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Application of molecular markers (RAPD, AFLP and Microsatellites) to Iberian pig genotype characterization

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SUMMARY - Use of molecular markers is specially interesting for the genetic study of the *Iberian* pig, whose morphologic and physiologic variability prevents the knowledge of his populational structure. Crossbreeding, mainly with *Duroc*, is a common practice to increase the carcass performance which leads to the attainment of worse quality cured products. Molecular analysis techniques allow the estimation of genetic variability and divergence between species and populations, and thus they can be used for phylogenetic studies, conservation programs and control of the genetic origin of products. This work presents the results obtained with the application of three different techniques of DNA polymorphisms detection to samples from different populations of *Iberian* pigs. The RAPD technique was used for the identification of diagnostic markers which allowed the detection of *Duroc* genes in *Iberian* pig samples. AFLP technique was used for the attainment of specific markers of one *Iberian* pig population, with a high inbreeding level, in order to study its possible utilization in a conservation program. Finally, seven populations have been analysed with 19 microsatellite markers. The analysis of these polymorphic loci genotypes enabled the estimation of different parameters of genetic diversity (heterozygosity, genetic distances).

Key words: Molecular markers, genetic diversity, *Iberian* pig.

RESUME - *"Application des marqueurs moléculaires (RAPD, AFLP et microsatellites) à la caractérisation génotypique du porc ibérique". L'utilisation de marqueurs moléculaires présente un intérêt spécial pour l'étude du porc ibérique, dont la variabilité morphologique et physiologique empêche la connaissance de la structure de sa population. Le croisement systématique avec d'autres races pour augmenter la production est une pratique habituelle, qui conduit à l'obtention de produits secs de qualité inférieure. Les techniques d'analyse moléculaire permettent d'estimer la variabilité et divergence génétique des espèces et populations, donc elles peuvent être utilisées pour des études phylogénétiques, programmes de conservation et contrôle de l'origine génétique des produits. Dans ce travail on montre les résultats obtenus avec l'application de trois techniques différentes de détection de polymorphisme ADN sur des échantillons de plusieurs populations de porc ibérique. La technique RAPD a été employée pour l'identification de marqueurs de diagnostic qui permettent de détecter des gènes Duroc dans les échantillons de porcs de type ibérique. La technique AFLP a été utilisée pour l'obtention de marqueurs spécifiques d'une population de porc ibérique, avec un haut niveau de consanguinité, à l'objet d'étudier son utilisation possible dans un programme de conservation. Finalement, ces populations ont été étudiées au moyen de l'analyse de 19 marqueurs type microsatellite. L'analyse des génotypes de ces loci polymorphiques permet l'estimation de différents paramètres de diversité génétique (hétérozygotie, distances génétiques).*

Mots-clés : *Marqueurs moléculaires, diversité génétique, porc ibérique.*

Introduction

Although the development of the high quality cured products market has made possible a noteworthy increase of the *Iberian* pig production, from the genetic point of view this population presents several problems. There are traditional strains of *Iberian* pig joint with commercial populations mostly of doubtful origin. The study of genetic relations among all them, as well as the establishment of conservation programs for those strains which are in a worse condition, is limited by the lack of genealogical control. The widespread use of crosses with *Duroc* constitutes a risk of introgression of genes from this breed. In addition, the products made from pure and crossed animals have to compete in the market, and so their objective differentiation is interesting for consumers, industry and for breeders.

The present work summarizes the application of molecular biology techniques to solve some of the problems mentioned. Results obtained with three different techniques of DNA polymorphism detection (RAPD, AFLP and microsatellites) are shown.

Genetic study of strains

Iberian pigs display a high variability, not only in their morphology and physiology, but also in their production (Silió *et al.*, 1996; Benito *et al.*, 1998) and reproduction (Pérez-Enciso *et al.*, 1992). However, there is no knowledge about the genetic variability within and between these populations, due to the lack of strict genealogical records. The application of molecular methods (Avise, 1994; Frankham, 1995) to studies of genetic diversity makes it possible to estimate the genetic variability in commercial and traditional strains, the genetic distances between them and their heterozygosity. This kind of analysis are suitable for the successful achievement of conservation programs and for the orientation of the genetic improvement in *Iberian* pigs.

With this aim, a preliminary study has been made of some herds ascribed to the genealogical control. Seventy animals have been genotyped, males and females, proceeding from seven herds members of the breeders' association (AECERIBER). Four of them belong to the chestnut type: *Retinto* from the public herds *Valdesequera* and *Censyra, Retinto Entrepelado* (with scarce hair) and *Retinto Mamellado* (with appendages hanging on the neck). Two of them belong to the black hairless type from different geographic origins: Guadiana (*Lampiño G)* and La Serena *(Lampiño S)*. The last one *Torbiscal* is a red coated line, obtained in 1963 by blending four traditional strains (Rodrigáñez *et al*., 1998).

We have used 19 microsatellite markers, fluorescently labelled, some of which are included in the panel proposed by the FAO for variability studies in porcine breeds, and others were selected on previous analyses of our group, according to their informativeness on *Iberian* populations. The markers used were: CGA, IGF1, S0068, S0071, S0090, S0106, S0228, SW1057, SW1111, SW210, SW240, SW2419, SW632, SW72, SW787, SW857, SW874, SW911 y SW936 (US Pig Gene Mapping Program; http://www.genome.iastate.edu).

The main advantage of microsatellites is the high level of polymorphism they show. In this work the mean number of alleles was 7.42, with a rank of variation between 2 (S0228) and 19 alleles (CGA). The mean heterozygosity of each locus for all the individuals together swings from 0.075 to 0.900. The heterozygosity of each population for the total of markers swings from 0.523 (*Lampiño G*) to 0.661 (*Retinto C*).

Genetic distances between populations have been calculated with the Reynolds estimator (Reynolds *et al.*, 1983) and the results are shown in Table 1. The dendrogram obtained from these distances with the UPGMA method (Weir, 1996) is displayed in Fig. 1. These results show two main and well differentiated branches. One of them includes the black hairless herds (*Lampiños S* and *G*), and the red strain *Torbiscal*. This similarity can be explained because the two black hairless strains have contributed to the foundation of *Torbiscal.* (Rodrigáñez *et al.*, 1998). The chestnut coated populations are grouped in the other main branch. Though it is necessary to improve the precision of these distances by the genotyping of a higher number of microsatellites, these results open the way of the future characterization of other *Iberian* pig populations with unknown genetic origin.

Table 1. Genetic distances (Reynolds *et al.*, 1983) estimated among seven *Iberian* pig populations: 1) *Retinto* C, 2) *Lampiño S,* 3) *Lampiño G*, 4) *Retinto Mamellado*, 5) *Retinto Entrepelado*, 6) *Torbiscal*, 7) *Retinto V*

Population	1	2	3	4	5	6	7
1	-						
2	0.2196	-					
3	0.2822	0.2254	-				
4	0.1744	0.2561	0.3245	-			
5	0.1346	0.2160	0.2726	0.1388	-		
6	0.2296	0.2044	0.2171	0.1964	0.1399	-	
7	0.1975	0.2505	0.3488	0.2258	0.1296	0.2661	-

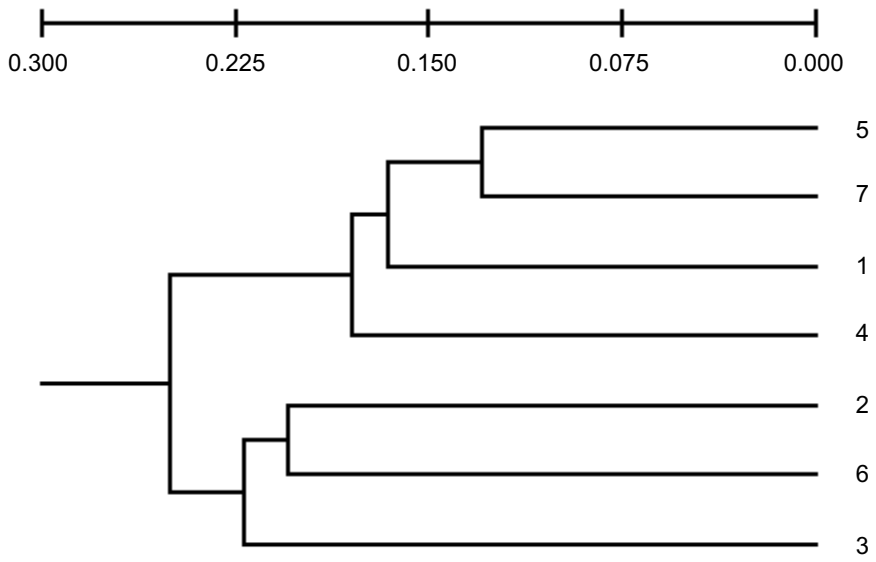

Fig. 1. Dendrogram of genetic distances among the *Iberian* pig populations analysed.

Marker assisted conservation

Some of the traditional types of the *Iberian* pig breed suffered a generalized rejection from the breeders during the crisis period of the breed, mainly due to their extremely fat carcasses (black hairless) and also because some of them did not respond exactly to the racial type. As the few existing populations of these types are in danger of extinction, the establishment of conservation programs from the surviving animals could be considered interesting. Without pedigree information, the use of molecular markers should make the choice of the founder animals in the program easier and it would also minimize the losses of genetic variability in the first generations (Toro *et al.*, 1998).

In order to verify the efficiency of the use of molecular information in the prediction of coancestry, ten animals of the strain *Lampiño G* have been used. This strain is conserved as a closed population with genealogical control since 1945 (Rodrigáñez *et al.*, 1997). For this objective we have used AFLP markers. These are restriction fragments which are obtained by means of genomic DNA digestion and selective amplification by PCR. Amplification products are analysed in denaturing polyacrylamide gels and visualized by autoradiography (Vos *et al.*, 1995). AFLP are markers of the type presence/absence of band, in which it is not possible to differentiate the heterozygote genotype.

AFLP technique was tested with 12 different primer combinations in the selective PCR. Out of 1721 bands obtained, 63 presented polymorphism in the *Lampiño G* strain. The polymorphic fragments were scored as 1 for presence and 0 for absence. Similarity matrices between individuals were calculated with the Rogers and Tanimoto coefficient (1960), within the computer program NTSYS-PC version 1.80 (Rohlf, 1993). Coancestry coefficients between the genotyped animals were calculated from the genealogical information.

Figure 2 shows the values of the similarity (RT) and coancestry (F) coefficients between all the pairs of individuals, and the correlation between them. The statistical significance of this correlation was verified with the Mantel permutation test (1967). The results confirm that, in absence of pedigree information, genetic markers could be useful to estimate relationships between individuals. With this objective, dominant markers (AFLP and RAPD) have a disadvantage, due to the lack of precision in the allele frequency estimation. Lynch and Milligan (1994) suggested that this type of applications must exclude the markers with extreme frequencies. Following this suggestion, a second analysis was made, in which only markers with frequencies ranging from 0.3 to 0.7 were used. The correlation obtained between the similarity and coancestry coefficients was then slightly higher: 0.611 (based on 38 AFLP markers).

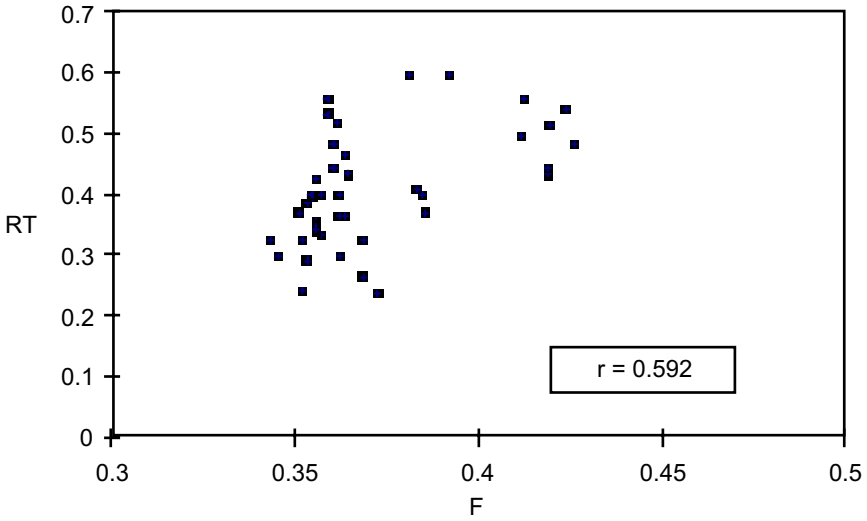

Fig. 2. Correlation between coancestry coefficients (F) calculated from the pedigree information and similarity coefficients (RT), calculated from AFLP markers, in 10 *Iberian* pigs.

Diagnostic marker identification

The traditional characterization of *Iberian* pigs has been based only upon morphological criteria of adaptation to the breed standard. This allows the identification of *Iberian* animals crossed 50% with *Duroc*. But animals with 25% or less of *Duroc* genes cannot undoubtedly be identified by morphological criteria. Regarding the cured products, the rules of the existing Denomination of origin demands a minimum proportion of *Iberian* genes, and there are also some breeders specialized in the production of purebred *Iberian* pig products. Nevertheless, there are not yet objective methods to verify the genetic origin of carcasses or cured products.

There are therefore, several reasons to develop objective methods, which by means of diagnostic markers will allow the differentiation of genetic types (purebred and crossbred in different percentages) in the *Iberian* pig production field. The unequivocal identification of pure and crossed animals could avoid the introgression of genes from foreign populations in the *Iberian* breed. On the other hand, it makes possible the detection of frauds in the cured product market.

Samples of 447 purebred animals have been used for the search of diagnostic markers, belonging to 23 different herds. Out of them, 15 are *Iberian* pig herds, owned by breeders associated to AECERIBER, and eight are *Duroc* pig herds. There were also samples obtained from 13 crossed animals (50% *Iberian* x *Duroc*). The RAPD technique was used, by testing 295 ten base pairs primers, corresponding to Operon Technologies kits A, B, C, E, F, H, N, R, T, W, X, Y, Z, AA and AB. This technique has shown its usefulness as differential diagnostic of bovine populations (Gwakisa *et al.*, 1994; Kemp and Teale, 1994). First of all, a bulked analysis was performed, by mixing equal quantities of genomic DNA from different *Duroc* populations. One *Torbiscal* strain sample was used as *Iberian* breed control. After the detection of each candidate marker in the bulked analysis, it was confirmed analysing all the available individual samples.

Nine *Duroc* markers were detected, which are shown in Table 2. As can be seen, those markers have very different frequencies, ranging from 13 to 93%, being the sizes of the amplification fragments comprised in the rank of 500 to 1700 base pairs. One of the markers detected is specific for one of the *Duroc* lines analysed. None of those markers can be detected in other available pig breeds (*Landrace* and *Large White*).

The results have been verified with DNA samples of crossbred animals *Iberian* x *Duroc*. The application of the technique to cured hams with two years of treatment has been also checked. This combination of specific markers is useful for the discrimination of purebred and 75% crossbred animal samples with a negligible probability of error. However, the search of

diagnostic markers is not yet finished, because it is still not possible to differentiate individual samples with less than 25% of *Duroc* genes. For this purpose, very interesting to defend the herdbook, it is necessary to identify a higher number of markers, or polymorphisms with 100% frequency, which have not been detected by now.

Table 2. RAPD diagnostic markers of the *Duroc* genome, which allow the differentiation of crossbred and purebred *Iberian* pigs

Primer	Sequence (5' - 3')	Marker size (bp)	Marker frequency (%) in *Duroc*
OPA – 10	GTGATCGCAG	1800	13
OPA – 19	CAAACGTCGG	800	77
OPE – 11	GAGTCTCAGG	550	93
OPE – 14	TGCGGCTGAG	600	92
OPR – 04	CCCGTAGCAC	1500	42
OPR – 09	TGAGCACGAG	500	38
OPW – 02	ACCCCGCCAA	700	51
OPY – O2	CATCGCCGCA	950	82
OPAA – 17	GAGCCCGACT	700	81

We have also detected seven RAPD markers specific for the *Iberian* genome. These markers, observed only in *Iberian* samples, have not been detected in any of the other analysed breeds *(Duroc, Landrace, L. White*). The frequency of these *Iberian* markers is being analysed actually.

Six Y chromosome markers were also found, which are porcine specific, as they have not been detected in other studied species: bovine, ovine, murine and human (Castellanos *et al.*, 1996).

Relative advantages of genetic markers

The three applications explained, although not yet finished, are examples of the usefulness of molecular techniques to solve genetic problems in extensive pig populations. Any of them, or a combination between them could be used for the characterization of individual or populational genotypes, though with different efficiency and cost.

Microsatellites are codominant markers, highly polymorphic and localized in genetic maps of farm animals (1200 in pig). Actually, the genotyping of microsatellite markers is performed automatically and with a low cost, due to the use of multiplex techniques, which allow the analysis of several microsatellites in the same reaction. The main disadvantage is that they are not a random sample of the genome (short tandem repeats) and they are scarce in some chromosomic regions. Those are the usual markers in populational studies as in economic trait loci mapping.

The interest of AFLP method is the possibility of amplification of a high number of DNA fragments potentially polymorphic. The power of this technique has contributed to its success in the comparison of varieties from cultivated vegetal species. The use of this type of marker in domestic animals is still incipient, but the automatization and the next inclusion of these markers in the genetic maps will make their use easier and more profitable, mainly in genomic regions where there is a lack of microsatellite type markers. The disadvantage of AFLP markers is its dominant nature.

Finally, RAPD markers are generally used because of their simple genotyping method and the near unlimited number of primers which could be tested. Those markers are also dominant and could present repeatability problems. They are suitable for the search of diagnostic markers, because of the possibility to analyse DNA mixtures from several individuals.

The study of DNA polymorphisms is a very active working area that note a lasting progress, which could be useful for our Mediterranean pig populations.

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