

EVIDENCE OF GENETIC HYBRIDIZATION OF THE WILD BOAR AND THE INDIGENOUS BLACK PIG IN NORTHERN GREECE

George P. Laliotis^{1*}, Meni Avdi²

¹ Research Institute of Animal Science, Hellenic Agricultural Organization "Demeter", Paralimni Giannitsa, 58100 Pella

² Laboratory of Physiology of Reproduction of Farm Animals, Department of Animal Production, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

* Corresponding author: George P. Laliotis, e-mail: glaliotis@rias.gr.

Original scientific paper

Abstract: In Greece both the black indigenous pig and the wild boar are considered as species of valuable genetic diversity while their products achieve a valuable market price. However, many crop damages are recorded, with farmers to claim that wild boar hybrids are responsible. On the other hand, black pig classification is based on phenotypic characteristics, which does not ensure breed's homogeneity in case of hybridization. Using the PCR-RFLP methodology, pig samples (n=135) from different rearing situations (feral boars, semi-extensive black pigs and extensive wild boars) were examined in order to identify whether or not hybridization exists. In the examined feral population of wild boar a 26% of hybrids was noted, while in the case of the extensive farming population of wild pigs a hybridization of 11.76% was observed. Interestingly, in both cases of the examined black pigs' populations, a mentionable hybridization with wild boar was observed, reflecting probably an implemented breeding practice or uncontrolled mating with wild boars. A pivotal level (5-7%) of inbreeding rate was also noted in the examined populations. The immediate removal of hybrids from all the examined populations should be achieved, in order to prevent and eliminate further introgression, genetic depression and loss of genetic diversity for both populations of wild boar and black pig. Finally, the applied methodology may be used by state authorities or certifying organizations to test, control or inspect farms rearing wild boar or black pig populations in order to record and eliminate hybridization events between them.

Key words: *Sus scrofa*, wild boar, *Sus domestica*, hybridization, PCR-RFLPs, genes

Introduction

Over the past decades animal production has developed a strong focus on high-yielding breeds and breeds that mainly offer high economic turn over. As a consequence, highly specialized traits in domestic animal breeds often became an obstacle in high-input-based farming systems (Mendelsohn, 2003; Tisdell, 2003), leading to a progressive replacement of traditional multipurpose breeds with high-yielding breeds (Ugarte et al., 2001; Zander et al., 2013). However, nowadays due to the high concern of consumers to healthier and of better quality livestock products, animal production trends have changed from a high-input economic systems to a more sustainable base characterized by a resource-driven activity bound to local conditions and environments. Thus, the effort of the global community is targeted at preserving the natural sources' biodiversity, existing among them the animal genetic resources. Many countries have put into force measures, laws or funds in order to protect and to preserve the autochthonous local breeds. Greece is one of these countries, which runs specific measures for the preservation of indigenous breeds with a total funding of twenty five millions euro for the period 2014-2020.

In Greece apart from the industrialized breeds used in the intensive pig farms, two populations of pigs are also exist; the feral population of wild pigs (*Sus scrofa scrofa*) and the population of black pig (*Sus scrofa domestica*), an autochthonous domesticated Greek pig breed (Laliotis, 2001, Laliotis et al., 2017). The wild boar is considered as very popular game specie (Acevedo et al. 2007). In Greece, is present in almost all mainland apart from Attica, Evian and islands, as its habitat is usually oak, chestnut or coniferous forests (Tsachalidis and Hadzisterkotis, 2009). In addition, wild boar's meat is considered of high quality and as a result a higher price in market is achieved. During the past decades its population was under restriction. Thus, hunting has been and still remains permitted for a certain period while a specific permissible game limit per hunter is implemented. In addition, wild boar settlements were established across the mainland of Greece firstly to protect the specie and secondly to restock and re-introduce pure specimens of the wild breed in its natural habitat for hunting purposes. Nowadays, an increase of its population is observed (Beskardes et al. 2010). However, a lot of damages in crops caused by wild boar populations have been recorded, while many farmers claim that theses damages are a result of pig hybrids (crossbreeds between wild boar and domesticated free-ranging pigs) and not actually from wild boar. On the other hand, the Greek black pig is a product of natural selection that was able to adapt to different and harsh environmental conditions. It is usually bred under semi-intensive systems, and the breed is considered under threat, rendering it on the list of endangered autochthonous breeds (Laliotis, 2001; Laliotis et al., 2017).

The discrimination of Greek breeds until today is based on phenotypic characteristics (Rogdakis, 2002). In the meantime, due to the cross breeding that

have taken place and the lack of preservation of pedigree books, there is difficulty in the objective and unambiguous classification of any individual animal into a certain breed. Simultaneously, payments concerning the aid to farmers that rare local breeds or wild species requires the confirmation of the breed /specie of the reared animal. As a result of the aforementioned, doubts about the correct and objective control implemented by public or private sector auditors on farms breeding rare animals are being raised, when only phenotypic characteristics are included in the inspection control.

The advent of novel DNA technology assisted the association of certain genome loci or single genes with the discrimination between species. One of these genes is the gene encoding the melanocortin-1 receptor (MC1R). The MC1R regulates melanogenesis in mammals within the mammalian melanocyte and the hair follicle. Common variations (polymorphisms) in the MC1R gene are associated with normal differences in skin and hair colour. At molecular level, the MC1R gene has been well studied in many eutherian species, among them human, rat and pig (Valverde *et al.* 1995; Box *et al.* 1997; Ollivier and Sellier 1982; Robbins *et al.* 1993). According to Kijas *et al.* (1998), a unique MC1R allele (E+) has been identified in the European wild boar (*Sus scrofa scrofa*) that is not found in any of the domestic breeds (*Sus scrofa domestica*).

The aim of the present study was to implement the genotyping procedure of the E locus of the MC1R gene on different pig sampling situations and specifically samples from i) feral boars, ii) black pigs reared under an semi- extensive system and iii) from wild pigs reared under an extensive system in order to: a) genetically test if the sampled animals objectively belong to the wild specie or the domesticated that farmers claim, b) to check if wild boar hybridization exists in the examined situations and c) to provide useful information to public and private sector concerning the inspection and certification of wild pig discrimination, which in the future may serve as a tool for the undoubted audit control.

Materials and Methods

Animals-Sampling

For the purposes of the present study the following sampling procedures were implemented for further analysis:

a) During the hunting period of wild boar in Greece, (15 September – 21 January) hair samples from fifty three (53) games of wild pigs (fifty sows and three boars) were collected (hereafter Case A). Samples were taken from different locations of North-eastern Greece.

b) Blood samples from the animals of two farms rearing the indigenous black Greek pig breed were collected (hereafter Case B1 and Case B2). The farms were located in Northern Greece and implement a semi-extensive livestock production system. The farm of “Case B1” was located near a forest area and twenty eight (28)

animals (twenty six sows and two boars) were sampled, while in the “Case B2” the respective animals were thirty seven (37) animals (thirty four sows and three boars).

c) Hair samples from the seventeen (17) animals (fifteen sows and two boar) of a farm rearing, under an extensive system, a small population of wild boar in Northern Greece, which is also serves for reintroducing wild boar in its natural habitat for hunting purposes, were collected (hereafter “Case C”).

DNA extraction and Genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was followed for genotyping the E locus of MC1R gene according to *Kijas et al. (1998)*. Briefly, DNA was extracted from blood or hair roots using the Nucleospin blood or tissue kits (Macherey-Nagel, Germany) according to manufacturer instructions and then was electrophorized to ensure the integrity of the DNA samples.

For the PCR reaction approximately 150 ng of genomic DNA was used as template and amplified in a final volume of 50 μ L containing 100 nM from each primer, 2 mM dNTPs and 1 unit MyTaqTM DNA Polymerase (Bioline). The PCR amplification conditions are shown in Table 1. Then, 25 μ L of each PCR product was digested in a total volume of 40 μ L, containing 10 U of the appropriate restriction enzyme (Table 1), 4 μ L of restriction buffer, and 10.2 μ L of ddH₂O for 2 hours at 37 °C. Restriction fragments were examined by electrophoresis on 2.5% agarose gel.

Table 1. Primers, PCR protocol, and restriction enzyme used at the present study for the molecular analysis of the MC1R gene

Gene	Primers (5' → 3')		PCR conditions	Restriction Enzyme
	Forward	Reverse		
MC1R (AF326520) c. 914C>T	RGTGCCTGGAGGTGTCCAT	CGCCAGATGGCCGCGATGGACCG	*94°C for 5 minutes *35 cycles: 94°C for 45 seconds 55°C for 45 seconds 72°C for 45 seconds *72°C for 50 minutes	<i>Bsp</i> HI (37°C, 120 min)

Statistical Analysis

Genotype frequencies, allele frequencies and Hardy-Weinberg equilibrium estimations were calculated using PopGene Software v. 1.32 (Yeh et al., 1997). The effective number (N_e) and the inbreeding rate (ΔF) of each flock were estimated using the following equations (Falconer and Mackay, 1989):

$$(a) N_e = (4 * \text{males} * \text{females}) / (\text{males} + \text{females}),$$

$$(b) \Delta F = 1 / 2N_e.$$

Results

The allelic and genotypic frequencies of the examined gene are presented in Table 2. Two alleles (A and B) and three genotypes, namely E^+/E^+ , E^+/E^- and E^-/E^- were identified in the examined cases. Specifically, in “Case A” 39 animals were found to carry the E^+/E^+ genotype, while 14 animals the E^+/E^- genotype. It should be noted that two of the three male samples were found to be heterozygous for the analysed gene locus.

Table 2. Genotype distribution, allele frequencies and Hardy-Weinberg status of the examined gene locus.

Genotype	Observed Genotypes				Expected Genotypes			
	Case A	Case B1	Case B2	Case C	Case A	Case B1	Case B2	Case C
E^+/E^+	39 (73.58%)	-	1 (2.70%)	14 (88.24%)	39.92	25.08	0.68	14.06
E^+/E^-	14 (2 ♂) (26.42%)	3 (♀) (10.71%)	8 (2 ♂) (21.62%)	2 (1 ♂) (11.76%)	12.15	2.84	8.65	1.88
E^-/E^-	-	25 (89.82)	28 (75.68%)	-	0.93	0.08	27.68	0.06
Allelic Frequencies	p= 0.87 q= 0.13	p=0.05 q=0.95	p= 0.14 q=0.86	p= 0.94 q= 0.06	HWE			
					P>0.05			

In “Case B1”, three animals (females) found to be heterozygotes (E^+/E^- genotype), while the rest of the animals carried the homozygote genotype E^-/E^- . In the case B2 28 animals carried the E^-/E^- genotype, 8 animals (6 females and two males) the E^+/E^- genotype and one animal (male) the E^+/E^+ genotype.

Regarding “Case C” almost all animals found to have the genotype E^+/E^+ apart from one female that found carrying the E^+/E^- genotype. The analysed gene locus was not found consistent with the Hardy-Weinberg Equilibrium ($P>0.05$) in none of the examined populations.

As far as it concerns the effective number of the population, in “Case B1” found to be $N_e = 7$, while the inbreeding rate (ΔF) was 0.07 (7 %). The respective parameters for “Case B2” and “Case C” were estimated as $N_e = 11$; $\Delta F = 0.05$ (5%) and $N_e = 7$; $\Delta F = 0.07$ (7 %), respectively.

Discussion

Both the indigenous pig breeds and the wild boar populations are considered as “pool” of valuable genetic diversity. The replacement of indigenous breeds by foreign improved breeds with greater yields led to dramatically diminish of their number, to a threat of extinction and to a loss of genetic diversity. In addition the wild boar declined significantly in Europe at the beginning of the 20th Century, rendering its population under threat (*Massei et al., 2015*). Many countries, including Greece, put into force measures and funds for the conservation of indigenous breeds, while wild life have been funded in the past as a tool for the conservation and re-colonization of the wild specie populations (i.e. wild boar).

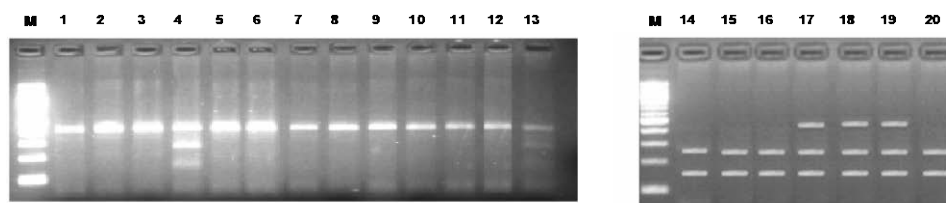


Figure 1. Genotyping analysis of the MC1R gene in the studied populations (representative samples). Wild boar (428 kb): samples: 1-3 and 6-12; Hybrids (428 kb; 256 kb; 172 kb): samples 4; 13 and 17-19; Black pig (256 kb; 172 kb): 14-16 and 20.

However, in Greece, any attempt of controlling and ensuring the rearing of a certain indigenous or wild population is accomplished through phenotypic (morphological) characteristics (i.e. coat colour, ear shape, etc.). This fact poses major risks, firstly due its subjective criteria and secondly due to the fact that many farmers cross breed their flocks with other improved (domesticated) breeds, rendering the certification and classification of the reared animal into a pure breed or population not an easy procedure. Such an example forms the breeding of the indigenous black pig and the wild boar in Greece. Herein, different rearing cases of wild boar and black pig were examined by means of their bred/population genetic purity or their hybridization using the implementation of a PCR-RFLP technique as an easy tool for checking, certifying and classifying such pig individuals.

From the observed results, none of the examined population was consistent with Hardy-Weinberg equilibrium, probably due to the small number of the observed heterozygotes. In the feral wild boar population (“Case A”) the 26% of

the examined animals found to be hybrids, meaning that hybridization of wild boar population with domesticated pig individuals had been taken place. Although two of the three analysed game males found to be hybrids, the fact itself raises serious questions at two levels; firstly at what extent these males have led to a genetic introgression of the wild population, taking into consideration the uncontrolled, and secondly suspicious are arising whether or not farmers that breeding animals belonging to the wild boar or the black pig keep the genetic pure of their livestock.

In all examined cases of farmed black pigs (B1, B2) a worth noting number of hybrid animals were detected (Table 2). In the “Case B1”, hybridization may be due to the implement livestock system (semi-extensive) near forest area, where wild boars may be mating more easily with the domesticated indigenous Greek breed. The cross-breeding between wild boar and free-ranging pigs or local domestic breeds (mainly Greek black pig) is a common practice in many wild boar farms in Greece (*Papatsiros et al., 2012*). The fact that in “Case B2” both wild and hybrids animals were detected may reflect an implementing breeding strategy, as firstly two mature hybrid males were detected and secondly the surrounding area of the livestock (semi-extensive fenced system) was not adjacent to any forest area. The low prolificacy performance of the black pig breed reflects to a narrow economic income. In order the farmers to cope with the aforementioned they tend to apply their own breeding strategies without any scientific assist, which in some cases may even include crossing with commercial breeds or wild boar in order to succeed higher production rates or higher value of their final meat product (*Laliotis et al., 2017*). Although crossbreeding potentially enhances production traits, it simultaneously threatens the heritage status of the indigenous breed or wild populations. As a consequence, hybrid males should not be used in mating or immediately should be removed and substituted by pure bred males in order to ensure breed’s genetic purity. Otherwise, if the male hybrids will be retained, then conservation of the flock as nucleus of black pig breed is under threat.

The same breeding management should be implemented in the “Case C” where two cross bred animals between wild boar and domesticated pig was observed. The reported cross breeding animals should probably be due to the free range breeding system that is followed, where uncontrolled mating is more easily to be occurred. However, this results in a continuous cross breeding of wild boar. Besides, the genetic purity of the wild species is normally desirable per se because the mixing of gene pools of formerly distinct taxa can lead to genetic homogenization and the extinction of rarer species (*Largiadèr, 2007*). In addition, hybridization can cause problems without breeding depression and mal-adaptation to a local environment (*Rhymer and Simberloff, 1996*). As some farmers might not be aware that they maintain hybrids among their herds, the hybrids should immediately be removed in order to ensure the purity of the wild species. Moreover, the future progeny of females should be checked in order to ensure the birth of piglets belonging to the wild boar specie. Apart from the aforementioned questions are raised regarding the

financial aid that farmers receive for retaining pure bred nucleus of wild boar or black pig. A proposal may be farmers who receive any fund for this purpose should be paid accordingly to the percentage of the genetically certified pure bred animals that rare plus a penalty regarding the introgression of the populations.

In addition, inbreeding rates in the examined farms ("cases B1, B2 and C") were found to be at a small but pivotal level ($5\% < \Delta F < 7\%$). However, further measures (i.e. mating with non-relatives) should be implemented in order to prevent the inbreeding depression and the occurrence of the genetic drift.

To sum up, four different wild boar or indigenous domesticated black pig populations were investigated in order to find out whether or not hybridization between species exists. Using a simple and easy implemented DNA technique we concluded that a notable percentage of specimens belonged to a cross-bred hybrids existed, rendering the conservation either of indigenous pig breed or the wild boar population under risk. Thus, the evidence of hybridization of the wild boar in the Northern Greece exists and confirms the respective reports of farmers concerning crops' damages by pig hybrids. However, a broader study recording all the areas of wild boar habitat should be undertaken in order to specify the extent of the hybridization. On the other hand, interestingly, hybridization also exists in the black pig population, rendering its homogeneity under risk. The applied method could be useful for State authorities or other certifying organizations in order to test, control or inspect farms that run under specific certification standards or specific funding aids for rearing the autochthonous Greek pig breed (black pig) or the wild boar.

Dokazi genetske hibridizacije u populacijama divlje svinje i autohtone crne svinje u severnoj Grčkoj

George P. Laliotis, Meni Avdi

Rezime

U Grčkoj, autohtona crna svinja i divlja svinja se smatraju vrstama dragocene genetičke raznovrsnosti, dok njihovi proizvodi postižu visoku tržišnu cenu. Međutim, zabeležene su brojne štete na usevima, a poljoprivrednici tvrde da su za to odgovorni hibridi divljih svinja. S druge strane, klasifikacija crne svinje bazirana je na fenotipskim karakteristikama, što ne obezbeđuje homogenost rase u slučaju hibridizacije. Koristeći metodologiju PCR-RFLP, ispitani su uzorci svinja ($n = 135$) iz različitih situacija u odgoju (svinje iz divljine/prirode), crne svinje iz polu-intenzivnog sistema gajenja i divlje svinje iz ekstenzivnog) kako bi se identifikovalo da li postoji hibridizacija. U ispitivanoj populaciji divlje populacije

divljih svinja zabeleženo je 26% hibrida, dok je u slučaju ekstenzivne gajene populacije divljih svinja zabeležena hibridizacija od 11,76%. Interesantno je da je u oba slučaja ispitane populacije crnih svinja posmatrana hibridizacija sa divljim svinjama, što je odraz verovatno sprovedene prakse oplemenjivanja ili nekontrolisanog parenja sa divljim svinjama. Ključni nivo (5-7%) stepena inbidinga takođe je zabeležen u ispitanim populacijama. Hibridi iz svih ispitanih populacija bi trebalo da budu odmah uklonjeni, kako bi se sprečila i eliminisala dalja introgresija, genetska depresija i gubitak genetičke raznovrsnosti za obe populacije divlje i crne svinje. Najzad, primenjenu metodologiju mogu koristiti državni organi ili organizacije za sertifikaciju, testiranje, kontrolu ili inspekciju farmi koje uzgajaju populacije divlje svinje ili crne svinje kako bi zabeležile i eliminisale događaje hibridizacije između njih.

Ključne reči: *Sus scrofa*, divlja svinja, *Sus domestica*, hibridizacija, PCR-RFLPs, geni

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Received 19 February 2018; accepted for publication 24 April 2018