphic Information Content (PIC), heterozygosity: observed (Ho), expected (He), Shannon's information index (I), Fixation index (F), and F (Null)—Null allele frequency estimated; Table S2: Fixation index (F_{IS}, F_{IT}, F_{ST}), gene flow (Nm), genetic diversity at each locus (G_{ST}), total expected heterozygosity (H_T), and Jost's estimate of differentiation (D) among Arabian horse population; Table S3: Hardy Weinberg (HW) equilibrium test in all studied microsatellite loci by Arabian horse lineages; Table S4: Analysis of molecular variance (AMOVA) and F-Statistic of Arabian horse lineages based on genotyping of 13 microsatellite markers; Table S5: The Arabian horse individuals' assignment into 3 clusters depending on Q value at K = 3; Table S6: Pairwise F_{ST} statistics (below diagonal) and Nei's genetic distances (above diagonal) among nine Arabian horse lineages.

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