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To cite this article: Carmelisa Valluzzi, Andrea Rando & Paola Di Gregorio (2019) Genetic variability of Nero Lucano pig breed at *IGF2*, *LEP*, *MC4R*, *PIK3C3*, *RYR1* and *VRTN* loci, Italian Journal of Animal Science, 18:1, 1321-1326, DOI: [10.1080/1828051X.2019.1649606](https://doi.org/10.1080/1828051X.2019.1649606)

To link to this article: <https://doi.org/10.1080/1828051X.2019.1649606>



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Published online: 26 Aug 2019.



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Genetic variability of Nero Lucano pig breed at *IGF2*, *LEP*, *MC4R*, *PIK3C3*, *RYR1* and *VRTN* loci

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ABSTRACT

The Nero Lucano pig is a native breed of Southern Italy which thanks to the joint action of Basilicata Region Institutions, University of Basilicata and breeders returned to populate the area of origin. In order to characterise and to monitor the variability present in the population, we genotyped 229 animals at 12 polymorphic loci located in the following genes: *IGF2*, *LEP*, *MC4R*, *PIK3C3*, *RYR1* and *VRTN*. According to the results three loci (*IGF2* 209G>C, *PIK3C3* 2058A>G and *RYR1* 1843C>T) did not show variability, while the others showed genotype distributions in agreement with Hardy-Weinberg equilibrium and a minor allele frequency ranging from 0.022 for *MC4R* 892A to 0.479 for *PIK3C3* 2604T alleles. The *IGF2*, *MC4R* and *VRTN* loci were characterised by very low frequencies (from 0.02 to 0.05) of the alleles that are associated with favourable productive characteristics in cosmopolitan breeds.

HIGHLIGHTS

- Analyses of the genetic variability of Nero Lucano pig population useful for meat production selection plans.
- The *IGF2*, *MC4R* and *VRTN* loci of Nero Lucano pig show very low frequencies of alleles associated with positive effects on meat production.
- The Nero Lucano pig can be considered as free from Malignant Hyperthermia, a positive result for the quality of cured meat products.

ARTICLE HISTORY

Received 24 April 2019
Revised 21 June 2019
Accepted 24 July 2019

KEYWORDS

Italian autochthonous pig;
DNA polymorphism; linkage disequilibrium



Introduction

The Nero Lucano (NL) pig is an ancient native black breed that inhabited forests and countryside of Basilicata region (Italian Southern Apennines) since 1800 (Stanga 1915). During the last century the population was reduced to few animals. In recent years the need to protect the biodiversity of the animal world and the policies for the recovery and protection of breeds in danger of extinction were strongly encouraged in several countries by consumer demand of products linked to the territory and easily traceable (Pulina 2011). The action of recovery of the NL pig by Basilicata Region Institutions, University of Basilicata and breeders arose from this background and started from the collection and random mating of the few remained individuals in one pilot farm. Next, at least 1 boar and 5 sows were distributed to the herds of 18 guardian-breeders. At present, the number of NL pigs is about 3000 individuals reared in the two provinces

(Potenza and Matera) of Basilicata. These animals are recorded in the 'Registro Anagrafico dei Tipi Genetici Autoctoni della Specie Suina' (Italian Registrar for Autochthonous Swine Breeds).

The NL pig is able to exploit marginal areas and the quality of its cured meat products is strongly appreciated. On the other hand, both production and reproduction traits, such as average daily gain, carcass quality and litter size are characterised by very low values and, therefore, need to be improved.

The aim of this study was to evaluate the genetic variability of the NL pig at some of the loci whose polymorphisms are associated with effects on production traits: the insulin-like growth factor 2 (*IGF2*) gene, SSC2p1.7 (Jeon et al. 1999; Nezer et al. 1999); the leptin (*LEP*) gene, SSC18q13-q21 (Cepica et al. 1999); the melanocortin-4 receptor (*MC4R*) gene, SSC1q22-q27 (Kim et al. 2000); the phosphatidylinositol 3-kinase catalytic subunit type 3 (*PIK3C3*) gene, SSC6q22-q23

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(Kim et al. 2005a); the ryanodin receptor 1 (*RYR1*) gene, SSC6q12 (Chowdhary et al. 1994) and the vertnin (*VRTN*) gene, SSC7 (Mikawa et al. 2011).

Materials and methods

The experimental procedures followed the requirements of the European Community Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes (14G00036).

DNA samples were obtained from 229 NL breeding pigs, 18 males and 211 females, reared in the farms of the 18 guardian-breeders. At the time of sample collection, the analysed individual represented about 70% of the total NL population. Genomic DNA was extracted from whole blood using NucleoSpin DNA QuickPure kit (Macherey Nagel, Düren, Germany). All samples were genotyped by means of PCR or PCR-RFLP at the following polymorphic loci; *IGF2* -366G>A,

-209G>C -225C>G and -182T>C (Aslan et al. 2012), *LEP* 2728G>A, and 3469T>C (Stratil et al. 1997; Kennes et al. 2001), *MC4R* 892A>G (Fan et al. 2009), *PIK3C3* 2058A>G, and 2604C>T (Kim et al. 2005a, 2005b), *RYR1* 1843C>T (Fujii et al. 1991) and *VRTN* 20311-20312ins291, and 19034A>C (Fan et al. 2013). Typing was performed according to the literature by using primers and restriction enzymes shown in Table 1.

Linkage disequilibrium and haplotypes were analysed by using HAPLOVIEW software version 4.2 (Barrett et al. 2005).

Phylogenetic trees and average heterozygosities were obtained by using the web version of POPTREE2 software (Takezaki et al. 2014).

Results and discussion

All sampled animals were homozygous at *IGF2* -209G>C, *PIK3C3* 2058A>G and *RYR1* 1843C>T loci

Table 1. Primer sequences and restriction enzymes used for genotyping Nero Lucano pig DNA samples.

Gene	Polymorphic site	Primers (5'→3')	Restriction enzymes
<i>IGF2</i> ^a	-366G>A	F: CCTCTGCTCTCGCCACATC R: GTAGCGGCTGGGATGAGTG	<i>PstI</i>
	-225C>G	F: CAGGTTGCCCCAGTTTACAG	<i>Eco0109I</i>
	-209G>C	R: TTCTCCACTCCGACGCAG	<i>Mbol</i>
	-182T>C		<i>Dra III</i>
<i>LEP</i> ^b	2728G>A	F: GGTTGGGCAGGGAGTTCA R: ACAAAACCTGGCACTATGGCT	<i>HindIII</i>
	3469T>C	F: CACGCCAGCCCAAGGAGTT R: GTTCCATCTCCAGCTCACCGC	<i>Hinfl</i>
<i>MC4R</i> ^c	892A>G	F: GCCATAGCCAAGAACAAGA R: AAATGGGGACAGAGGAGAC	<i>TaqI</i>
<i>PIK3C3</i> ^d	2058A>G	F: TGTGATGTCAATGCCAGTTA R: TACTAACAGGTGGAAATGCTC	<i>BsrSI</i>
	2604C>T	F: GAAGAGGCAGTCCATTACATAC R: GTGTGGCAGAGGTTTGA	<i>NlaIII</i>
<i>RYR1</i> ^e	8413C>T	F: GTTCCCTGTGTGTGCAATGGTG R: ATTACTGAGAAGCTGCTCCCTGGCC	<i>CfoI</i>
<i>VRTN</i> ^f	19034A>C	F: TGACCCCTGACAAAAGCAT R: AGTTGGCTGTAGACGGTCC	<i>MaellI</i>
	del/ins291	F: GAGGCCAACCCATCTACCA R: GCATTCATTCACCCCTTGG	-

^aAslan et al. (2012);

^bKennes et al. (2001); Stratil et al. (1997);

^cFan et al. (2009);

^dKim et al. (2005a, 2005b);

^eFujii et al. (1991);

^fFan et al. (2013).

Table 2. Genotype distribution and allele frequencies at nine polymorphic sites in Nero Lucano pig.

Gene	Polymorphic site	N	Genotypes		Allele frequencies		χ^2	p-value	
<i>IGF2</i>	-366G>A	227	GG = 0	GA = 18	AA = 209	fG = 0.0396	fA = 0.9604	1.0590	.3034
	-225C>G	229	CC = 0	CG = 18	GG = 211	fC = 0.0393	fG = 0.9607	1.0590	.3034
	-182T>C	226	TT = 0	TC = 18	CC = 208	fT = 0.0398	fC = 0.9602	1.0590	.3034
<i>LEP</i>	2728G>A	227	GG = 29	GA = 120	AA = 78	fG = 0.3921	fA = 0.6079	2.7900	.0948
	3469T>C	227	TT = 103	TC = 103	CC = 21	fT = 0.6806	fC = 0.3194	0.3740	.5408
<i>MC4R</i>	892A>G	227	AA = 0	AG = 10	GG = 217	fA = 0.0220	fG = 0.9780	1.0050	.3161
<i>PIK3C3</i>	2604C>T	218	CC = 53	CT = 121	TT = 44	fC = 0.5206	fT = 0.4794	2.5610	.1035
<i>VRTN</i>	19034 A>C	217	AA = 195	AC = 22	CC = 0	fA = 0.9493	fC = 0.0507	1.0480	.3059
	del/ins291	217	del/del = 195	del/ins = 22	ins/ins = 0	fdel = 0.9493	fins = 0.0507	1.0480	.3059

for: G, G and C alleles, respectively. According to the absence of the *RYR1* 1843T allele in the analysed individuals, the NL pig breed can be considered as free from malignant hyperthermia and, therefore, from pale, soft, exudative (PSE) myopathy (Ilie et al. 2014). This result is extremely positive since the greatest part of meat produced by this population is used for cured products.

Table 2 shows the results obtained for the other analysed loci. According to the χ^2 values the genotype distributions were in Hardy-Weinberg equilibrium. Furthermore, the *PIK3C3* 2604C>T polymorphisms were characterised by the highest level of variability, with a heterozygosity of 0.5. In Duroc breed, Hirose et al. (2011) observed that the *PIK3C3* 2604C allele was associated with increased average daily gain, backfat thickness and intramuscular fat. In crosses between Korean native and Landrace pigs the *PIK3C3* 2604C allele was associated with positive effects on body weight and backfat (Kim et al. 2005b). On the contrary, the lowest MAF value was observed for the *MC4R* A allele (0.022) which, according to several authors, is associated with increasing daily gain, higher lean meat percentage and lowest backfat thickness in different breeds (Fan et al. 2009; Jokubka et al. 2006; Davoli et al. 2012).

Linkage disequilibrium analysis of the three *IGF2* polymorphic sites showed D' and r^2 values equal to 1.0 for all two loci pairwise comparisons. As a consequence, only two of the eight possible haplotypes were inferred: A = A-G-C (0.962) and B = G-C-T (0.038). The B haplotype should correspond to the HAP1 haplotype which was associated with lower backfat thickness in Large White pigs (Aslan et al. 2012). In NL pig the B haplotype was associated with lower intramuscular fat, higher *Longissimus lumborum* and *Psoas* weight, muscle drip loss and polyunsaturated acids content (Simonetti et al. 2017). Furthermore, a complete linkage disequilibrium was observed for the two *VRTN* polymorphic sites ($D' = 1$ and $r^2 = 1$) with the presence of only two of the four possible haplotypes: del-A (0.949) and ins-C (0.051). The same complete linkage disequilibrium and the same haplotypes were observed in Sutai, Duroc, Landrace and Large White breeds (Fan et al. 2013). The two polymorphisms are located within an active promoter and the ins-C haplotype is responsible for a higher expression level of the *VRTN* gene associated with an increase in the number of thoracic vertebrae (Fan et al. 2013). This haplotype was also associated with higher carcass length and teat number in different breeds (Nakano et al. 2014; Yang et al. 2016; Dall'Olio et al. 2018).

Table 3. Comparison among allele frequencies at nine polymorphic sites in five pig breeds.

Breeds	IGF2			LEP			MC4R			PIK3C3			RYR1			VRTN		
	-366G>A	-182T>C		2728G>A	3469T>C		892A>G	2604C>T		1843C>T		del/ins291		19034A>C				
	G	A	T	G	A	T	A	C	T	C	T	del	ins	A	C			
Nero Lucano	0.040	0.960	0.040	0.392	0.608	0.680	0.022	0.320	0.479	1	0	0.949	0.051	0.949	0.051			
Pietrain	0.990 ^a	0.010 ^a	0.990 ^a	na	na	0.750 ^c	0.076 ^e	0.250 ^c	na	0.971 ^h	0.029 ^h	0.500 ^j	0.500 ^j	na	na			
Duroc	0.964 ^a	0.036 ^a	0.956 ^a	0.910 ^b	0.090 ^b	0.768 ^d	0.879 ^d	0.232 ^d	0.715 ^d	0.917 ⁱ	0.083 ⁱ	0.460 ^k	0.540 ^k	0.460 ^k	0.540 ^k			
Large White	0.400 ^a	0.600 ^a	0.321 ^a	na	na	0.820 ^c	0.694 ^f	0.180 ^c	0.425 ^g	0.981 ⁱ	0.019 ⁱ	0.340 ^k	0.660 ^k	0.340 ^k	0.660 ^k			
Landrace	na	na	na	0.850 ^b	0.150 ^b	0.940 ^b	0.188 ^f	0.060 ^b	0.600 ^g	0.972 ⁱ	0.028 ⁱ	0.290 ^k	0.710 ^k	0.290 ^k	0.710 ^k			

^aAslan et al. (2012);
^bKennes et al. (2001);
^cStratil et al. (1997);
^dHirose et al. (2014);
^ePiorkowska et al. (2010);
^fDavoli et al. (2012);
^gKim et al. (2005b);
^hIlie et al. (2014);
ⁱRuan et al. (2013);
^jBurgos et al. (2012);
^kFan et al. (2013);
 Data for *IGF2* -209G>C, *IGF2* -225C>G and *PIK3C3* 2058A>G are not shown since unavailable for all cosmopolitan breeds.
 na: not available.

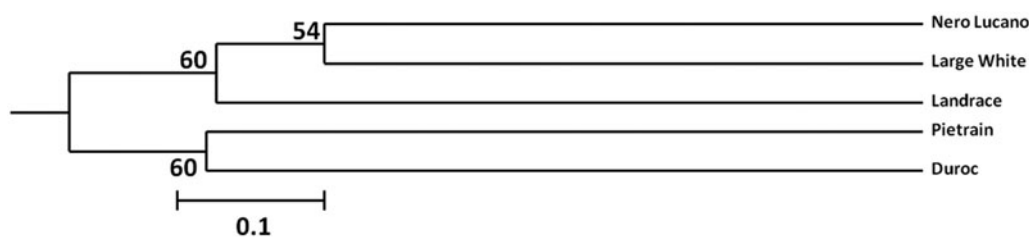


Figure 1. UPGMA phylogenetic tree obtained according to genetic distances (Nei et al. 1983) estimated by considering the allele frequencies at the *IGF2*, *LEP*, *MC4R*, *PIK3C3*, *RYR1* and *VRTN* loci of Nero Lucano, Large White, Landrace, Pietrain and Duroc pig breeds. Bootstrap values (1000 replicates) are reported on the nodes.

Finally, results obtained for the two *LEP* polymorphic sites showed all the four possible haplotypes in partial linkage disequilibrium ($D' = 0.78$ and $r^2 = 0.45$). Results of the effects of the different haplotypes on some meat production traits (average daily gain, backfat thickness lean meat, feed intake, growth) are conflicting probably because the detected association depends on the analysed population (Kennes et al. 2001; Urban et al. 2002; Szydlowski et al. 2004; Bauer et al. 2006; De Oliveira Peixoto et al. 2006). As a consequence, the effects of the variability at the *LEP* gene on meat quality and carcass traits should be also analysed in the NL pig population.

Table 3 shows the comparison between the allele frequencies calculated according to the typing results of the NL pig and the data available in literature for the cosmopolitan (Pietrain, Duroc, Large White and Landrace) breeds (Stratil et al. 1997; Kennes et al. 2001; Kim et al. 2005a; Piorkowska et al. 2010; Aslan et al. 2012; Burgos et al. 2012; Davoli et al. 2012; Ruan et al. 2013; Fan et al. 2013; Hirose et al. 2014; Ilie et al. 2014). As a whole, the average heterozygosity of the NL pig population, at the 12 considered loci, showed a very low value of 0.157. Pairwise comparisons with the other breeds showed that the NL pig population is, in any case, characterised by the lowest average heterozygosity (not shown).

Data in Table 3 were also used to estimate genetic distances (Nei et al. 1983) and the generated unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree (Figure 1) shows that the NL pig population is more similar to the Large White breed. This result could be explained by the historical data on the Nero Lucano pig. In fact, at the beginning of the nineteenth century, Cavallina Lucana and York pigs, considered the ancestors of NL and Large White pigs, respectively, were crossed in order to obtain a heavier pig.

The results of this research could be exploited both to preserve the actual variability by preventing the

loss of rare alleles and to start breeding plans to enhance the NL population by increasing the frequency of alleles associated with positive effects on meat production (Russo et al. 2007).

Conclusions

This study was performed to analyse the genetic variability of the NL pig breed at some loci whose polymorphisms are associated, according to literature, with effects on production traits. The analysed individuals were characterised by good levels of variability only for three of the considered loci. The other loci showed a low or null level of variability. In particular, the *IGF2*, *MC4R* and *VRTN* loci were characterised by very low frequencies (from 0.02 to 0.05) of the alleles that, according to the literature, are associated with positive effects on some meat production traits. On the contrary, the *RYR1* locus was monomorphic for the favourable 1843C allele in the analysed individuals, with the consequence that the NL pig can be considered free from Malignant Hyperthermia. This result is extremely positive both for wild or semi-wild rearing conditions and for quality of cured meat products.

Acknowledgements

We thank the Basilicata Region Breeders Association (Associazione Regionale Allevatori, Basilicata) for collaboration in blood samples collection and relationship with breeders.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by PSR of the Basilicata Region 2014–2020, Misura 10, sottomisura 10.2. “Conservazione e uso sostenibile delle risorse genetiche in agricoltura” (Conservation and sustainable use of genetic resources in

agriculture), project “(TGA) - Standardizzazione, stabilizzazione e valorizzazione dei tipi genetici autoctoni suini, ovicapri ed equini” (TGA - Standardization, stabilization and enhancement of native pig, sheep, goat and equine genetic types); CUP C36C18000160006.

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