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Substantial Hybridisation Between Wild Boars (*Sus scrofa scrofa*) and East Balkan Pigs (*Sus scrofa f. domestica*) in Natural Environment As a Result of Semi-wild Rearing in Bulgaria

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ABSTRACT

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The East Balkan pig (EBP) is a traditional domestic pig breed in Bulgaria managed in semi-wild conditions and well-adapted to the continental climate and rearing on pastures. From the genetical and historical point of view it is important to preserve this ancient breed. However, over the last several years, a dramatic decline of EBP herds has been observed. Moreover, introgression between EBP and wild boar in Bulgaria (WBB) is very likely to have occurred. In this study we used a set of 10 microsatellites and the polymerase chain reaction–restriction fragment length polymorphism method on melanocortin-1 receptor (*MC1R*) polymorphisms to study the degree of hybridisation between WBB, EBP, and a commercial pig breed (CPB). *MC1R* results identified WBB-EBP hybrids and the analysis of the microsatellite data with a Bayesian assignment approach and the Discriminant Analysis of Principal Components revealed a low genetic differentiation between WBB and EBP and a high amount of introgression of WBB into EBP. A mentionable introgression of CPB into EBP was also revealed. It is apparent that the traditional rearing system of EBP, which is hundreds of years old, has led to a permanent hybridisation between WBB and EBP. In our opinion, the preservation of the semi-wild rearing system is a prerequisite for the conservation of this old, indigenous pig breed and its genetic composition, as the semi-wild rearing system allows the continuous introgression with WBB. Moreover, the introgression of commercial breeds into EBP or WBB should be prevented. Due to the bidirectional gene flow these hybridisation events would have negative consequences (i.e. loss of viability and adaptation) for the wild boars as well as for the indigenous pig breed.

Keywords: microsatellites; PCR-RFLP; melanocortin-1 receptor; introgression; local pig breed; commercial pig breed

The East Balkan (Iztochno-Balkanska) pig (*Sus scrofa f. domestica)* (EBP) is an indigenous breed in Bulgaria. EBP is characterized by middle-sized to big heads with a long muzzle, short necks, small and almost standing ears, middle long and very strong legs, and sharp, smooth and rough bristles,

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along the middle backline, like a comb. Although the dominant coat colour is black, individuals with spotted or yellowish brown coats (called "fox" type) are also observed (Dimitrov and Dimitrova 1994). This breed is well-adapted to the extreme continental climate and reared in wooded areas as well as on pastures. The herds predominantly feed on natural food sources as they have been reared outdoors for centuries in small (20–30 individuals) or in large groups (200–300 pigs) (Hlebarov 1921). Due to this rearing condition introgression between EBP and Bulgarian wild boars (WBB) is very likely to have occurred (Genov et al. 1991; Nikolov et al. 2009). Evidences of hybridisation between wild boars and domestic pigs have been found in Slovenia, in Western Balkan regions, and in Italy (Scandura et al. 2008; Fontanesi et al. 2014), Belgium and Luxembourg (Frantz et al. 2013), Great Britain (Frantz et al. 2012), Greece (Koutsogiannouli et al. 2010), and Croatia (Sprem et al. 2014) based on the analysis of genetic markers (microsatellites, mitochondrial DNA, and coat colour linked genes). The plausible causes for hybridisation are various. Frantz et al. (2012) assumed that farmers release farmed wild boars that had been crossed with domestic pigs in captivity. The semi-wild rearing conditions also lead to hybridisation between wild boars and indigenous domestic pigs (Canu et al. 2014; Sprem et al. 2014). In order to preserve the genetic integrity of small and rare domestic breeds, the analysis and monitoring of introgression is crucial (Sprem et al. 2014).

According to historical records, EBP was crossed with imported pig breeds (Berkshire, Bulgarian White, Coloured German Swine, and Mangalica) (Georgiev and Benkov 1964a, b). In 1952, EBP was the most common pig race in Bulgaria. However 30 years later the race was close to extinction. In 2006 a conservation program was started with 3200 individuals divided into numerous herds. In spite of this effort to conserve this breed, a dramatic decline of EBP herds has been observed in Bulgaria over the past several years (1858 individuals in 2009 and 1216 in 2012) due to administrative restrictions and intensification of livestock breeding which makes other pig breeds more profitable than autochthonous ones (Nakev et al. 2011; Nakev and Kulev 2013).

Two types of genetic markers (microsatellites and melanocortin-1 receptor) are frequently used to detect the hybridisation between wild boars and domestic pigs (e.g. Scandura et al. 2011a; Frantz et al. 2012, 2013). Microsatellite loci are very useful to analyze gene flow and admixture even between closely related populations due to their high level of polymorphism and mutation rate (Roy et al. 1994) and they have been widely used in combination with Bayesian clustering and admixture analysis (Pritchard et al. 2000) in order to analyze hybridisation between wild boars and different pig breeds (e.g. Scandura et al. 2011a, b; Frantz et al. 2012; Sprem et al. 2014).

In mammals, the melanocortin-1 receptor (*MC1R*) regulates melanogenesis within the melanocyte and the hair follicle. Common variations (polymorphisms) in the *MC1R* gene are associated with natural differences in skin and hair colour. To date, several distinct *MC1R* alleles have been identified (E^+, E^{D1}, E^{D2}, E^P, and *e*) which are associated with different colour phenotypes in pigs (Fajardo et al. 2008; Fontanesi et al. 2014). E^+ is associated with the wild-type coat colour in European wild boar (*Sus scrofa scrofa*), and is not found in any of the domestic breeds (Kijas et al. 1998) except in the European breed Mangalica (Fang et al. 2009). The analysis of the *MC1R* gene is widely used to search for hybrids between wild boar and domestic pigs (Ciobanu et al. 2001; Fajardo et al. 2008; Koutsogiannouli et al. 2010; Frantz et al. 2013; Fontanesi et al. 2014).

In the present study we used microsatellites and *MC1R* to (*i*) study the genetic composition of the EBP and (*ii*) unravel the hybridisation patterns between WBB, EBP, and commercial pigs (CPB) in order to understand the genetic history of the indigenous EBP, which helps design a sound and sustainable management strategy for EBP.

MATERIAL AND METHODS

Study populations. A set of 291 WBB was included in this study. We used DNA of 289 WBB individuals analyzed by Nikolov et al. (2009) and two additional samples from Rila Mountain population provided by hunters in 2009. EBP samples (n = 23) were received from local farmers. Sampling locations and number of individuals per population are shown in Figure 1. In order to assess the genetic relationship between EBP and CPB, tissue samples (n = 36) from the crossbreed between German Landrace and Pietrain pig (n = 33),

doi: 10.17221/49/2015-CJAS

Schwabian-Hall pig (n = 1), Pietrain (n = 1), and German Landrace (n = 1) were also included. The samples were provided by German farmers in 2009.

For subsequent analysis (Bayesian clustering and Discriminant Analysis of Principal Components (DAPC)), the entire sample set was divided in four groups. One group included all EBP samples, one consisted of CPB, and the wild boar individuals were split in two groups according to the results of Nikolov et al. (2009), reporting two main genetic groups in Bulgaria (north group (NWG) and south group (SWG)) for WBB (Figure 1).

DNA isolation and genotyping. Total genomic DNA was extracted from liver of WBB, EBP, and CPB individuals. Ten polymorphic and independent microsatellites (Rohrer et al. 1994; Lowden et al. 2002) were selected for this study (Supplementary Table S1). Details of DNA extraction, PCR protocol, and genotyping procedures are described in Nikolov et al. (2009).

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis was used to distinguish the wild type allele (E^+) from domestic variants. *MC1R* exon fragments were obtained by PCR, using two locus-specific pairs of primer. The first one (*MERL1* and *EPIG2*) amplified a 428 bp product (Kijas et al. 1998). The amplification reactions and the PCR profile were carried out as described by Kijas et al. (1998). PCR products were digested by *Bsp*HI (Fermentas, Germany) restriction enzyme. According to Kijas et al. (1998), the PCR products from wild boars are not digested, while products of hybrids and pure domestic pigs show specific banding pattern with three or two bands, respectively. In order to test all samples for the presence of wild Boar × Duroc/Large Black/Meishan hybrids (Koutsogiannouli et al. 2010) we used the primers *EPIG1* and *EPIG3* amplifying a 405 bp fragment of the *MC1R* gene containing the polymorphism at codon 240. The fragment was digested with the enzyme *Bst*UI (New England BioLabs, USA). Digestion of this fragment with *Bst*UI produces two bands for one *MC1R* allele except for allele *e* (recessive red), which is an allele of European origin that occurs in the Duroc, Large Black, and Meishan breeds

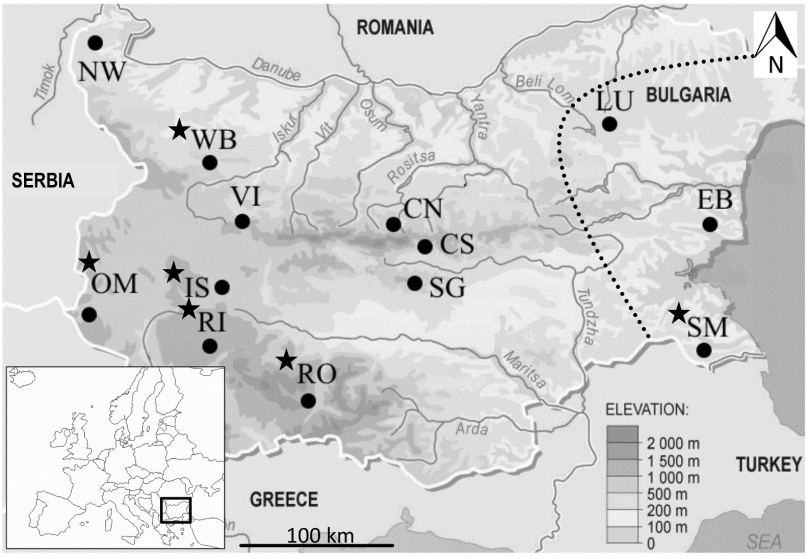

Figure 1. Thirteen sampling locations of wild boars in Bulgaria (abbreviations and number of individuals are indicated in brackets): north west (NW, n = 19), Ludogorie (LU, n = 24), west Balkan (WB, n = 20), Vitinja range (VI, n = 16), central Balkan – north part (CN, n = 20), central Balkan – south part (CS, n = 29), Strandja Mountain (SM, n = 28), east Balkan (EB, n = 24), Sredna Gora (SG, n = 25), Iskur range (IS, n = 27), Osogovo Mountain (OM, n = 23), Rila Mountain (RI, n = 14), and Rhodope Mountain (RO, n = 22). According to Nikolov et al. (2009) there are two main genetic wild boar groups (WBB) in Bulgaria: the north group (NWG) and the south group (SWG). Populations of SWG (RO, OM, IS, RI) and populations including individuals (17 animals from SM and WB) which belong to the south group (Nikolov et al. 2009) are marked with an asterisk. The area east of the black dotted line (administrative districts of Burgas, Varna, and Shumen) represents the territory where East Balkan pigs (EBP) are reared. EBP tissue samples (n = 23) for analysis were provided by local farmers from SM, LU, and EB

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(Kijas et al. 1998). All digested PCR products were visualized by gel electrophoresis according to Koutsogiannouli et al. (2008).

Data analysis. The FSTAT Version 2.9.3 program package (www.unil.ch/izea/softwares/fstat.html) was used for calculating the total number of alleles (N_{al}), the expected and observed heterozygosities (H_e, H_o), and allelic richness (A_r).

Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were estimated in each of the four groups using GENEPOP (Version 3.3) (Raymond and Rousset 1995) with probability tests carried out using the Markov chain method with 100 000 iterations and 1000 dememorization steps (Guo and Thompson 1992). F_{ST} (Weir and Cockerham 1984) values between SWG, NWG, CPB, and EBP were calculated with the same software. MICRO-CHECKER (Version 2.2.3) (Van Oosterhout et al. 2004) was used to test the data set for genotyping errors and for the presence of null alleles.

To infer the individual genetic ancestry we conducted the Bayesian clustering method as proposed by Pritchard et al. (2000) using the version of STRUCTURE (Version 2.3.4) according to Hubisz et al. (2009). It is advisable to use this approach of STRUCTURE if genetic divergence between analyzed individuals is low (Hubisz et al. 2009), which was detected between WBB and EBP samples in preliminary tests using STRUCTURE. The admixture model, correlated allele frequencies model, and locprior option were chosen in order to reveal the number of distinct genetic clusters (K). We tested K from 1 to 10 with 10 iterations (50 000 burn-in, 500 000 Markov chain Monte Carlo replicates in each run) to assess convergence of ln Pr(X/K). The numbers of clusters present were then determined from posterior probabilities of K and additionally by an *ad hoc* statistic K based on the rate of change in the log probability of data (Evanno et al. 2005) using the software STRUCTURE HARVESTER (Version 0.6.94) (Earl and von Holdt 2012). Since Bayesian clustering techniques may produce biased results when faced with unequal sample sizes (Puechmaille 2016), we verified the result of STRUCTURE with the multivariate approach DAPC (Jombart et al. 2010), which is more insensitive when sampling is uneven (Puechmaille 2016). The DAPC was implemented in the ADEGENET package in R software Version 3.1.2 (www.R-project.org) and was used to visualize the genetic structure of NWG, SWG, EBP, and CPB.

RESULTS

Genetic diversity and differentiation. MICRO-CHECKER (Version 2.2.3) (Van Oosterhout et al. 2004) did not detect any genotyping errors and null alleles among the data set. Genotyping with 10 microsatellite loci of NWG, SWG, EBP, and CPB samples showed 117, 104, 77, and 69 different alleles, respectively. Expected (H_e) and observed heterozygosity (H_o) varied from H_e = 0.64 (CPB) to H_e = 0.73 (SWG) and from H_o = 0.58 (EBP) to H_o = 0.63 (SWG), allelic richness (A_r) varied from A_r = 6.4 (CPB) to A_r = 7.80 (SWG) (Supplementary Table S1). All of the pairwise estimates of F_{ST} were significant ($P < 0.001$) with the highest F_{ST} values between NWG and CPB (F_{ST} = 0.104) and SWG and CPB (F_{ST} = 0.081) and the lowest between SWG and NWG (F_{ST} = 0.038) and SWG and EBP (F_{ST} = 0.040) (Supplementary Table S2).

Linkage and Hardy-Weinberg equilibrium. The test for linkage disequilibrium revealed twelve cases (SWG: 2; NWG: 6; EBG 2; CPB: 2) of linkage dis-

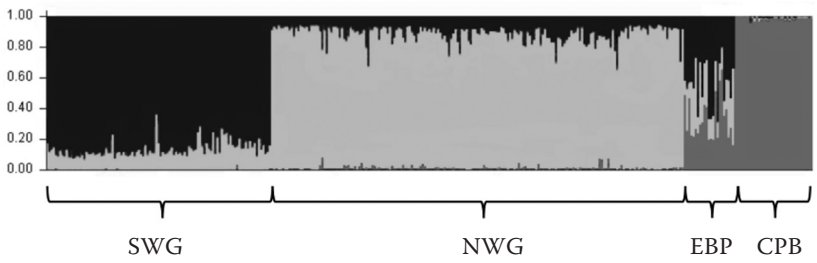

Figure 2. Bayesian clustering analysis with STRUCTURE program (Version 2.3.4.) (Pritchard et al. 2000; Hubisz et al. 2009) revealed three clusters expressed by black, dark grey, and light grey colour
SWG = south group, NWG = north group, EBP = East Balkan pigs, CPB = commercial pig breed

doi: 10.17221/49/2015-CJAS

Table 1. Representation of the STRUCTURE program (Version 2.3.4.) (Pritchard et al. 2000; Hubisz et al. 2009) results (assignment to cluster 1–3, in %) for sampled wild boars (north group – NWG, south group – SWG), East Balkan pig (EBP), and commercial pig breed (CPB); absolute number of sampled individuals per group (*n*); the three columns on the right show absolute number of wild boar in Bulgaria, hybrids, and EBP/CPB genotypes as detected by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method (in parentheses as %)

Groups	*n*	Bayesian method			PCR-RFLP		
		cluster 1	cluster 2	cluster 3	E^+/E^+	E^+/E^P	E^P/E^P
SWG	103	11.2	88.4	0.4	102 (99.0)	1 (1.0)	–
NWG	188	85.8	13.5	0.7	143 (76.1)	32 (17.0)	13 (6.9.0)
EBP	23	20.0	44.2	35.9	5 (22.0)	9 (39.0)	9 (39.0)
CPB	36	0.1	0.5	99.4	–	–	36 (100)

equilibrium after Bonferroni correction ($P < 0.005$). Consequently the data set was scrutinized intensively but no systematic linkage could be detected.

Tests on Hardy-Weinberg equilibrium for each locus in each group revealed two deviations in SWG, EBP, and CPB and three deviations in NWG after sequential Bonferroni correction (Supplementary Table S1).

Bayesian clustering and admixture analysis. Application of the clustering method using STRUCTURE followed by ΔK calculation according to Evanno et al. (2005) produced three clusters (Figure 2, Supplementary Figure S2, Table 1). The largest proportion of genome of SWG wild boars was attributed to cluster 2 (88%), the NWG wild boars were assigned with a greater posterior probability (86%) to cluster 1, and CPB individuals formed a separate cluster 3 (99%). The samples of EBP showed a strong signal of admixture and were portioned between the three clusters (20, 44, and 36% respectively) (Figure 2, Table 1).

DAPC detected an intermediary status of EBP between the wild boar groups (SWG and NWG) and the domestic group CPB (Figure 3).

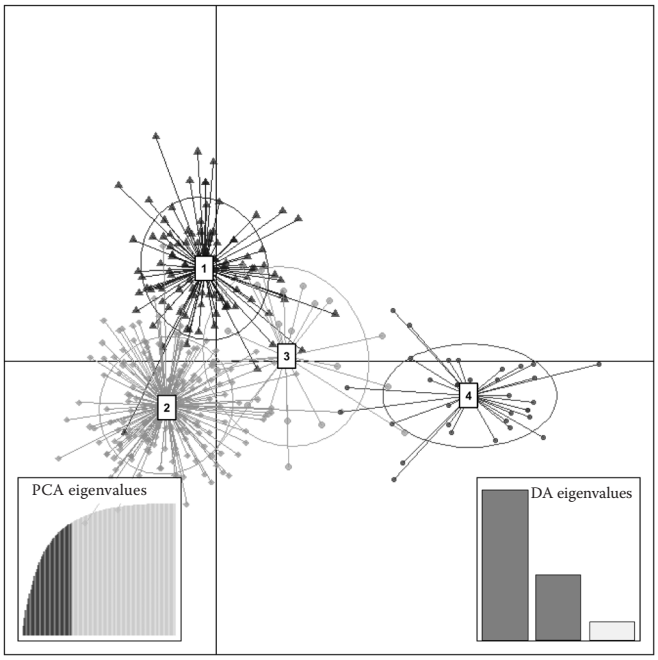

Figure 3. Discriminant Analysis of Principal Components (DAPC) scatterplot: 40 principal components of Principal Component Analysis (PCA) were retained in order to explain approximately 90% of the total variation of the data set analyzed in this study. The obtained graph represents the individuals as dots, triangles, and squares, the groups (1 = south group (SWG), 2 = north group (NWG), 3 = East Balkan pigs (EBP), and 4 = commercial pig breed (CPB) are shown as inertia ellipses. Eigenvalues of the analysis are displayed in inset

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Analysis of genetic variation with RFLP (MC1R gene). The treatment of PCR products with *Bsp*HI showed no digestion for wild boars (allele E^+), while the samples of the reared breeds (EBP, CPB) and hybrids (hybrids within EBP and hybrids within the two WBB groups) were digested, producing two (allele E^P) and three DNA bands (allele E^+ and E^P), respectively (Supplementary Figure S1). Nine of the 23 analyzed EBP samples had the genotype E^P/E^P, five had the genotype E^+/E^+, and nine were defined as hybrids with the alleles E^+ and E^P. Within the NWG, 13 individuals (6.9%) were detected with the genotype E^P/E^P and 32 (17%) showed admixed ancestry with the genotype E^+/E^P (Table 1). Within SWG one individual out of 103 (1.0%) showed the hybrid genotype (Table 1). The majority of hybrids and E^P/E^P genotypes within NWG were found at sampling sites East Balkan (EB), Ludogorie (LU), and Strandja Mountain (SM), in populations on the territory where EBP are reared (data not shown). The analysis for detecting the recessive red allele by digesting (enzyme *Bst*UI) the PCR products amplified with the primers *EPIG1* and *EPIG3* revealed no *e* allele in all samples. Thus, no admixture with Duroc, Large Black, and Meishan could be detected.

DISCUSSION

In this study we used microsatellite data and the PCR-RFLP method based on *MC1R* mutations to study the genetic constitution of EBP by considering introgression of WBB and CPB. PCR-RFLP revealed a high degree of recent bidirectional hybridisation events among wild boars and EBP with 17% hybrid genotypes E^+/E^P in the NWG group and 39% hybrid genotypes in EBP. Even pure WBB genotypes E^+/E^+ were detected in EBP (22%) and pure E^P/E^P genotypes were revealed in the wild boar group NWG (7%). These results confirmed the suggestion of Genov et al. (1991) and the hypothesis of Nikolov et al. (2009) about the possible introgression between EBP and wild boars in Bulgaria. However, the exclusive use of the *MC1R* gene marker could overestimate the introgression rate from EBP into WBB as mentioned by Fontanesi et al. (2014).

The existence of hybrids and pure WBB among reared EBP herds is an expected finding, since WBB and EBP are morphologically difficult to differentiate (Dimitrov and Dimitrova 1994). Due to the similar anatomy of WBB and EBP (Dimitrov and Dimitrova 1994) and the diverse coat colour of this pig breed (Hlebarov 1921) some farmers might not be aware that they maintain hybrids and even WBB among their herds. To our knowledge hybridisation is also tolerated by numerous breeders in order to increase profitability (Rusev and Stojnov, pers. comm.).

The analysis of the microsatellite data using both Bayesian clustering and DAPC clearly revealed a mentionable introgression of WBB and CPB into EBP, whereas the proportion of WBB gene pool in EBP predominates. According to Puechmaille (2016), Bayesian clustering may be biased and deliver wrong estimates of the number of clusters with uneven sample sizes. As the visualization of the population genetic structure with the DAPC is in concordance with the STRUCTURE result, a bias associated with uneven sampling is unlikely.

The population genetic indices H_o and H_e are similar among all analyzed genetic groups and in accordance with those reported by Druml et al. (2012) for local Balkan pig breeds in Austria (Mangalica, $A_r = 3.8$, $H_o = 0.58$, and $H_e = 0.54$), Croatia (Black Slovonian, $A_r = 5.4$, $H_o = 0.59$, and $H_e = 0.64$ and Turopoljski Lug, $A_r = 3.3$, $H_o = 0.38$, and $H_e = 0.37$), Serbia (Mangalica, $A_r = 3.9$, $H_o = 0.58$, and $H_e = 0.54$), and Bosnia-Herzegovina (Bosnian mountain pig, $A_r = 4.2$, $H_o = 0.62$, and $H_e = 0.58$). The observed mean allelic richness in EBP ($A_r = 7.70$) is substantially higher than in breeds studied by Druml et al. (2012) which may be explained by historical genetic introgression with wild boars.

The detected introgression from CPB into EBP can be attributed to the breeding history and corresponds with the results reported by Hirata et al. (2015). Additionally it is evident, that the traditional rearing system of EBP which is hundreds of years old, has led to a permanent introgression between WBB and this breed, resulting in a very high genetic similarity. However, this does not apply for many other rare breeds. For instance, Sprem et al. (2014) found a clear genetic differentiation between WBB and reared pigs.

CONCLUSION

The results of our study provide valuable information in regard to conservation of the EBP breed in

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Bulgaria. This breed is well-adapted to the extreme climate conditions of pastures, it is disease resistant, produces high quality meat, and is suitable for organic farming. For these reasons, it is of utmost importance to preserve this ancient breed.

The rearing of EBP in semi-wild conditions has resulted in permanent introgression with wild boars which has consequently shaped the genetic constitution of EBP and thus contributed to its unique characteristics. For EBP it is therefore especially important to support and preserve the traditional rearing system.

Our results indicate a bidirectional gene flow between wild boars and EBP. Furthermore, EBP represents an intermediate genepool of wild and commercial pigs. Further introgression of commercial breeds into EBP and of course into wild boar populations could have negative consequences for their genetic integrity (i.e. loss of viability and adaptation, less resistance to diseases). This underlines the importance of preserving the genetic composition of both EBP and wild boar and further hybridisation with commercial breeds should be prevented during pig farming. Beside this, periodical genetic monitoring of wild boars as well as of EBP populations in Bulgaria would be an effective approach to detect such undesirable hybridisation events and secure the extraordinary status of the East Balkan pig breed.

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