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in

De Pedro E.J. (ed.), Cabezas A.B. (ed.).
7th International Symposium on the Mediterranean Pig

Zaragoza : CIHEAM

Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 101

2012

pages 31-37

Article available on line / Article disponible en ligne à l'adresse :

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To cite this article / Pour citer cet article

Martínez Martínez A., Landi Periatí V., Martínez Domínguez M., Poto Remacha A., Peinado Ramón B., Pons A., Barba Capote C., Carril González-Barros J.A., Méndez Tur Y., Vega-Pla J.L., Delgado Bermejo J.V. **Genetic characterization of autochthonous pig breeds from Spain with microsatellite markers.** In : De Pedro E.J. (ed.), Cabezas A.B. (ed.). *7th International Symposium on the Mediterranean Pig*. Zaragoza : CIHEAM, 2012. p. 31-37 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 101)



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Genetic characterization of autochthonous pig breeds from Spain with microsatellite markers

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Abstract. The European pig genetic resources are essentially formed from breeds belonging to two ancient strains: the Celtic type in north-central Europe and the Mediterranean type in the south. Both types were represented in Spain by native breeds, but today only the Iberian pig is completely developed. Other autochthonous breeds are rare, endangered or extinct. Population structure and genetic diversity in the Spanish native breeds of pigs Ibérico (ten varieties), Chato Murciano, Negro Canario, Negro de Formentera e Ibiza, Celta and Euskal Txerria were analyzed by typing 25 microsatellite markers recommended by the Food and Agriculture Organization of the United Nations. Within and between breed genetic variation was estimated by heterozygosities, F-statistics estimate, genetic distances and assignment tests. The results of this study confirm that Spanish local breeds of pigs represent a very interesting reservoir of allelic diversity, even though the current levels of inbreeding observed in some of the studied breeds. Therefore, appropriate conservation efforts should be undertaken, such as adopting strategies aimed at minimizing inbreeding, to avoid further losses of genetic diversity.

Keywords. Spanish local pigs – Genetic relationships – Conservation – Genetic structure.

Caractérisation des races autochtones porcines espagnoles avec des marqueurs microsatellites

Résumé. La plupart des variétés européennes sont principalement de deux souches: le type Celte du Nord et d'Europe centrale et le type du sud de la Méditerranée. Les deux types sont représentés en Espagne par des races de porcs autochtones, et bien que certaines variétés de porcs ibériques soient pleinement mises en œuvre, d'autres variétés et la plupart des autres races sont fortement menacées. Nous avons étudié la structure et la diversité génétique des races de porcs espagnoles: Ibérico (une dizaine de variétés), Chato Murciano, Negro Canario, Negro Mallorquin, Negro de Formentera et Ibiza, Celta et Txerria Euskal ont été analysés avec 24 microsatellites recommandés par la FAO. La variation génétique au sein des races et entre les races a été étudiée par analyse d'hétérozygotie, par les indices de fixation, les distances génétiques et les études génétiques et l'affectation des individus aux populations. Les résultats confirment que ces animaux sont un sujet très intéressant de diversité génétique, en dépit des niveaux élevés de consanguinité observés dans certaines races. Cette étude est une première étape qui fournit des informations génétiques sur l'état de ces importantes ressources génétiques et en tenant compte de cette information, les programmes de conservation doivent être mis en œuvre dans certaines races pour éviter de nouvelles pertes de diversité génétique.

Mots-clés. Races porcines espagnoles – Relations génétiques – Conservation – Structure génétique.

I – Introduction

Until the 1950s, swine production in Southern Europe was essentially based on native breeds raised under extensive systems, often in specific ecosystems integrating forest lands (Vicente and Alés, 2006). The intensification of agriculture that took place after that time caused major changes to pig breeding, with traditional systems being replaced by intensive production based on a reduced number of exotic breeds, while native breeds were progressively abandoned and became virtually extinct (Gama, 2006). The European pig genetic resources are essentially formed from breeds belonging to two ancient strains: the Celtic type in north-central Europe and the Mediterranean type in the south (Porter and Tebbit, 1993). Both types were represented in Spain by native breeds, but today only some varieties of the Iberian pig are completely developed (Retinto and Entrepelado). Other autochthonous breeds are rare, endangered or extinct. Currently, 7 native breeds of pigs are recognized in Spain (i.e., Ibérico, Chato Murciano, Celta, Negro Canario, Negro Mallorquín, EuskalTxerria and Gochu Asturcelta).

Detailed knowledge of population structure among and within breeds of livestock is essential for establishing conservation priorities and strategies (Caballero and Toro, 2002). Microsatellite markers have proved extremely useful for the analysis of population structure and relationships, and have been widely used for genetic characterization of several species and populations including European pig breeds (Laval *et al.*, 2000; Martínez *et al.*, 2000; San Cristobal *et al.*, 2006; Vicente *et al.*, 2008). Nevertheless, information on native breeds of pigs is still scarce, even though they possess unique characteristics in terms of adaptation, hardiness, and quality of products. In this study we used microsatellite markers i) to evaluate the degree and pattern of genetic variability in 6 native breed and one not officially recognised pig population currently used in Spain, and ii) to assess with different statistical tools the integrity and degree of admixture of these native swine breeds.

II – Materials and methods

Individual blood or hair samples were collected from 593 representative animals of the 15 populations under analysis. Specifically, the breeds studied and corresponding sample sizes are shown in Table 1.

DNA was extracted with Chelex 100 (Bio-Rad Laboratories, Alcobendas, Spain) and proteinase-K (Qbiogen, Illkirch, France), as described by Walsh *et al.* (1991). A panel of 24 microsatellite markers was established, according to the recommendations of the Food and Agriculture Organization of the United Nations and the International Society for Animal Genetics (FAO, 2004): *IGF1*, *S0002*, *S0005*, *S0026*, *S0068*, *S0090*, *S0101*, *S0155*, *S0178*, *S0215*, *S0225*, *S0226*, *S0227*, *S0228*, *S0355*, *S0386*, *SW24*, *SW72*, *SW240*, *SW632*, *SW857*, *SW911*, *SW936*, *SW951*. Microsatellite markers were amplified in multiplex polymerase chain reactions (PCRs) using fluorescence-labelled primers according the methodology used by Vicente *et al.*, 2008. PCR products were separated by electrophoresis on a ABI 377XL instrument (Applied Biosystems, Foster City, CA) according to manufacturer recommendations and allele sizing was accomplished by using the internal size standard GeneScan-400HD ROX (Applied Biosystems, Warrington, UK).

Results of electrophoresis were read directly and interpreted with GeneScan and Genotyper software (Applied Biosystems, Applied Biosystems B.V.), respectively. A first approach to within-breed diversity was investigated by calculating the mean number of alleles, observed and unbiased expected estimates of heterozygosity per population (Nei, 1987) and their standard deviations using "Microsatellite Toolkit" software (Park, 2001). F_{ST} , F_{IT} statistic and within-breed inbreeding coefficient (F_{IS}) (Weir and Cockerham, 1984) were estimated with a 95% confidence interval, determined by 10000 bootstraps across loci using "Genetix" v4.04 (Belkhir *et al.*, 2003). Genetic divergence among breeds was estimated through D_A genetic distances (Nei *et al.*, 1983), with the "Populations" software (Langella, 1999).

The "Structure" v.2.1 software (Pritchard *et al.*, 2000) was used to investigate the genetic structure of the 16 populations, in order to identify population substructure and admixture. Runs of 300000 iterations after a burn-in period of 100000 iterations were performed for each K, to determine the most probable number of clusters, as inferred from the observed genotypic data. The "Distruct" v.1.1 software (<http://rosenberglab.bioinformatics.med.umich.edu/distruct.html>) was used to obtain a graphical display of individual membership coefficients in each ancestral population.

Table 1. Number of samples analysed (N), mean number of alleles per locus (MNA), average expected heterozygosity (He), average observed heterozygosity (Ho) and their standard deviations, inbreeding coefficient (F_{is}) with their confidence interval and number of loci with deviations from Hardy-Weinberg equilibrium (HWE) (p<0.05) estimated with 24 microsatellites in 16 Spanish pig populations

Population	N	Mna (sd)	He ¹ (sd)	Ho (sd)	Fis (ic)	Hwed
Retinto portugués ²	14	3.75 (1.62)	0.521 (0.046)	0.551 (0.027)	-0.059 (-0.201 - -0.009)*	0
Retinto ²	88	6.38 (2.81)	0.545 (0.047)	0.508 (0.011)	0.068 (0.025 - 0.099)*	7
Entrepelado ²	73	6.21 (2.48)	0.564 (0.051)	0.543 (0.012)	0.036 (-0.008 - 0.066)	7
Mamellado ²	7	3.46 (1.69)	0.509 (0.058)	0.520 (0.039)	-0.023 (-0.300 - 0.007)	0
Silvela ²	14	4.00 (1.56)	0.568 (0.047)	0.510 (0.028)	0.106 (-0.038 - 0.154)	1
Dorado gaditano ²	7	3.29 (1.33)	0.495 (0.055)	0.500 (0.039)	-0.012 (-0.299 - 0.054)	1
Torbiscal ²	60	5.00 (2.50)	0.527 (0.051)	0.465 (0.013)	0.118 (0.066 - 0.151)*	7
Negro de los Pedroches ²	29	4.21 (1.41)	0.561 (0.040)	0.554 (0.020)	0.012 (-0.101 - 0.076)	2
Lampiño ²	59	6.08 (2.99)	0.573 (0.048)	0.533 (0.013)	0.070 (0.012 - 0.111)*	6
Manchado de Jabugo ²	41	3.38 (1.64)	0.388 (0.051)	0.397 (0.016)	-0.021 (-0.088 - 0.019)	3
Chato murciano	64	4.04 (1.27)	0.515 (0.042)	0.521 (0.013)	0.056 (0.011 - 0.090)*	14
Negro canario	27	3.96 (1.76)	0.474 (0.049)	0.475 (0.020)	-0.001 (-0.110 - 0.059)	1
Negro de Formentera e Ibiza	20	3.88 (1.62)	0.415 (0.044)	0.356 (0.022)	0.146 (-0.011 - 0.215)	6
Negro mallorquin	17	4.42 (1.50)	0.562 (0.032)	0.516 (0.025)	0.084 (-0.035 - 0.132)	4
Euskaltxerria	46	3.00 (1.47)	0.369 (0.052)	0.382 (0.015)	-0.034 (-0.092 - 0.002)	1
Celta	27	4.54 (1.79)	0.596 (0.047)	0.496 (0.021)	0.171 (0.079 - 0.219)*	0
Average		4.35 ± 1.05	0.511 ± 0.068	0.489 ± 0.061	0.045 ± 0.069	3.75 ± 3.86

¹Nei, 1987; ²Ibérico varieties.

III – Results and discussion

Information on pig native breeds is very scarce, even though they possess unique characteristics in terms of adaptation, hardiness, and quality of products, and could thus represent an interesting reservoir of genetic diversity (Cañón *et al.*, 2006; Foulley *et al.*, 2006; Peter *et al.*, 2007), in addition to being a major source of between-breed diversity. Knowledge of the structure of a livestock population in terms of sources of variability among and within breeds is essential for establishing conservation priorities and strategies (Caballero and Toro, 2002), with the long-term objective of maintaining genetic diversity for future generations (Notter, 1999). In the particular case of native breeds that have nearly become extinct, the possible existence of bottlenecks or admixture with other breeds in the recent past warrants special attention. Therefore, an assessment of their genetic diversity and possible relationships with other breeds represents a major step toward the development of conservation and improvement programs. Microsatellite markers are particularly suitable for genetic diversity studies, because of their large number, distribution throughout the genome, high level of

polymorphism, codominant inheritance, neutrality with respect to selection, and easy automation of analytical procedures (Cañón *et al.*, 2001).

Although many of the breeds included in this study have been previously characterized genetically to some extent together with other breeds (Martínez *et al.*, 2000; Martínez *et al.*, 2003; Peinado *et al.*, 2003; Peinado *et al.*, 2006; Martínez *et al.*, 2007), a comprehensive analysis of the genetic diversity present in Spain and a source of important pig genetic resources has never been made. This study includes 6 of the 7 officially recognised local breeds and one population recently defined: Negro de Formentera e Ibiza. All the populations studied are endangered with the exception of two varieties of the Ibérico (Retinto and Entrepelado). Euskal Txerria and Celta breeds are representative of the Celtic trunk.

The total number of alleles found for the 24 microsatellite markers was 267, and polymorphisms in all loci were observed in most of the breeds. The number of alleles per locus ranged between 5 (S0215) and 25 (S0005), with a global mean for the 24 markers of 11.13 alleles per locus. This value is slightly superior to the range found in other European breeds of pigs (Laval *et al.*, 2000; San Cristobal *et al.*, 2006; Vicente *et al.*, 2008).

The within-breed analyses (Table 1) indicated that the mean number of alleles per locus was greatest in Retinto (6.38) and least in Euskaltxerria (3.00) with a mean number of alleles per locus over all populations of 4.35 ± 1.05 . The average expected heterozygosity ranged between 0.596 in Celta and 0.369 for the Euskaltxerria breed. The proportion of loci that were not in HWE ($P < 0.05$) in each of the breeds analyzed ranged between 0 (Retinto Portugués, Mamellado, Celta) and 14 (Chato Murciano). Significant F_{IS} values were found in several populations, suggesting deviations from Hardy–Weinberg proportions might be due to consanguineous mating in those populations more endangered. Retinto, Torbiscal, Lampiño, Chato Murciano and Celta showed heterozygote deficit, while Retinto Portugués showed an excess of heterozygotes.

The F statistics showed a clear structure of the Spanish pig breeds. These statistics with their corresponding 95% confidence intervals obtained with 1000 bootstraps over loci were $F_{IS} = 0.055$ (0.030 – 0.085), $F_{IT} = 0.254$ (0.226 – 0.283) and $F_{ST} = 0.211$ (0.193 – 0.230). The large among-breed fixation index (F_{ST}) was significantly different from 0, indicating a clear structuring of the Spanish pig populations from the point of view of neutral genetic variability. This value is in agreement with the studies of Laval *et al.* (2000) and SanCristobal *et al.* (2006) on European breeds, but it markedly exceeds most previous studies of pig diversity (Martínez *et al.*, 2000; Yang *et al.*, 2003; Vicente *et al.*, 2008).

The graphical representation by Neighbor-joining of genetic distances between populations, calculated by the D_A distance, is shown in Fig. 1.

Low bootstrap values were obtained as it was expected when a high number of populations is analyzed (Felsenstein, 2004), and has been previously observed in the analysis of closely related domestic breeds with different genetic distances (Laval *et al.*, 2000; Martínez *et al.*, 2000; San Cristobal *et al.*, 2006; Vicente *et al.*, 2008). Nevertheless, all the Ibérico varieties formed a cluster while the rest of the breeds clustered separately. In the NJ tree, large branch lengths either corresponded to populations with low within population diversity. For example, the Euskaltxerria and the Negro Canario had very low observed and mean numbers of alleles and were found in the longest branches.

Bayesian clustering methods allow for the assignment of individuals to groups based on their genetic similarity and provide information on the number of ancestral populations underlying the observed genetic diversity. We tested values of K ranging from 2 to 16. The $\ln \Pr(X|K)$ increased sharply between $K = 2$ and $K = 13$, and stabilized between $K=13$ and $K=16$. These results would thus indicate that the appropriate value of K would be 13.

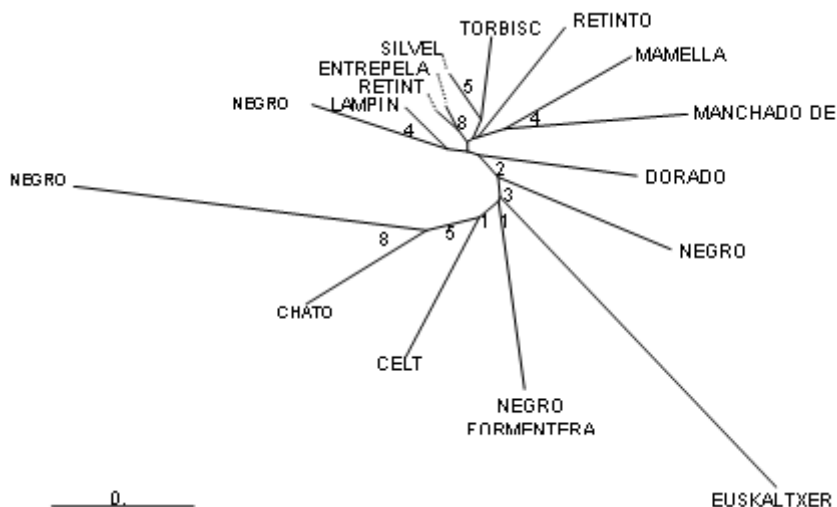


Fig. 1. Neighbour-joining dendrogram of 16 Spanish pig populations based on D_A (Nei 1983). Numbers are the percentage bootstrap values from 1000 replications of resampled loci.

The proportional membership of individual genotypes in the different clusters (Figure 2) indicates that the Iberian pig contains a high within-breed biodiversity as was previously reported by Martínez *et al.* (2000).

There are Ibérico varieties completely defined such as Retinto Portugués, Torbiscal, Negro de los Pedroches, Lampiño and Manchado de Jabugo. The rest of the Ibérico varieties belong to the “Red trunk” and it was difficult to separate them. Nevertheless, it was possible to discriminate between the Ibérico and the rest of the Spanish breeds. The Spanish breeds Iberian pig, Chato Murciano, Negro Canario, Negro de Formentera e Ibiza, Negro Mallorquín, Euskal Txerria and Celta showed that there was very little admixture between them, which are now quite distinct from each other. This result is in line with the F_{ST} estimate and confirms the distinctiveness of and low gene flow between the swine breeds analyzed, contrasting with results obtained when Structure was applied, for example, to horse (Vega-Pla *et al.*, 2006) and sheep (Quiroz *et al.*, 2008) populations, where admixture of breeds is very common.

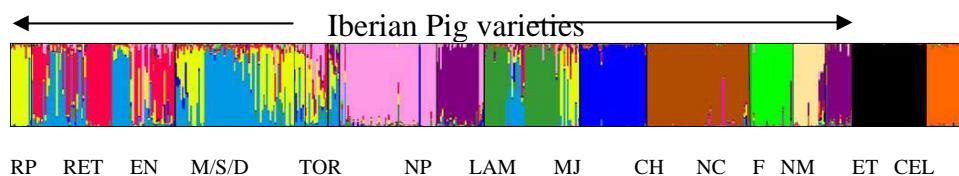


Fig. 2. Population structure of 16 Spanish pig populations using the model-based STRUCTURE software, where each animal is represented by a single vertical line divided into K colours, where K is the number of clusters assumed and the colours show the estimated individual proportions of cluster membership. Results are shown for K=13.

IV – Conclusions

There is a high porcine genetic diversity in Spain. The breeds sampled had a high level of differentiation, and most of them showed signs of accumulated inbreeding. Some of the breeds included in this study are involved in recent recovering processes and the results reported here may serve as useful indicators in setting conservation priorities, taking into consideration both among-population diversity and within-population variability, in addition to information on traits of current or potential economic importance, including adaptation.

Acknowledgements

The authors wish to acknowledge the financial support received by the Project RZ00-15 of the Programa Nacional de Recursos y Tecnologías Agroalimentarias of Ministerio de Ciencia y Tecnología of Spain.

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