

CHARACTERISATION OF GENETIC VARIATION IN THE PIG BREEDS OF CHINA AND EUROPE - THE PIGBIODIV2 PROJECT

CARACTERIZACIÓN DE LA VARIABILIDAD GENÉTICA EN LAS RAZAS DE CERDOS CHINOS Y EUROPEOS. EL PROYECTO PIGBIODIV2

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PALABRAS CLAVE ADICIONALES

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SUMMARY

The PigBioDiv2 project will evaluate and compare genetic diversity among the pig breeds of China and Europe. At least 50 Chinese breeds will be sampled and compared with 59 European breeds previously sampled in the PigBioDiv1 project. The European pig biodiversity database will be extended to at least 100 breeds, one third of the world's total number of pig breeds.

Relationships among breeds will be determined by estimating genetic distance based on micro-satellite markers and haplotypic relationships from mtDNA and Y-chromosome polymorphisms. The project will also determine the relationship between general measures of genetic diversity, such as genetic distance, and functional differences among breeds by characterising

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trait gene loci (type I markers) and QTL regions. Measures of diversity derived from anonymous markers (microsatellites) will be compared to measures derived from the trait genes. New approaches using DNA marker (genetic) data to identify genes involved in functional differences between breeds will also be developed.

RESUMEN

El proyecto PigBioDiv2 evaluará y comparará la diversidad genética entre las razas de China y de Europa. Al menos 50 razas chinas serán muestreadas y comparadas con 59 razas europeas previamente estudiadas en el proyecto PigBioDiv1. La base de datos sobre la biodiversidad porcina europea se extenderá hasta al menos 100 razas, un tercio del total de las razas porcinas europeas. Serán determinadas las relaciones entre razas por medio de la estimación de las distancias genéticas basadas en marcadores microsatélites y relaciones haplotípicas desde los polimorfismos del ADN mitocondrial y del cromosoma Y. El proyecto también determinará las relaciones entre medidas generales de la diversidad genética, tales como la distancia genética, y diferencias funcionales entre razas por la caracterización de TGL (marcadores tipo I) y regiones QTL. Las medidas de la diversidad derivadas desde marcadores anónimos (microsatélites) serán comparadas con las medidas derivadas desde los genes de caracteres. También serán desarrollados nuevos avances utilizando datos de marcadores (genéticos) del ADN para identificar genes relacionados con diferencias funcionales entre razas.

INTRODUCTION

The critical evaluation of livestock genetic resources and conservation of key populations will be important factors in enabling the agriculture and food

industries to respond to future changes in consumer needs. The European pig industry currently relies on a limited number of breeds but in spite of the dominance of these breeds there remain a large number of traditional breeds in Europe (33 percent of the FAO Inventory). A similar situation exists in China, a country which also has a very large number of traditional breeds (34 percent of the FAO Inventory) many of which are unique to the region. These traditional breeds provide potential sources of novel genetic variants which may be valuable to producers, supplying the diversity which will allow exploitation of future market opportunities and the creation of sustainable agricultural systems for the 21st century. Traditional breeds also represent a potential resource for the improvement of commercial lines.

The first European pig biodiversity project (PigBioDiv1) characterised genetic diversity for 59 lines and breeds of European pig using anonymous DNA markers (microsatellites and AFLPs). The project (which was completed in December 2000) has provided key steps in facilitating and rationalising the maintenance of genetic diversity in European pig populations. A DNA bank of European breeds has been created and samples are available for further genetic studies. Applications to access the samples are considered according to the terms of the Material Transfer Agreement developed for these studies (Cardellino, 2003). Information on samples, genotypes and results are held on the European pig diversity database (http://databases.roslin.ac.uk/pig_biodiv) which was also created as part of the project. The second project

(PigBioDiv2) will build on the results of PigBioDiv1 and extend the work both geographically and technically. The overall aim of the new project is to extend the characterisation of pig biodiversity by sampling a relatively large proportion of Chinese pig breeds and comparing these populations with those in Europe. A second important aim is to ensure that this effort builds on the existing European database establishing continuity and ensuring data compatibility. This second aim is essential if genetic distance data is to be useful for the conservation of biodiversity. A third aim is to investigate how general measures of genomic diversity (e.g. genetic distances calculated using anonymous DNA markers) are related to functional genetic differences between breeds. An important component of this objective will be to determine whether DNA marker and genetic distance data could be used to identify specific genes or quantitative trait loci (QTL) that give rise to significant breed differences.

New molecular biology tools will be utilised to enhance the characterisation of diversity and new methodologies will be established that can identify functionally different alleles at important loci – the loci that are of most interest for both conservation and the sustainability of biodiversity. Statistical analysis of microsatellite genotypes as well as of mitochondrial and Y-chromosome sequences and specific genes associated with breed determining traits, such as coat colour, will provide estimates of breed relatedness and of the extent of the genetic diversity among the Chinese and European populations.

SELECTION OF BREEDS TO BE EVALUATED

The Chinese pig breeds to be sampled will be selected on the basis of the information held in the FAO and EAAP diversity databases. The first EC pig biodiversity project (PigBioDiv1) sampled a range of breeds, including 25 rare or traditional European breeds together with several commercial lines based on the Landrace (12 lines), Large White (9 lines), Piétrain (4 lines), Duroc (3 lines) and Hampshire (2 lines) breeds. At least 50 Chinese breeds will be added to these during the PigBioDiv2 project. The breeds chosen will represent the widest possible range of phenotypes available with respect to important production characteristics (growth performance, reproduction and the production of finished products e.g. lean content, fatness, meat quality, skin characteristics for leather production). The historical and cultural importance of the breed may also be taken into account. Sampling design and specification of sampling procedures will be determined following FAO guidelines for diversity studies and taking into account the lessons learned from the PigBioDiv1 project. Phenotypic measurements on breeds will be obtained if already collected previously (i.e. it is not proposed to measure phenotypes for this project) for the maximum number of breeds possible. Where possible, measurements on individual animals will be stored on the database; alternatively breed averages will be used. Suitable traits might include mean age at slaughter, weight at slaughter, number of piglets born per parity and age at weaning.

BREED RELATIONSHIPS AND
VARIABILITY DETERMINED USING
MICROSATELLITE MARKERS

Genetic relationships between the Chinese and European pig breeds will be determined by typing the same microsatellite markers that were used in PigBioDiv1 (for marker details see <http://www.projects.roslin.ac.uk/pigbiodiv/markers.html>) on the 50 Chinese breeds being sampled for PigBioDiv2. In order to ensure that the new genotyping results are comparable with those obtained in the PigBioDiv1 project a pilot study will be carried out. This will facilitate the transfer of genotyping protocols to the new participating laboratories. Each of the laboratories responsible for genotyping will type all of the breeds for different sets of microsatellites, to eliminate the problem of genotyping variation between laboratories (as recommended by PigBioDiv1). Based on the results from PigBioDiv1 and those of the pilot study microsatellite markers will be evaluated on and selected for robustness and reliability (ease of amplification and typing, together with ease of scoring). Mutation rate at individual loci also impacts on the degree of usefulness of a marker for determining population relationships. If the mutation rate is high then this causes loss of relationship information since any degree of similarity between alleles has been lost. Markers will also, therefore, be evaluated on their informativity for determining breed relationships. In addition to genotyping individual animals some bulked genotyping will also be undertaken. The new project provides

potentially for a wider range of alleles which may make this an interesting approach. The lower potential cost is also of significant interest for diversity studies in general, although the first project established that there are still some problems with data interpretation that require further exploration (Groenen, 2003).

Initial estimates of genetic distance will be based on the measures used in the PigBioDiv1 project (Nei, 1972; Reynolds *et al.*, 1983). In addition, further estimates of genetic distance will be obtained that have been corrected for genetic drift (Relethford, 1996; Gaggiotti and Excoffier, 2000). Drift can artificially inflate the estimate of genetic distance obtained making a breed appear genetically unique when in fact the difference is due to the breed being less genetically variable than others. Drift is also a factor that must be accounted for when selecting breeds for conservation based on measures such as the Weitzman diversity statistic (Thaon d'Arnoldi *et al.*, 1998). This measure seems to have a low correlation with within-population allelic richness, i.e. the more homozygous breeds made a greater contribution to the diversity statistic (SanCristobal *et al.*, 2002, SanCristobal *et al.*, 2003). Further research is warranted, to explore the impact of drift on diversity measures and to directly compare these general measures of genetic diversity with information derived from specific genomic regions known to contribute to phenotypic differences among breeds. This new project provides an excellent opportunity to further investigate this issue.

MITOCHONDRIAL DNA DIVERSITY

Mitochondrial DNA is maternally inherited and multiple copies (100-1000) are present in each cell. This makes it particularly useful for tracing the maternal lineages in populations. Giuffra *et al.* (2000) and Kijas and Andersson (2001) have shown that distinct European and Asian mtDNA clades exist in domestic pigs, reflecting that pigs have been domesticated both from European and Asian Wild Boar subspecies. Furthermore, the presence of Asian mtDNA types in some European domestic pigs most likely reflects introgression of Asian germplasm into Europe during the 17th and 18th century. The average sequence difference between the European and Asian mtDNA (based on the near complete sequence) is 1.2 percent (Kijas and Andersson, 2001). Analysing mtDNA variation together with Y-chromosome diversity will allow the origin of 50 European and 50 Chinese breeds to be traced as well as female- versus male-mediated introgression to be assessed.

Four variable positions in the cytochrome B gene will be scored which allows the major mtDNA clades to be distinguished. This will be carried out on 40 animals from each of 50 European and 50 Chinese breeds. In addition, a part of the hypervariable D-loop from 400 individuals will be sequenced to identify novel variants. A comprehensive phylogenetic tree will be constructed on the basis of these sequence data.

Y-CHROMOSOME DIVERSITY

The mammalian Y chromosome

consists of a euchromatic non-recombining region (NRY), which makes up 95 percent of its length, flanked by two pseudoautosomal regions (Cooke *et al.*, 1985). Aside from the absence of recombination, NRY differs from all other nuclear chromosomes by its male specificity, its common ancestry and persistent meiotic relationship with the X chromosome, and the tendency of its genes to degenerate during evolution. The NRY contains genes which have been shown to be involved in male sex determination (*SRY*) (Sinclair *et al.*, 1990), spermatogenesis and determination of stature (Lahn and Page, 1997). Considerable nucleotide variation has been found throughout the NRY in humans. Biallelic variation (SNPs) has been described in the human *SRY* region (Whitfield *et al.*, 1995), the *RPS4Y* (Bergen *et al.*, 1999), *DFFRY* (Sun *et al.*, 1999), *DBY*, *SMCY* and *UTY1* genes, as well as other sites dispersed throughout the NRY region (Vollrath *et al.*, 1992). In human populations these polymorphic genes have been shown to be evolutionarily neutral (have not been subject to significant selection) and hence provide a valuable system for the reconstruction of the history of the species. In contrast, little is known about the pig NRY as very few genes or markers in this region of the pig genome have been identified until recently (Quilter *et al.*, 2002). As part of the PigBioDiv2 project the pig NRY will be screened for polymorphisms and an assessment made of whether genes in this region can be regarded as evolutionarily neutral in the pig. The pattern of diversity observed on the pig Y chromosome could have arisen as a result of drift

(limited effective population size), and hence be neutral, or may be the result of selection. The diversity observed will allow these hypotheses to be tested using approaches such as coalescent simulation. Simulations could be constructed to emulate, for example, a selective sweep and the diversity expected under this model is then compared to that observed. In addition, the genealogical relationship among Y chromosome haplotypes both within- and between- breeds will also be constructed. As with the mtDNA this will elucidate breed histories and origins and will also allow the extent of male-mediated introgression to be determined.

DIVERSITY AND PHENOTYPIC VARIATION

Breeds of livestock are genetically distinct for physical characteristics, such as coat colour, performance and other commercially important traits (e.g. product quality, growth efficiency and disease resistance). These characteristics make different breeds of livestock valuable in their own right and also for the particular alleles they may possess. However, the genes and alleles that underlie phenotypic differences are for the most part unknown. Some progress has been made in the identification of genes controlling colour variation and QTL mapping studies are beginning to locate genomic regions associated with breed differences in performance and other traits. This may ultimately lead to the identification of the genes of value. However, mapping studies require the production of F2 crosses between pairs of breeds,

followed by the measurement of the F2 population for the traits of interest and genotyping of markers across the genome. It is, therefore, not practical to use the mapping approach to assess the genes controlling differences for more than a few pairs of breeds.

In most diversity studies genetic markers are used to assess overall genetic differences among breeds. The results of such studies can be used to determine between and within breed variation, elucidate the relationships between breeds and identify the genetically most distinct breeds and so may contribute to decisions on the allocation of resources to breed conservation. However, these studies do not presently identify the particular genomic regions that contribute to the differentiation between breeds at the phenotypic level. Hence they cannot be used to identify the genes that make breeds distinct and which may be of value in breeding programmes. The PigBioDiv2 project will focus on making a link between diversity and discovery of functional variation by 1) utilising existing type I markers associated with variation in phenotypic traits to characterise the breeds and 2) by developing new tools that can help identify genomic regions that contribute to a breed's unique characteristics without the need to measure and genotype large numbers of animals in a mapping experiment.

TRAIT GENES (TYPE I MARKERS) FOR CHARACTERISATION OF BIODIVERSITY

Variation (polymorphism) in several specific genes has now been shown to

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explain phenotypic variation in pigs. These include the well known examples of *KIT* and *MC1R* for coat colour (Kijas *et al.*, 2001, Kijas *et al.*, 1998; Marklund *et al.*, 1998), *CRC1* (Halo-thane) and *PRKAG3* (*RN*) for meat quality (Fujii *et al.*, 1991; Milan *et al.*, 2000; Ciobanu *et al.*, 2001b), *MC4R* for appetite and growth (Kim *et al.*, 2000), *ESR* for litter size (Short *et al.*, 1997) and *FUT1* for disease resistance (Meijerink *et al.*, 2000, Frydendahl *et al.*, 2003). It has been proposed that the addition of such markers is a useful complement to the study of diversity using anonymous markers (such as microsatellites) as the variation in allele frequency may provide information related to functional differences between breeds (Ciobanu *et al.*, 2001a). This type of marker is thus most likely to represent genes that are breed-differentiating. Introgression and past migrations between populations can mean that phylogenies derived from other markers do not reflect true population history. In contrast, phylogenies obtained from breed-differentiating genes, e.g. coat colour genes, will reflect true population history because they will not be affected by introgression events, since positive selection for the locus will have ensured that particular alleles remained fixed within breeds (Ting *et al.*, 2000). Phylogenies derived from these genes will be valuable points of reference in assessing the utility of other marker types to determine breed histories. The comparison of results obtained from different marker types and the evaluation of correlations among markers will form an important component of the PigBioDiv2 project,

with a major objective being the evaluation of diversity measures as valid or useful measures of functional genetic differences among breeds.

For PigBioDiv2 thirty type I markers located throughout the genome will be selected for genotyping on 50 breeds (25 European and 25 Chinese). This will enable the direct comparison of diversity measures obtained from anonymous microsatellite markers with those obtained from coding (type I) loci. The markers will be selected based on their known or predicted impact on traits that are of interest in Chinese breeds such as prolificacy, growth and fatness, disease resistance, meat quality (including marbling) and coat colour.

GENOMIC REGIONS CONTAINING GENES RESPONSIBLE FOR FUNCTIONAL VARIATION

PigBioDiv 2 will also help to advance the utility of diversity studies by developing a tool that can identify genomic regions that contribute to a breed's unique characteristics without the need to measure and genotype large numbers of animals in a mapping experiment. The potential to do this arises because current breeds have been shaped by past selection. Where genes of moderate or large effect (i.e. those that could be detected in a mapping study) have been fixed in the process of breed selection, they will leave the trace of this past selection in the genome (Kohn *et al.*, 2000). Thus if genetic analysis can be used to identify these *footprints* of selection, it will point to the regions of the genome that are important in shaping breed charac-

teristics. Where selection on an allele has occurred, it will have moved an initially rare allele (originally there may have been only a single allele, representing the founding mutation) through the population to fixation. This allele will carry with it a section of chromosome carrying alleles that are in linkage disequilibrium. (In the extreme case, selection will drag with it a section of the gamete in which the original mutation occurred). This can result in a section of chromosome in which there is little variation within a breed, although if other breeds are fixed for alternative alleles, there may be substantial differences between breeds. Where breeds have not been affected by selection in a particular region, variation will not have been reduced other than by the effects of genetic drift. Thus if genetic variation around a gene, where selection has contributed to breed characteristics, is examined there should be reducing variation within breed, possibly accompanied by increasing variation between breed, as the gene of interest is approached. Consequently, if regions showing this pattern can be identified, they could point to genes of importance.

The PigBioDiv2 project will focus on demonstrating the feasibility of such an approach with pig breeds. The objective will be to focus on genomic regions where selection is known to have taken place in order to characterise the size of the resulting footprint, the density of markers required to identify the footprint and to establish the analysis methodology required to identify the footprint. Four genomic regions will be studied: a region around the *KIT* locus, on chromosome 8, and QTL regions that have been identified

on chromosomes 2, 4 and 7. The *KIT* locus has been identified as responsible for colour variation in pigs, in particular harbouring the dominant white (in Large White and Landrace breeds) and belted alleles (in Hampshire), with the wild-type allele present in coloured pigs (such as the Duroc and Large Black). The mutations responsible for these different alleles have been characterised in European breeds and the results obtained to date show that it is likely that the individual mutations producing the white and belted phenotypes have only occurred once. Comparisons will be made with Chinese white, spotted, belted and black pig breeds. It is likely that there would be three major clades, representing the white, belted and coloured phenotypes. Major differentiation between the clades would be expected at and around the *KIT* locus, with limited variation at this locus within the white and belted clades (representing descendants of a single allele) and more variation in the coloured clade, which should represent a sample of the variation in the original wild boar population. The QTL regions on chromosomes 2, 4 and 7 are associated with performance traits, such as growth and fatness, and have been confirmed in many studies including those utilising the Chinese Meishan breed (De Koning *et al.*, 1999; Walling *et al.*, 2000; Amarger *et al.*, 2002).

Ultimately the methods developed may be applied to scan the complete genomes of a series of diverse breeds. The anticipated development of large numbers of SNP markers in livestock together with methods to rapidly genotype large numbers of such markers will allow the approach to be

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applied on a large scale. This will allow large areas of the genome to be scanned for novel *footprints* representing previously unknown genes of importance and to assess breeds for the novel alleles they may contain.

CONCLUSIONS

The PigBioDiv2 project will expand on the first EC pig biodiversity project (PigBioDiv1) both geographically and technically. By adding 50 Chinese breeds to the previously sampled 59 European breeds the project will provide a wider global perspective on pig biodiversity. As a result the European pig biodiversity database will be greatly enhanced. New molecular

markers (e.g. mtDNA and Y-chromosome polymorphisms) will provide fresh insights into European and Chinese pig breed histories, and comparisons between different marker types will shed light on how population demography, as well as evolutionary forces such as selection, have affected different regions of the genome. A major focus of the PigBioDiv2 project will be on assessing functional differences among breeds, based either on individual trait loci or by scanning regions of the genome containing genes that are likely to have been under selection. If successful, this would be a significant step forward in guiding diversity studies towards new criteria and measurements on which livestock conservation decisions should be based.

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