APPROACHES AND CHALLENGES IN MEASURING GENETIC DIVERSITY IN PIGS[1]

AVANCES Y RETOS EN LA MEDICIÓN DE LA DIVERSIDAD GENÉTICA EN LOS CERDOS

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SUMMARY

While the number of diverse genetic breeds of pigs may exceed 600 worldwide, there is a limited amount of information to assess their genetic and functional diversity. Efforts have primarily been conducted to examine genetic diversity using anonymous markers and to a more limited extent individual gene markers and mtDNA. This paper discusses the methods used to date and the need to examine other methods to more fully understand not only the genetic diversity but the functional diversity of different pig breeds.

RESUMEN

Aunque el número de razas genéticas de cerdos distintas excede de las 600 en todo el mundo, hay una disposición limitada de información para acceder al conocimiento de su diversidad genética y funcional. Los esfuerzos deben ir preliminarmente conducidos a examinar la diversidad genética utilizando marcadores anónimos y con una extensión más limitada a marcadores génicos individuales y ADNmt. Este trabajo discute los métodos utilizados hasta la fecha y la necesidad de examinar otros métodos para un conocimiento más profundo no sólo la de la diversidad genética, sino la diversidad funcional de las distintas razas porcinas.

INTRODUCTION

The pig was one of the first animals likely to have been domesticated over 5,000 years ago (Rothschild and Ruvinsky, 1998). To date there are likely over 600 breeds or lines worldwide of which the most reside in China and Europe and over 200 are considered endangered (Ollivier *et al*.,

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2001). Considerable differences appear to exist both morphologically and physiologically between the various domestic breeds and their wild boar ancestors from Europe and Asia (Giuffra *et al*., 2000). These changes in the domestic pig have reflected the rather *plastic* nature of the pig and humankind's ability to genetically manipulate it to fit certain needs and markets.

A large percentage of pig breeds are now in danger of extinction and others are threatened by inefficient use and loss due to crossbreeding. Efforts to determine the level of such risk are underway, especially in Europe but also in other parts of the world. In Europe, at the European Association for Animal Production, an Animal Genetic Data Bank has been established as a repository to record such breeds and to assess risk (see Simianer and Meyer, 2003, in this Proceedings). Along with assessment of risk is the consideration of which breeds should be preserved. Certainly there is the increased interest in this approach and there is some support by private organizations and governmental programs. Ruane (1999) has provided a set of criteria to be considered when choosing a specific breed for a conservation program. The degree of endangerment and genetic uniqueness of the breed are two of seven essential criteria discussed. However, while breeds have cultural and historical value, from an economic point of view, the functional diversity for a set of important economic traits should be considered the most important criterion. Efforts therefore to determine both genetic diversity and functional diversity should be used to help in the determination of breed differences and in determining those which must be preserved.

The objectives of this paper are to review in general terms ways to measure genetic diversity among and within pig breeds and to discuss methods to quantify diversity and relate it to functional importance.

METHODS TO DETERMINE GENETIC DIVERSITY

Over the past ten years considerable improvements in molecular genetics have led to the development of genetic maps of many organisms. These advances in molecular biology have made it possible to develop comprehensive genetic linkage maps in the pig (e.g. Archibald *et al*., 1995; Rohrer *et al*., 1994; 1996). To date, over 6,000 genes and anonymous markers have been added to the gene map of the pig (see www.thearkdb.org or http://iowa.thearkdb.org)

In addition to identifying and mapping genes and markers, animal geneticists have begun to search for the individual genes that affect traits of interest in the pig. Since the earliest quantitative trait loci (QTL) scan in pigs by Andersson *et al*. (1994) many others have followed and a number of regions are now identified (see review by Bidanel and Rothschild, 2002). In addition, the candidate gene approach (Rothschild and Soller, 1997) has been employed and many candidate genes have been shown to be associated with traits of interest (Rothschild and Plastow, 1999). These QTL and

candidate genes form the basis of comparison of genetics related to functional differences.

Key issues to discuss include but are not limited to 1) What is genetic homology/diversity? 2) How is it best measured? 3) What is functional diversity and how is it best assessed? Some examination of what has occurred in other fields may be useful for discussion purposes. Evolutionists are interested in knowing if two species are related. Therefore, interest is in evolutionary changes and knowing that the genes are the same is not enough. Given two DNA sequences, a researcher can ask the question: How much evolutionary change has occurred between these two sequences? Seemingly this appears to be a simple question but the answer may prove elusive. Researchers can use observed differences and the simplest measure of distance is to count the number of nucleotides that differ between the two sequences. This approach has been used in livestock (Giuffra *et al*., 2000; Kijas and Andersson, 2001) to examine evolutionary changes in the pig. However, there are potential problems with such an approach because if change has been common then the same site may have undergone repeated substitution. So as more time passes, the number of differences between two sequences becomes a less accurate estimate of the actual number of substitutions that truly occurred. As a general rule animal geneticists and breeders have a different problem in that they are not only interested in homology but also genetic and functional diversity, even so the same types of concern exist relative to comparisons.

A number of genetic methods exist to measure genetic diversity. These include comparison of anonymous markers such as microsatellites, minisatellites, and amplified fragment length polymorphisms (AFLPs), gene markers/SNPs (single nucleotide polymorphisms), large scale or directed sequencing, mitochondrial genotyping, and Y chromosome genotyping. Minisatellites are the preferred method in human studies and allow for seeing expansion and hence direction of evolution. Mitochondrial (mtDNA) genotyping has also been used to look for different female lineages and Y chromosome genotyping has also been used for measuring ethnic differences. Dealing with only 2 narrow genomic regions, Y and mtDNA, give some insight into possible phylogenetic origins, among the many others that SNPs in autosomal regions might indicate.

In livestock early measures of diversity were associated with protein polymorphisms measured on 2D gels. More recently, several recommendations have been made for genetic diversity studies (Barker *et al*., 1998). These recommendations include use of 2-5 microsatellites per chromosome and genotyping of 50 animals (25 of each sex) that are unrelated. Breeds that differ in the frequency of alleles at these loci are declared *different or diverse* after determination of genetic relationships or genetic distances between breeds (Barker *et al*., 1998). Examples of these approaches include an European Community project that was recently completed to evaluate the genetic diversity of European pig resources (considering more than 50

breeds) using primarily microsatellites and AFLP markers (Laval *et al*., 2000; San Cristobal *et al*., 2002). For details see http://databases.roslin.ac.uk/pigbiodiv. However, just as with evolutionary comparisons of gene sequence differences, the number of differences between two microsatellite sequences, as time passes, becomes a less accurate estimate of the actual number of substitutions that truly occurred. Certainly, given the accepted rate of mutation in microsatellites (10^{-4}) it is possible that mutations may change in one direction and then back again, confounding such comparisons further.

Another important question to ask is do microsatellites measure functional diversity? For major genes like Halothane (HAL), RN (PRKAG3) and *E.coli* K88 resistance we have examples of genetic and functional diversity. For other traits in which major genes do not appear to exist would sequence differences or polymorphisms within proteins or SNPs within genes (introns or exons) be a better measure of genetic and functional diversity than microsatellites? Today use of SNPs within genes, especially those shown to be associated with traits of interest, may be a better measure of functional differences. This approach has been recommended and employed but on a limited basis for both genes and mtDNA.

Ciobanu *et al*. (2001) examined animals from two local Romanian pig breeds, Mangalitsa and Bazna. Polymorphism was assessed at nine genes known to cause phenotypic variation, were potentially involved in trait differences or were putative candidate genes. The traits they considered were disease resistance, growth, coat color, meat quality and prolificacy. Ciobanu *et al*. (2001) found significant differences in five of the ten characterized polymorphisms and they concluded the observed allele frequency differences were related to gene function and the phenotype of the breed. A limitation in such studies is that the number of animals in such populations is usually small

Candidate gene approaches can be combined with QTL scans for traits that are economically important and could be a better approach to measure of functional diversity [e.g. new mutations with PRKAG3 affecting meat quality (Ciobanu *et al*., 2001)]. The use of exotic or local or country breeds in scans is less likely due to cost of the QTL scan and such scans represent more limited sampling of the breeds. When more is known about the genes of interest then anonymous markers will be irrelevant. The challenge is to pick the right genes and compare breeds. The European Community Pig Biodiversity II project PigBioDiv 2 (QLRT-2001-01059) has accepted the idea that real gene differences are important and has expanded to include SNPs (see Blott *et al*., 2003, in the present proceeding).

NEW DIRECTIONS AND NEEDS FOR FUTURE STUDY

Technical problems with use of many of these types of markers do exist. These methods (PCR-RFLP, microsatellites, and AFLPs) are constrained generally by gel electro-

phoresis resulting in low throughput. Use of microsatellites or SNPs requires previous identification of a polymorphism. Recognition of the polymorphisms is based on size separation and correlating bands among labs can be difficult and differences could be misleading. This has certainly been the situation for pig microsatellite genotyping between labs where using control DNAs showed that the range of allele size usually differed by less than one bp between PigMaP and PigBioDiv labs, though for 2 microsatellites out of 26 differences exceeded 2 bp (Ollivier, personal communication).

One approach designed to overcome many of these limitations is the use of hybridization-based methods using nucleic acids fixed to solid-state surfaces. An example of such an approach is the use of DNA chips for genotyping for SNPs. This technique is again limited by first knowing the genes and SNPs and then also by cost. An alternative approach that uses parts of genes but also the random nature of variation is the use of so called Diversity Array Technology (DArT™). This approach uses methods similar to a combination of RFLP analysis and array technology to measure gene expression and has been used in examining differences in rice (Jaccoud *et al*., 2001). This technology certainly has promise because genes can later be identified that are associated with differences and function inferred or later studied.

Whole genome sequencing has yet to be undertaken for the pig but efforts are underway to begin in the near future. Such results could lead to whole genome SNP discovery and with lowering of genotyping costs a new DNA genotyping chip may have possibilities for diversity studies. It is likely that technology may advance rapidly once sequencing is underway.

Some examination of the larger picture is also required. First if breeds are found to be different which methods should be used to choose animals that represent unrelated samples of a particular breed. Clearly, selection of representative animals from a breed depends on availability of animals both within and across families. The use of microsatellites to determine more accurately the relatedness with family and to make decisions on mating pairs for breed preservation is quite appropriate. Additionally, the use of specific SNPs to represent trait diversity could be included as ways to select diverse animals.

Researchers should consider the needs and solutions required for future trait gene mapping and diversity studies. These include, but are not limited to:

1) Available gene (allele) frequency screens - will tests work in all populations?.

2) DNA from large phenotyped populations of diverse breeds.

3) Ability to accomplish high density (throughput) genotyping - HDG.

4) Inexpensive, high throughput phenotyping.

5) Development of advanced statistical analysis tools (bioinformatics).

6) Large scale gene expression screens - chips and arrays.

7) Real financial investment in diversity data.

Point 4 requires more discussion. The ability to measure functional

diversity relies on phenotypic traits that are accurate and easily collected. Animal scientists will need to assist in this process so that data are reliable and phenotyping costs can be reduced.

Other issues also cloud the picture of future research and the results that are likely to come from it. Background genetics (epistasis) makes single gene comparison's difficult and limited on a per gene/breed basis. In the future, large-scale studies involving hundreds or thousands of genes would provide the ability to look for functional diversity among interacting genes and gene pathways. Since discovery of functional gene differences are the preferred endpoint of diversity studies then protection of intellectual property (IP) and exclusive *vs* non-exclusive use of IP from diversity studies may be an issue. Certainly maintaining a real public and scientific interest in diversity issues will be challenging.

CONCLUSIONS

Diversity studies have come along way in attempting to measure genetic differences in pigs from a variety of breeds and countries. The use of anonymous markers has limitations but has been useful to date and has value in measuring relatedness within breeds. Gene differences will be better than anonymous markers for determining both genetic and functional diversity. Their use will require considerable new information about many more genes than those presently know to be associated with traits of interest. New technologies such as array methods or those resulting from large scale sequencing may revolutionize approaches for determining genetic and functional diversity. Researchers should consider new technologies as they approach the challenges ahead.

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