

Short communication

MITOCHONDRIAL DIVERSITY OF THE EAST BALKAN SWINE (*SUS SCROFA F. DOMESTICA*) IN SOUTH-EASTERN BULGARIA

PALOVA Nadezhda¹, YANKOVA Iskra², NEOV Boyko², HRISTOV Peter^{2*},
RADOSLAVOV Georgi²

¹Scientific Center of Agriculture, Agricultural Academy, Sredets, Bulgaria; ²Department of Animal Diversity and Resources, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria

(Received 07 April, Accepted 22 May 2019)

The East Balkan Swine (EBS) is the only preserved local swine breed in Bulgaria and one of the few indigenous pig breeds in Europe. The EBS is distributed in the region of Eastern Balkan Mountains and the Strandja Mountain. To reveal the breed's genetic profile, we analyzed 50 purebred individuals according to mitochondrial DNA (D-loop region, HVR1) and sequence analysis in the Scientific Center of Agriculture (Sredets region) in the country.

The obtained results show the presence of four haplotypes: three Asian specific haplotypes (H1, H2, and H3) and the European specific E1a1. The haplotypes H2 (6 %) and H3 (2 %) were newly described and were branched from the basic clade H1 (90 %). All haplotypes belong to the Asiatic clade A (98 %), except one sample assigned to the European haplogroup E1 (2 %) in contrast to samples from East North Bulgaria where Asiatic and European clades were with almost equal distribution. The coexistence of two mtDNA clades in EBS in Bulgaria may be related to the source of the pig populations and/or the historical crossbreeding with imported pigs.

In conclusion, due to its native origin, the East Balkan Swine may be the only possible option for a solution to the exhaustion of the beneficial genetic variation of available cultural breeds. With its participation, high-productive populations can be restored and established after a long and purposeful selection.

Key words: East Bulgarian Swine, D loop region, genetic diversity

INTRODUCTION

The East Balkan Swine (EBS) is the only preserved aboriginal pig breed in Bulgaria and one of the few indigenous pig breeds in Europe. The EBS is spread in the region of the Eastern Stara Planina Mountain and the northern slopes of the Strandja Mountain, dating back more than 2500 years [1]. It was formed mainly under the influence of

*Corresponding author: e-mail: peter_hristoff@abv.bg

natural selection, with very little human intervention. Therefore, it can live in nature without much human care and control. It has a well-developed herd instinct, a sense of self-preservation and protection from enemies (mainly jackals and wolves), and great stamina in long transitions through rough terrains.

The genetic diversity of the EBS has been investigated based on mitochondrial DNA analysis [2]. According to Hirata et al.[2] the characteristic for this breed is a mixed mitochondrial profile, i.e. both European clade E1 (61.3%) and Asiatic clade A (38.7%) co-exist. The authors identified a predominance of the Asiatic clade A in South-Eastern Bulgarian population. They discuss these differences as the relict of historical pig translocation, closest to the Anatolian pig population. Other study of EBSs and wild boar based on microsatellite analysis also showed a difference between North and South Bulgarian wild boar populations [3]. Because of the small number of EBS, the genetic structure showed a mixed profile with wild boar populations.

To preserve the breed and its unique morphological features, the Breeding and Storage Association of the EBS (ARSIS) has implemented a breeding policy in several areas: to maintain, preserve the breed and preserve the EBS as a genetic reserve, by increasing the efficiency of breeding the EBS in its natural environment [4].

According to data from the ARSIS of the Eastern Balkan Pig in 2016, the total number of pigs which had been a subject of selection control was 1127, of which 323 (8 herds) from Shumen district, 576 (7 herds) from Varna district and 228 (3 herds) from Bourgas district.

Strict following of the Breeding Program and ensuring rational use of the natural resources of Northeastern Stara Planina and Strandzha-Sakar Mountains create conditions for the production of high quality meat, delicatessens, and certified products. The implementation of the breeding program ensures the desired controlled genetic state of the breed, using the principles of pure breeding on the basis of maintenance selection.

Due to the valuable biological and economic qualities – high adaptability, healthy constitution, resistance to diseases and excellent quality characteristics of meat and fats, the breed is of particular interest mainly in two directions – as a gene pool and for the production of certified organic products [5,6].

Therefore, the aim of the present study is to reveal the mtDNA haplotypes of EBS reared in South-Eastern Bulgaria (Sredets), based on mitochondrial DNA (D-loop region, HVR1).

MATERIALS AND METHODS

Hair sample collection and DNA extractions

Hair samples were collected from 50 purebred unrelated individuals from the EBS in 2018. All animals had undergone selection control by the Breeding and Storage

Association of the EBS. Total DNA was extracted from hair follicles from the tail by using a GeneMATRIX Tissue DNA purification kit (E3550, EURX, Gdansk, Poland) according to the manufacturer's instructions. The quality of the isolated DNA was checked by 1 % agarose gel electrophoresis stained with SimplySafe (E4600-01, EURX, Gdansk, Poland). The quantity of isolated DNA was measured spectrophotometrically. The isolated DNA was stored at – 20 °C prior to analysis.

PCR amplification and sequencing

Based on the Pig Reference Sequence EF545567 [7], a 638-bp-long fragment of the mtDNA D-loop region was amplified using the following primers: L15387 and H16108 [8]. All PCR reactions were performed with 10ng/μl DNA in a final volume of 50 μl (NZYTaq Colourless Master Mix, Cat No – MB040, NZYTech, Portugal). The PCR conditions were the following: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, primers hybridization at 50 °C for 30 s, elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. The amplified fragments were separated and visualized on 1% agarose gel electrophoresis. The successfully amplified products were purified with a PCR purification kit (Gene Matrix, PCR clean-up kit, EURX, Poland) and sequenced in both directions, using a PlateSeq kit (Eurofins Genomics Ebersberg, Germany).

Data processing and analysis

All 50 obtained DNA sequences were manually edited and aligned with the MEGA7 program [9], using the pig reference DNA sequence EF545567 [7]. The obtained sequences (about 648 bp) were deposited in the GenBank database of the National Biotechnology Information Center (NCBI) under GenBank accession numbers MK618760-MK618763. Sequences were analyzed by polymorphic single nucleotide polymorphism (SNPs) position, and haplogroups were determined according to [7]. Calculations of the statistical quantities for the DNA sequences, including the number of haplotypes and haplotype diversity, nucleotide diversity, number of polymorphic sites (p. s.), and Fu and Li's D and F tests were performed by using DnaSP 5.10.1 [10]. Phylogenetic relationships between mtDNA haplotypes were explored by a Reduce Median network, using NETWORK 5.0.0.0 (Fluxus Technology Ltd.) (available at <http://fluxusengineering.com>).

RESULTS

After proper processing, a fragment of 648 bp from the beginning of the D-loop region was used for analysis. It was observed that 49 sequences (98 %) belong to the Asiatic clade A and only one sequence (2 %) was assigned to the European clade E1 (Table 1). In general, four different haplotypes were detected. The Asiatic haplogroup A was present with three haplotypes: H1, H2, and H3. The H1 haplotype was with the

highest frequency (90 %). The H2 haplotype was observed with 6 % frequency, while the H3 haplotype was with the lowest frequency of 2 %. The European haplotype E1a1 was detected with the same frequency as the H3 (Table 1).

Table 1. Haplogroup sequence variation in East Balkan Swine. EF545567 was used as a reference sequence (Wu et al., 2007). Haplotypes were assigned by Wu et al., (2007).

HVR1	1	1	1	1	1	1	2	2	2	3	3	3	4	4	5	5	5	5	MtDNA clade	MtDNA haplotype	n
	0	2	3	4	5	5	4	7	9	0	2	8	5	7	0	6	7	7			
	9	4	1	4	2	7	0	8	3	5	2	9	1	3	0	8	2	4			
Ref. seq. n. EF 545567	C	A	A	T	T	G	C	C	G	T	T	T	T	C	G	A	T	G	Asiatic clade	D1a1	-
H1 MK618760	T	C	T	A	.	.	.	Asiatic clade	D1a1'	45
H2 MK618761	T	C	T	A	G	.	.	Asiatic clade	D1a1'	3
H3 MK618762	T	C	T	A	.	C	.	Asiatic clade	D1a1'	1
H4 MK618763	T	T	G	C	C	A	T	.	A	C	C	C	C	.	A	.	.	A	European clade	E1a1	1

'Denotes unique haplotype

In general, 16 polymorphic sites were observed in the population of EBS (Table 2). The Fu and Li's FL-D and FL-F tests showed a negative value, which is an indication for lower average heterozygosity than that of segregating sites. The biological interpretation of this negative value may be related to a recent selective sweep, a population expansion after a recent bottleneck, or a linkage to a swept gene.

Table 2. Haplotype diversity (H), nucleotide diversity (π_n), mean number of pair-wise differences (π), number of haplotypes (Hn), number of polymorphic sites (p. s.), and Fu and Li's D and F tests in East Balkan Swine.

Breed	H \pm SD	$\pi_n \pm$ SD	π	Hn	p. s.	Fu and Li's D test	Fu and Li's F test
East Balkan Swine	0.186 \pm 0.072	0.0011 \pm 0.002	0.701	4	16	-5.01379	-4.91225

In order to investigate the phylogenetic relationship of the haplotypes in the EBS, we used a reduce-median network (RM network) with 50 sequences (Figure 1). The median-joining (MJ) network based on the mtDNA haplotypes of the EBS in Bulgaria consisted mainly of two major mtDNA clades (the European clade E1 and the prevalent Asian clade A). The clustering of some EBS with Asian pigs indicates that Asian pigs were involved in the development of the EBS.

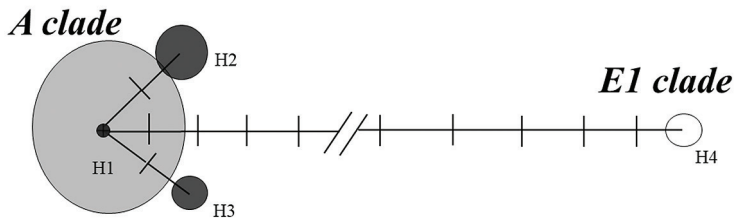


Figure 1. The Reduced-Median network of the mtDNA haplotypes in East Balkan Swine. The sequence variations and codes of the haplotypes are from Table 1.

DISCUSSION

The Balkans have served as a bridge for the movements of species that had been restricted to Anatolia during Pleistocene glaciations [12] because the Balkan region of Europe and Anatolia were connected by a land bridge during the last glaciation [13,14]. In this relation, some authors [15,16] proposed that the modern genetic structure of *S. scrofa* in Europe is the result of a post-glacial colonization from one or more southern refugia, including Iberia and the Balkans but excluding the Italian Peninsula where a highly divergent mitochondrial DNA (mtDNA) exists.

To reveal the genetic structure of the southern Balkan wild boar population in an effort to understand the historical effect of this region, Alexandri *et al.* [17] investigated a total of 200 wild boar samples from 18 locations in Greece and one location in Bulgaria based on mitochondrial DNA analysis. The obtained results showed that all samples studied from continental Greece and Bulgaria grouped into the European E1 clade, but in Bulgaria clade A also exists. These data showed that the Asiatic clade A was disseminated via migration processes from the Neolithic period (at least 8 000 BP). Specific for Europe both for wild boar and domestic pig is clade E1 [17]. The presence of this clade showed that the process of pig domestication in Europe may be associated with hybridization events between the native wild boar and the domesticated pig.

Other studies investigated the ESB based on mitochondrial DNA analysis [2] and microsatellite markers [3]. According to [2], characteristic for North Bulgaria is a mixed profile, i.e. both European clade E1 and Asiatic clade A co-exist. In contrast, in South Bulgaria the A clade is observed as dominant. Similar data about wild boar based on microsatellite analysis showed a clear difference between North and South populations [3]. A comparison of the EBS with Bulgarian wild boars and commercial pigs showed there that there has been a gene flow between these three groups.

The results of our study include all available maternal lineages from animals reared in The Scientific Center of Agriculture (Sredets) which are representative of the South group population of the EBS. As expected, considering other similar investigations, we have observed a prevalence of the Asiatic clade A (Fig. 1). We have identified

two additional new haplotypes, H2 and H3, branched from the basic clade H1. The dominance of the Asiatic H1 haplotype (90 %) is a reason for the low value of Fu and Li's FL-D and FL-F tests (Table 2).

A possible explanation of the dominance of the Asiatic A clade in our samples may be attributed to the bottleneck population structure of the first created pig stock (about 50 pigs) and the specific stochastic case in comparison to the North EBS population. These results may also be explained with the close geographical location to Anatolia where the Asiatic haplogroup A prevailed. Another possible explanation is associated with a possible gene flow with other European breeds like Berkshire, Bulgarian White, Coloured German Swine and Mangalica [2] in the EBS population distributed in the North.

CONCLUSION

The results of our study provide valuable information in regard to the conservation of the EBS breed in Bulgaria. Our data have confirmed a preliminary investigation about the mitochondrial genetic profile of the EBS which had shown that the Asiatic clade A is predominant in South-Eastern Bulgaria. In addition, we have identified two new haplotypes, H2 and H3, with a frequency of about 10 %. These data have also shown a lower mitochondrial diversity in comparison to the Northern Bulgarian EBS group. A possible explanation of these results may be associated with the bottleneck effect of the EBS or the typical conserved domestic pigs closest to Anatolian populations.

Acknowledgements

This work was supported by the National Scientific Fund of the Bulgarian Ministry of Education and Science, grant number H 21/32 2018.

Authors' contributions

PN obtained the samples. YI and NB carried out the molecular genetic studies. HP and RG participated in the sequence alignment and drafted the manuscript. PN, RG and HP participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Ivanova-Peneva SG, Stoykov A: East Balkan Swine in Bulgaria—an option for organic production. *Systems Development: Quality and Safety of Organic Livestock Products*. 2005, 97.
2. Hirata D, Doichev VD, Raichev EG, Palova NA, Nakev JL, Yordanov Y M, Masuda R: Genetic variation of the East Balkan Swine (*Sus scrofa*) in Bulgaria, revealed by mitochondrial DNA and Y chromosomal DNA. *Anim Genet* 2015, 46:209-212.
3. Nikolov IS, Stoeckle BC, Markov G, Kuehn R: Substantial hybridisation between wild boars (*Sus scrofa scrofa*) and East Balkan pigs (*Sus scrofa f. domestica*) in natural environment as a result of semiwild rearing in Bulgaria. *Czech J Anim Sci* 2017, 62:1–8.
4. Palova N: Evaluation of the basic fattening and slaughtering qualities of pigs of the East Balkan breed in natural conditions of cultivation, *Autoreferate* 2007, 7-29.
5. Marchev Y, Nedeva R, Nakev Zh, Ivanova-Peneva S, Gineva E, Palova N: Quality and fatty acid composition of the meat of pigs of East Balkan breed grown in different habitats. *Bul J Anim Husb* 2010, 5:48 – 56.
6. Nakev Zh, Marchev Y, Nedeva R, Palova N: Composition of the pig carcass of Eastern Balkan breeds cultivated by traditional technology; I. Composition of pig carcasses from East Balkan breeds grown in different regions of the country. *Agric Sci* 2012, 45:20-24.
7. Wu GS, Yao YG, Qu KX, Ding ZL, Li H, Palanichamy MG, Zhang YP: Population phylogenomic analysis of mitochondrial DNA in wild boars and domestic pigs revealed multiple domestication events in East Asia. *Genome Biol* 2007, 8:R245.
8. Larson G, Dobney K, Albarella U, Fang M, Matisoo-Smith E, Robins J, Rowley-Conwy P: Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. *Science* 2005, 307:1618-1621.
9. Kumar S, Stecher G, Tamura K: MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016, 33:1870-1874.
10. Librado P, Rozas J: DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 2009, 25:1451–1452.
11. Hewitt GM: Post-glacial recolonization of Europe. *Biol J Linn Soc* 1999, 68:87–112.
12. Hewitt GM: The genetic legacy of the Quaternary ice ages. *Nature* 2000, 405:907–913.
13. Tzedakis PC, Andrieu V, de Beaulieu JL, Crowhurst S, Follieri M, Hooghiemstra H, Magri D, Reille M, Sadori L, Shackleton NJ, Wilmstra TA: Comparison of terrestrial and marine records of changing climate of the last 500,000 years. *Earth Planet Sci Lett* 1997, 150:171–176.
14. Aksu AE, Hiscott RN, Yasar D: Oscillating Quaternary water levels of the Marmara Sea and vigorous outflow into the Aegean Sea from the Marmara Sea–Black Sea drainage corridor. *Mar Geol* 1999, 153:275–302.
15. Scandura M, Iacolina L, Apollonio M: Genetic diversity in the European wild boar *Sus scrofa*: phylogeography, population structure and wild · domestic hybridization. *Mammal Rev* 2011, 41:125–137.
16. Scandura M, Iacolina L, Crestanello B, Pecchioli E, Di Benedetto, F, Russo V, Davoli R, Apollonio M, Bertorelle G: Ancient vs. recent processes as factors shaping the genetic variation of the European wild boar: are the effects of the last glaciation still detectable? *Mol Ecol* 2008, 17:1745–1762.

17. Alexandri P, Triantafyllidis A, Papakostas S, Chatzinikos E, Platis P, Papageorgiou N, Triantaphyllidis C: The Balkans and the colonization of Europe: the post-glacial range expansion of the wild boar, *Sus scrofa*. *J Biogeogr* 2012, 39:713-723.

DIVERZITET MITOHONDRIJA KOD ISTOČNOBALKANSKE SVINJE (*SUS SROFA F. DOMESTICA*) U JUGOISTOČNOJ BUGARSKOJ

PALOVA Nadezhda, YANKOVA Iskra, NEOV Boyko, HRISTOV Peter, RADOSLAVOV Georgi

Istočnobalkanska svinja (IBS) je jedina sačuvana lokalna rasa svinja u Bugarskoj i jedna od nekoliko domaćih rasa u Evropi. IBS je rasprostranjena u regionu istočnobalkanskih planina i na planini Strandja. U cilju određivanja genetskog profila rase, u Naučnom institutu za poljoprivredu (Sredets region) obavili smo analize mitohondrijalne DNK (region D-loop, HVR1) sekvenci 50 čistokrvnih jedinki.

Dobijeni rezultati ukazali su na prisustvo četiri haplotipa: tri haplotipa koji su specifični za Aziju (H1, H3 i H3) i jednog specifičnog za Evropu, E1a1. Haplotipovi H2 (6%) i H3 (2%) su opisani prvi put i nastali su grananjem od osnovne klade H1 (90%). Svi haplotipovi pripadaju azijskoj grupi A (98%), sa uzuzetkom jednog uzorka koji se svrstan u evropsku haplogrupu E1 (2%), za razliku od uzoraka iz severoističnog regiona Bugarske gde su azijske i evropske grupe imale skoro istovetnu distribuciju. Koegzistencija dve mtDNK klade IBS Bugarske može da bude povezana sa izvorom populacije svinja i/ili sa ukrštanjem uveženih svinja u prošlosti.

Može se zaključiti da, zahvaljujući prirodnom poreklu, istočnobalkanska svinja može biti jedina moguća opcija za rešenje problema smanjenja korisnih genetičkih varijacija kod rasa svinja koje se danas koriste. Visoko produktivne populacije, mogu da budu obnovljene i ponovo uspostavljene posle duge i ciljane selekcije. Njihovim uključivanjem u programe dugoročne i ciljane selekcije mogu se obnoviti i uspostaviti visokoproduktivne populacije.