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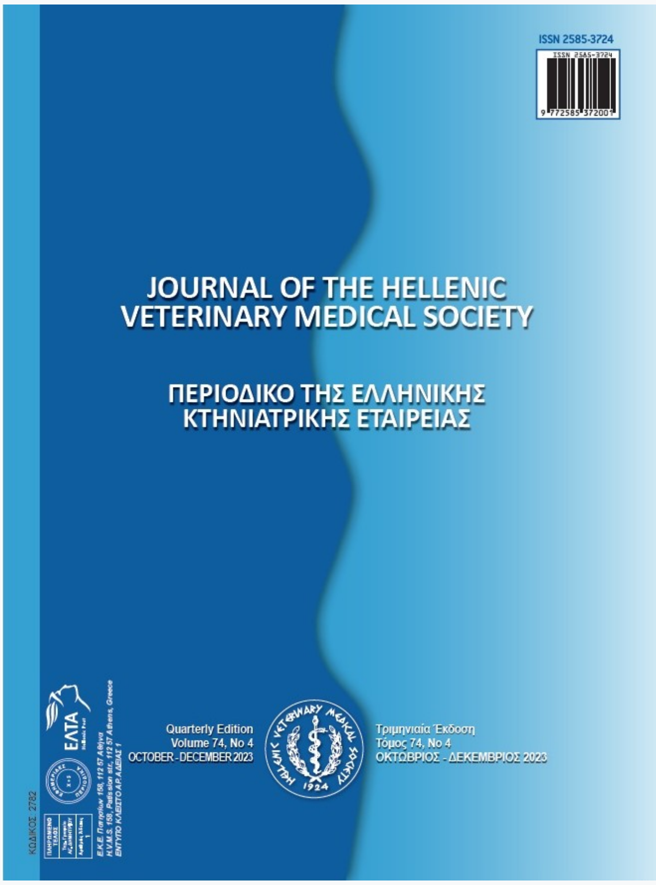

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Research article
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Identification of genetic polymorphisms in MC4R and GPX5 genes in the autochthonous Greek black pig breed

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ABSTRACT: The importance of local farm animal breeds as genetic reservoirs of valuable genetic variation is well established. Although these breeds are important for the maintenance of genetic diversity, most of them are now in danger of extinction. The autochthonous Greek black pig breed is the only traditional indigenous pig breed reared in Greece and is able to adapt to different and harsh environmental conditions. Recent studies have reported that gene polymorphisms in melanocortin 4 receptor (MC4R) and glutathione peroxidase 5 (GPX5) genes are associated with litter size in pig and can be used as genetic markers in gene assisted selection programs for the improvement of reproductive performance. The objective of this study was to investigate the existence of these polymorphisms in the autochthonous Greek black pig breed. One hundred sixty pigs raised in Greece were included in the study. DNA was extracted and genotyping was performed using RFLP - PCR. The molecular results revealed that for MC4R, genotype GG had a frequency of 0.37, GA 0.44 and AA 0.19, while the frequency of allele G was 0.56 and of A 0.44. For GPX5, genotype AA had a frequency of 0.19, AB 0.41 and BB 0.40, with frequencies of alleles A and B being 0.43 and 0.57, respectively. These data revealed that all genotypes of the two genes were present in the investigated population, indicating that these genes could be used for Marker-assisted selection programmes for the genetic improvement of reproductive characteristics of this breed.

Keywords: gene polymorphisms; pig; litter size; reproduction

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INTRODUCTION

One of the most important economically traits in pig production is reproductive rate and especially litter size. An increase in the number of pigs weaned per sow will increase economic returns for pig producers with minimal additional inputs and even a small or moderate increase in the litter size is associated with large profits for the farmers (Rothschild et al., 1996; Buske et al., 2006). Therefore, the genetical improvement of litter size in swine is of expanding interest for pig producers. Until recently the improvement of quantitative characteristics, including reproductive traits was mainly based on selection programmes, which take into consideration only phenotypical traits. However, these selection programmes, are laborious, expensive and especially in pig production time consuming.

During the last years, advances in molecular techniques can now be used in order to increase rate of response to selection and Marker-assisted selection (MAS), employed in conjunction with traditional selection methods, has been in progress to increase litter size in swine. It has been proposed that the candidate gene analyses can be used to identify individual genes, which are responsible for traits of economic importance. Litter size by means of total number born (TNB) and number born alive piglets/birth (NBA) directly controls the annual production of a sow and are therefore, considered very important characteristics by the swine producers (Gordon, 1997; Rothschild and Soller, 1997). Marker-assisted selection programmes are used as complementary to the traditional selection methods, in order to provide faster improvement in specific quantitative traits by associating a region of the DNA with a specific characteristic (Rothschild et al., 2000; Du et al., 2022; Wijesena et al., 2023).

Several genetic studies have revealed that in the porcine genome, many genes have been associated with various reproductive traits, including litter size and have been mapped on the porcine chromosomes. However, as at the moment there are only limited information provided and in conjunction with the fact that in many cases the results are inconsistent, there is an urgent need of further research in this area (Rothschild and Ruvinsky, 2010; Munoz et al., 2018).

Recent studies have reported that the glutathione peroxidase 5 gene (GPX5) on SSC7 is located in a chromosomal region in which several quantitative trait loci (QTL) for reproductive traits in swine, such as uterine capacity, ovulation rate and litter size have been detected. Linkage analyses of GPX5 showed that this gene is closely linked to the major histocompatibility complex (MHC), which has been suggested to have an effect on reproductive traits in swine (Vaiman et al., 1998; Buske et al., 2005; Kumar et al., 2021).

Furthermore, it has been reported that the melanocortin 4 receptor (MC4R) gene codes for a G-protein-coupled receptor and it was demonstrated to be important in the control of energy balance in humans and rodents. Energy balance is maintained by controlling energy intake, i.e. feed intake, and energy expenditure by physical activity and metabolism. The response of the melanocortin 4 receptor to leptin signalling can thus be considered as a link between feed intake and body weight and body composition (Marsh et al., 1999; Wikberg et al., 2000; Yin et al., 2019). Consequently, MC4R is a strong candidate for growth and body composition in pigs, as well as reproductive performance.

In order to improve the reproductive traits of the autochthonous Greek black pig breed the first step is to investigated the genetic polymorphisms of reproductive related genes and their associations with various reproductive traits. The autochthonous Greek black pig is the only traditional indigenous pig breed raised in Greece. Its domestication is lost in history and was raised in order to provide meat and leather to almost every Greek family living in urban regions till recently. It is a product of natural selection that was able to adapt to different and harsh environmental conditions. Currently, there are very few breeders in Greece raising this breed intensively and this breed is on the list of endangered autochthonous breeds.

As the identification of the genetic structure of this autochthonous pig breed is one of the main aspects for the conservation of this local pig breed, the aim of the present study was to establish GPX5 and MC4R genes allele and genotypes frequencies, in the autochthonous Greek black pig.

MATERIALS AND METHODS

Genotyping procedure

DNA was extracted from hair roots of one hundred sixty Greek black pigs using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to manufacturer's instructions. The integrity of the DNA samples was examined by electrophoresis through a 1% agarose gel. For genotyping of the MC4R gene a 483 bp fragment was amplified by PCR using the primer pair: 5′-ACAGTTAAGCGGGTTGGAAT-3′ and 5′-CAGGGGATAGCAACAGATGA-3′. PCR amplification was performed using approximately 300ng of genomic DNA as template, 200 nM primers each, 1 mM dNTPs and 1 unit Taq DNA Polymerase Recombinant in 25 μl total volume reaction. PCR conditions were 94°C for 3 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec and a final extension period at 72°C for 10 min. PCR products were digested using *TaqI* restriction enzyme and resolved by electrophoresis on 2% agarose gels, visualised with ethidium bromide and imaged under UV illumination. The G/A polymorphism in the porcine MC4R gene (Asp298Asn) resulted in 407- and 76-bp fragments (wildtype: G nucleotide, Asp298), while the absence of the TaqI cleavage site yielded a single 483-bp band (mutant: A nucleotide, Asn 298).

For genotyping of the GPX5 gene a 501 bp fragment was amplified by PCR using the primer pair: 5′-TTCATGTAGAACTTATTTCTG -3′ and 5′-TGACTTACCCATTCTTCAG-3′. PCR amplification was performed using approximately 300ng of genomic DNA as template, 200 nM primers each, 1 mM dNTPs and 1 unit Taq DNA Polymerase Recombinant in 25 μl total volume reaction. PCR conditions were 94°C for 3 min, 35 cycles of 94°C for 30 sec, 51°C for 30 sec, 72°C for 30 sec and a final extension period at 72°C for 10 min. PCR products were digested using *Hinfl* restriction enzyme. Using this method, the fragment length of AA genotype will be 298, 94, 53, 33 and 23bp, the fragment length of AB genotype will be 298, 234, 94, 64, 53, 33 and 23bp and the fragment length of BB genotype will be 234, 94, 64, 53, 33 and 23bp. The products were resolved by electrophoresis on a 2% agarose gels, visualised with ethidium bromide and imaged under UV illumination (the 23, 33, 53 and 64bp products are not visible).

Once genotypes were determined, allelic frequencies at each gene locus were calculated by gene counting. Deviations from Hardy-Weinberg equilibrium were examined for each locus using chi-squared tests.

RESULTS

As illustrated in Figures 1 and 2 (representative of some samples), PCR amplification of the MC4R and GPX5 genes was successful, using the conditions detailed in materials and methods. Furthermore, RFLP-PCR analysis performed in the PCR products, using the restriction enzymes for each SNP, as described in materials and methods, revealed the genotype of each animal, for each gene locus (Figures 3 and 4, representative of some animals, for each gene).

Genotypic and allelic frequencies estimated for the 160 examined pigs, for the two gene loci are presented in Table 1.

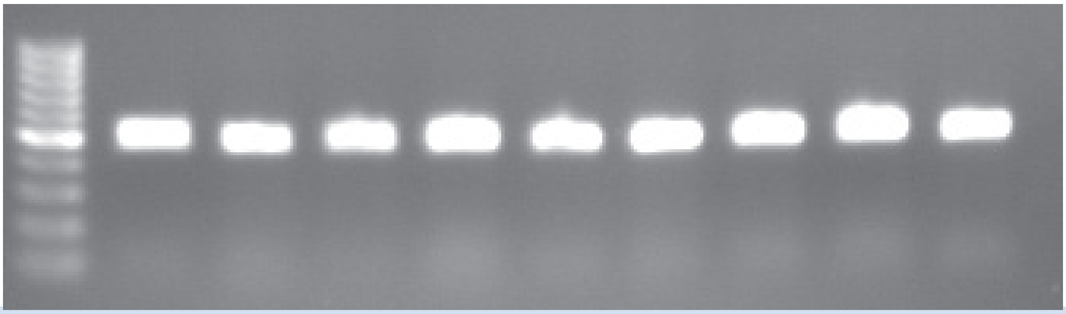

Fig. 1. PCR amplification of the MC4R gene locus.

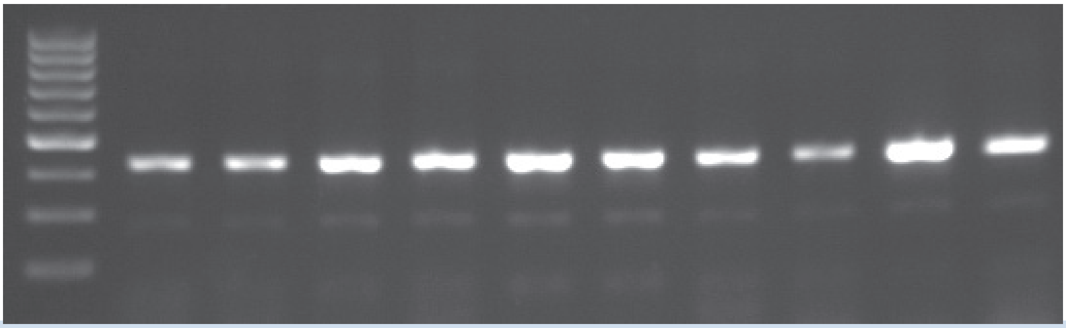

Fig. 2. PCR amplification of the GPX5 gene locus.

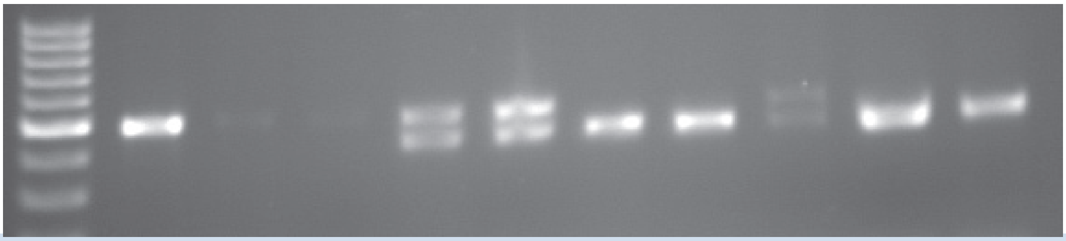

Fig. 3. RFLP-PCR analysis for the MC4R gene polymorphism. PCR products were digested with *TaqI*.

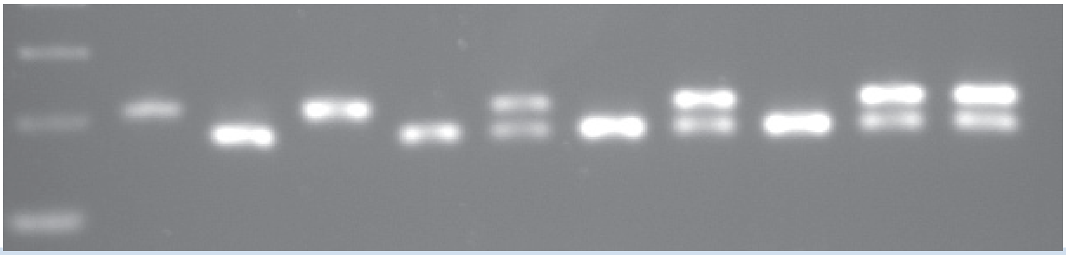

Fig. 4. RFLP-PCR analysis for the GPX5 gene polymorphism. PCR products were digested with *Hinfl*.

Table 1. Genotypic and allelic frequencies (%) in the two studied gene loci

Gene Locus	Allele		Genotypic Frequency (%)			Allelic frequency (%)	
	0	+	00	0+	++	0	+
MC4R	G	A	0.37	0.44	0.19	0.56	0.44
GPX5	A	B	0.19	0.41	0.40	0.43	0.57

As illustrated in Table 1, for MC4R, genotype GG had a frequency of 0.37, GA 0.44 and AA 0.19, while the frequency of allele G was 0.56 and of A 0.44.

For GPX5, genotype AA had a frequency of 0.19, AB 0.41 and BB 0.40, with frequencies of alleles A and B being 0.43 and 0.57, respectively. Both MC4R and GPX5 gene loci were found to be in Hardy-Weinberg equilibrium in the studied population.

DISCUSSION

It is now well established that the one of the main factors for a sustainable development is the conservation of animal genetic resources, in order to respond to changing conditions, such as the climate change, new or resurgent disease threats, new requirements on human nutrition and changing market demands or societal needs (Hoffman, 2010; Cortes et al., 2016; Cao et al., 2021; Martyniuk 2021).

As has been stated by the United Nations Food and Agricultural Organization, it is now well established the importance of local farm animal breeds in the maintenance of genetic diversity and also for the futured food supply (FAO, 2007). Apart from that, these domesticated farm animal breeds are also considered an important part of each country's cultural heritage. Pig breeds are of course of particular interest, as these animals are an important meat source in order to feed local populations since ancient times, particularly in the mountainous areas of the Mediterranean basin.

Although in Europe exists a large number of local pig breeds, the majority of the pigs which are breed in production systems belong to a few intensively selected breeds (Nidup and Moran, 2011), because of their bigger productivity and the higher profit which they provide to the producers, compared to local autochthonous breeds. Therefore, many of these local breeds are now in danger of extinction, and others are threatened by inefficient use or loss due to cross-breeding (FAO, 2006; Nidup and Moran, 2011). This has led to a severe loss of farm animal genetic resources, mainly due to poor agricultural policies, introduction of exotic germplasm, degradation of ecosystems and development of only few breeds (FAO, 1998).

In the swine industry, during the last years, there has been a significant reduction in the genetic variation among pig populations and a severe loss of useful and desirable traits as the result of the genetic selection of high-performance pig breeds, as well as the displacement of extensive production systems, leading to a dramatic decrease in gene pool. During the last decades, in most countries, pig production has changed from small holders to an intensive industry and large-scale production systems have achieved high levels of production performance, as a result of the introduction of high-yielding improved pig breeds under intensive production systems which have replaced the native local pig breeds. Therefore, only a few traditional forms of pig production and local autochthonous pig breeds exist in the developed countries. However, at the moment, several research projects are ongoing regarding the preservation and exploitation of local animal breeds, including big breeds, in order to reverse this negative trend and also in order to identify and reintroduce potentially important genetic traits that have been lost (Guastella et al., 2010). Furthermore, there is a growing awareness of the importance of these local breeds in terms of adaptive traits to specific environments and as genetic reservoirs, in addition to their historical or cultural value that plays a key role in the sustainable development of marginal rural areas (Cortes et al., 2016). It is therefore of crucial importance to implement policies focused on the conservation of genetic diversity and on the maintenance of distinct populations of local pig breeds. In addition to these genetic information and breed contributions to the conservation of genetic resources, other aspects must also be considered in the final decision for conservation, including the breed's regional importance and also its social, cultural, environmental and economic impact (Cortes et al., 2016).

In Greece, the autochthonous black pig breed is the only traditional indigenous pig breed raised for centuries. This breed is an important source of high-quality protein food, as it has an excellent quality of meat and of great organoleptic characteristics, which makes this breed special among all other pig breeds raised in Greece. Furthermore, it has a great adaptability, which makes this breed ideal for regions with harsh environmental conditions. However, at the moment, there is a threat regarding the persistence of this breed, because of the small numbers of the animals of this breed that exist and important measures must been take in order to save this breed, including genetic analyses. The objective of this study was therefore to investigate the genetic diversity of this breed, by investigating for the first time the genotypes of the MC4R and GPX5 gene polymorphisms. Since these two single nucleotide polymorphisms have been reportedly associated with reproductive traits, such as litter size, it was considered important to determine whether the desired genotypes were present in this breed.

Results presented in this study revealed that the allelic frequencies of the two investigated gene locus estimated in the present study were similar to those reported by previous studies (Buske et al. 2006; Jokubka et al. 2005). In particular, the frequencies alleles for the MC4R genotype were 0.56 and 0.44 respectively, while for the frequencies of alleles were 0.43 and 0.57 respectively. This suggests that the desired genotypes are present in this pig breed and would be very interesting to perform association studies between these SNPs and reproductive parameters. Therefore, further work is needed in order to confirm these associations in this local pig breed. Once confirmed, these SNPs could be incorporated in a larger panel of markers, in order to assist Greek breeders in selecting pigs with improved reproductive traits, such as litter size.

CONCLUSIONS

The autochthonous black pig plays an important social and cultural role in Greece and supports local economy, as it provides income to several Greek breeders. The results of this study revealed for the first time that the autochthonous black pig, despite its small population size has a high level of genetic variability, as evidenced by the genetic analysis of this study. This variability in alleles should be preserved for creating a gene pool to conserve this breed's characteristics for the future. In addition to the genetic considerations, the cultural and environmental importance of this breed should also be taken in consideration in future conservation programmes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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