Practical applications of molecular research on pigs

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What type of molecular technology?

Molecular technology can be used as an adjunct to conventional breeding programmes. The idea is that naturally occurring variation in DNA sequences (markers) can be detected in potential breeders. If this DNA variation has previously been correlated with variation in an economically important trait, such as growth or meat quality or susceptibility to disease, it can be used to assist or accelerate the choice or rejection of animals as parents. In some cases, this will merely slightly speed up the rate of improvement for traits which are already easy to select, like backfat. In other cases, it may make it possible to make early decisions on the choice of parents for difficult-to-measure but important traits, like meat quality, which can only be measured post-slaughter. This process has been termed marker assisted selection (MAS) and it is the underlying rationale for the type of molecular study in pigs which will be described.

It is also possible to directly alter the genetic make-up of pigs by transgenesis. Although there are important biomedical applications of transgenesis in pigs, for example for xenotransplantation research, there is not a single example, in pigs or any other domestic mammal or bird, where transgenesis has improved the productivity of animals in a commercial context. It is likely to be many years before this type of molecular intervention has any impact on commercial production in pigs.

Uses of this molecular technology

So what are the main applications, real or potential, of molecular marker technology in pig breeding? The diagnosis of inherited disease or susceptibility to disease is an important application where pig breeders and pig geneticists have been at the forefront of progress.

The ability to verify the parentage of animals is vitally important in animal breeding and molecular technology has now provided us with the ability to do this with great accuracy. With costs of parentage testing decreasing and the efficiency of detection of errors now very close to 100%, there may be a greater place for routine parentage testing in pig breeding. Errors can easily occur even in well managed systems and it will be important to detect and remediate these errors to get the full benefit of systems like PigBLUP, where performance information from relatives is important in estimating breeding values.

The conservation of genetic resources is an increasingly important issue to animal breeders and the general community alike. Concentration on a narrow spectrum of high performance breeds has led to the abandonment of lower performing breeds at a rate which is ringing alarm bells internationally. Molecular marker technology is now for the first time allowing us to independently and rigorously measure the diversity within

and between breeds and permitting the development of action plans which will hopefully permit the conservation of the greatest level of diversity for the least cost.

Molecular markers also permit the development of detailed genetic maps. These are of great intrinsic interest to geneticists as genetic maps open up new breeding opportunities previously unavailable. Practically genetic maps permit us for the first time to recognise at least some of the genes responsible for the variation in performance between animals. Such quantitative trait loci (QTL) studies in pigs are still in their infancy but there is promise in the early findings.

Diagnosis of genetic disease

The majority of genetic disorders that occur in animal (and human) populations are recessive. This means that animals with two copies of the defective gene can easily be recognised and prevented from breeding, if they are capable of breeding in the first place. However preventing these animals from breeding has little influence on the overall incidence of the genetic disease in successive generations. What is required is a method for detecting carriers (heterozygotes), since most or all affected progeny will be the result of carrier by carrier matings. These carriers can only be recognised by the production of an affected offspring (hardly an efficient mechanism) or by the recognition of the defect in the animals DNA. In this case, all that is required is a samples of the animals DNA from as little as a single hair, application of the molecular test and a decision can then be made whether to use the animal for breeding or not.

Additionally some genes have been recognised which confer resistance to infectious disease. *E coli F18 resistance* (ECF18R) has long been recognised as inherited but could only be diagnosed using complicated assays to test whether the bacteria bound to the gut lining of the pigs. Recently a Swiss group has identified the gene in pigs which appears to confer resistance. The porcine fucosyl transferase 1 (FUT1) gene, (originally cloned by a medical research group in Melbourne), causes a sugar to be attached to the surface of cells in the gut to which the bacteria bind. A non-functional form of the gene which does not allow attachment of this sugar provides resistance. Now it is possible to accurately identify animals which carry the resistance alleles using a DNA test.

Controlling inherited disorders

The groundbreaking application of molecular genotyping to control genetic disease in animals is provided by the *hal* gene in pigs. The defective n allele at this locus is responsible for a syndrome of abnormalities in homozygous animals, including an abnormal reaction to halothane anaesthesia, with muscle stiffening and an abnormal and frequently fatal increase in body temperature. These signs could also be induced by stress. However, of far greater commercial significance is the severe effect on meat quality, termed PSE (pale, soft and exudative) pork. The n allele reached quite high frequency in many breeds apparently because of a favourable effect on backfat thickness.

In the early 1990, a Canadian group cloned the gene involved and identified the single nucleotide change in the genetic code for this gene which was responsible for the entire syndrome of effects. After this it was simple matter to devise a simple genotyping

assay. With payment of very substantial royalties to the Canadian discovers and also to the company holding the rights to the PCR process, detection of nn and Nn pigs became a simple matter.

Hal testing in Australia

A group in the Queensland Department of Primary Industry gained a licence to provide this test and were so successful in identifying the unwanted n allele that after about four years, there was so little demand for testing that they relinquished their licence. The experience overseas has been similar and the *hal* gene went from being the single most important genetic defect in pigs to being of negligible concern. Breeders could either get rid of it altogether or sequester it within lines where it was deliberately maintained.

Inherited resistance to disease

Coincidentally the *hal* gene and the ECF18R gene are located very close to each other on pig chromosome 6. The favourable resistance allele at ECF18R is found with the unfavourable *hal* n allele in about 93% of cases. This means that elimination of the *hal* n allele will also have caused a strong reduction in the frequency of the favourable resistance allele. Since it is now possible to identify the favourable resistance allele by a DNA test, it may be desirable to check for its presence in breeding animals and to attempt deliberately to increase its frequency. However the importance in Australia of *E. coli* disease due to the F18 strain is not known and without this information, it is not possible to make a clear recommendation to use the test. The Swiss group are in the process of patenting their discovery so the test would be available under licence and subject to royalty payments.

Parentage verification

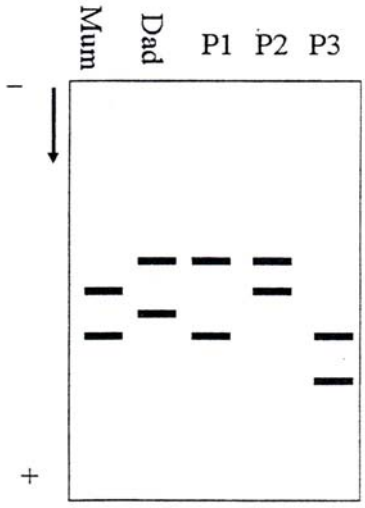
Another extremely important application of molecular marker technology is in parentage testing. Even in the best run breeding system (and even in humans), an error rate of about 10% or more is likely. This usually involves misidentification of sires. However recording errors can result in misidentification of dams and in some cases even of both parents. For example if a piglet were to change pens before it was earmarked, then the identity of both parents would be incorrectly recorded. In the past, blood group variation was used as a rather inefficient tool to detect pedigree inconsistencies. Now there is a new type of molecular marker, called a microsatellite, which allows virtually certain recognition of pedigree errors and in many cases will actually allow retrospective identification of the correct parents.

Microsatellites consist of short repeats of letters in the genetic code, such as CACACA, which range in repetition from about 10 to 50 or more. There are countless sites of these microsatellites in the chromosomes of an animal, with at least 60,000 sites of the CA repeat alone in pigs. Their most important feature is that the repeat number varies between animals. The microsatellite sites are highly variable (hyperpolymorphic) but inherited according to normal Mendelian genetic rules. With as few as 10 highly variable microsatellite markers, it is possible to recognise all pedigree errors. The cost of this type of pedigree checking is likely to continue to go down and in the future, it is probable that it will be routinely applied even in pig breeding, as well as sheep and

cattle breeding, where there is strong demand due to the difficulty of supervising or otherwise monitoring matings.

Microsatellites are detected as bands of different size which are separated in a high resolution gel by electrophoresis. In parentage testing, an allele (band) present in an offspring which is not present in a parent indicates a pedigree error. In Figure 1 below, the first two progeny (P1 and P2) have bands consistent with the designated parents. However P3 has one band which it could have inherited from its mother, but it could not have inherited the front running band from its father. This would be flagged as a paternity error, although there are other possible interpretations, for example if you had reason to suspect that piglets had escaped between pens.

Figure 1. - Parentage Testing



More recent technology for the analysis of microsatellites employs dye-labelling. In the system we use at the University of Sydney (an ABI 373), four dye colours are available. The red colour is reserved for the size standard, which permits the accurate measurement of the size of microsatellite alleles down to single nucleotide resolution. The three other colours, blue, green and yellow, are available for labelling the microsatellites. Software is available for automatically interpreting the output from this machine and storing the data for subsequent analysis. In our gene mapping work, we are analysing up to 100 or so loci for 500-600 animals, so keeping track of all the information becomes difficult, even with systems which automatically log and assist in interpreting the data.

We have successfully used porcine microsatellites for parentage testing in a collaborative project with Westmead Hospital, where we are assisting a transplantation research group is making an inbred line of pigs by successive generations of brother-sister mating. At one stage in the breeding programme, a gilt was kept in a pen with two of her littermate brothers (who were believed to be sexually immature). A deliberate mating was set up with one of these males, but it was suspected that the

other male had also mated with her. We were able to prove that this was so and to assign unambiguously the individual piglets from her first litter to the respective sires.

Determining breed relationships (biodiversity)

Conservation of domestic animal genetic resources is becoming an increasingly important issue. A major programme of the FAO is MoDAD (Measurement of Domestic Animal Diversity), which has the objective of determining breed relationships as well as assessing intrabreed variability for all major species of domestic animals. For the pig, both the International Society of Animal Genetics and the PiGMaP consortium have assisted by developing a panel of microsatellite markers to be genotyped on samples of all breeds. There are currently 27 microsatellites in this panel but at the recent ISAG meeting in Auckland, it was decided to increase this number to 55. Ideally a sample of 25 unrelated males and 25 unrelated females should be genotyped for each breed, but for many of the rare breeds, this is impossible.

During a stay in my laboratory in 1997, Professor Kui Li, from Huazhong Agricultural University in China, genotyped these 27 microsatelites on samples of 8 Chinese breeds. Although the samples from some of the breeds were very small and the results are accordingly unreliable for those breeds, it was nevertheless possible to get some feel for the relationships of the different breeds. We also have data on Australian commercial pigs for comparison with the Chinese breeds but have not yet completed the analyses.

Breed relationship (phylogeny) information can be used in various ways. For example, if a rare and endangered breed is shown to be closely related to a common breed, then it may be logical to abandon the rare breed or merge it with the common breed. If there are several rare breeds which are shown to be closely related, it may be most logical to merge them and manage them as a single population. On the other hand, if there is a rare breed which is very different from any other breed, then conservation resources should target that breed.

Constructing genetic maps

The most important practical application of molecular markers is in gene mapping and particularly in their use for detection/mapping genes affecting economically important traits.

Any genetic map must refer back to the chromosomes where the genes reside. Initially chromosomes were stained with dyes which coloured them uniformly along their length. With these techniques, it was possible to count the chromosomes (there are 38 in the pig) and to get some idea of the variation in their shapes, but it was not possible to reliably identify many individual chromosomes. We had the beginnings of a map, but it was a bit like making a map of the moon using your bare eyes for observation.

In the mid 1970s, new staining techniques were developed which caused distinguishable and repeatable patterns of light and dark bands to appear on the chromosomes. This finally permitted the unambiguous assignment of chromosomes to their individual partners and the development of a reliable system of chromosome identification. Even so, the standard karyotype for the pig was only established as

recently as 1988 (Gustavsson, I. (1988). Standard karyotype of the domestic pig. Committee for the Standardized Karyotype of the Domestic Pig. Hereditas 109, 151-157). To continue with our moon mapping analogy, we now had the benefit of a small telescope and could recognise the really big features but none of the detail.

The standardised karyotype could be converted into the first outline map, namely an ideogram, where only the landmark bands featured. The next job was to add features to this map and the important features we wanted to add were genes and other markers

Physical mapping

One very important technique for adding features to the chromosomal map is called in situ hybridisation. Here a specific cloned or amplified fragment of DNA, say from a gene of interest, is labelled and added to a chromosome preparation on slide. The labelled DNA is able to seek out and bind to the corresponding DNA in the chromosome and the position of the clone/gene can then be identified by the position of the label. Fluorescent labels are now generally used which can be directly visualised down the microscope, where the bright yellow label can be seen against the red stained chromosomes. By restaining the chromosomes later to bring out the bands and rephotographing them, it is possible to relate the position of the label to band positions on the ideogram. This is a very useful technique but the resolution is still not high. We can now put new features on our moon map, but the accuracy with which we map them is still very limited.

Markers and genes which have been physically mapped on porcine chromosome 2 illustrate the outcome of FISH (fluorescent in situ hybridisation). At best, the marker is mapped to a single band or interband and at worst, it is merely assigned to the chromosome. There are now many hundreds of genes which have been physically mapped in the pig using FISH or alternative methods.

Another way to add detail to the physical map of the pig is to relate it to the maps of other species. It has been possible for some time to obtain purified preparations of individual human chromosomes. Each individual chromosome can then be labelled with a fluorescent dye and hybridised to a preparation of porcine chromosomes. This ZOO-FISH technique permits recognition of the equivalent parts of chromosomes between species. More recently purified porcine chromosomes have become available and the technique can be applied in the reverse direction. For pig and human, the overall pattern of "relationship" between all chromosomes has been recognised.

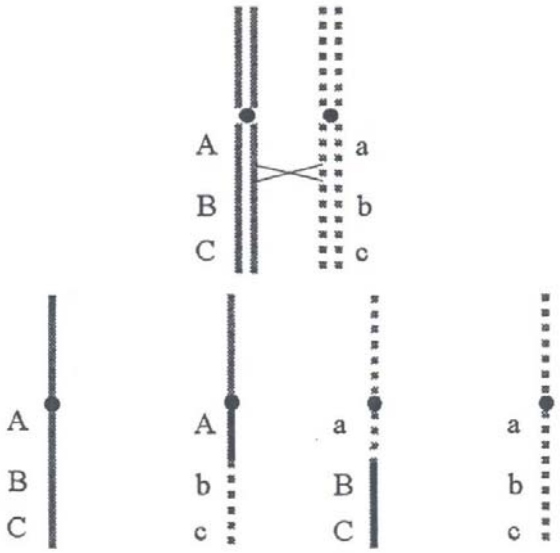
Very interesting you might say, but what is the use of this? Sometime in the next five years, the complete sequence of the human genome will become available. When this task is complete, it will be possible to recognise all 60,000 to 70,000 genes in a standard mammalian genome, the majority of them currently undiscovered. Many of these genes will be very relevant to animals and it would be of great interest to have at least some idea of where the equivalent genes reside in animal species. Our comparative map will allow for the immediate transfer of this information between information rich species like humans and information poor species like the pig. For example, let us say that we can map a gene which has an important and large effect on fat % to chromosome 2. We could immediately search among the human genes from the equivalent region to try to find likely candidates which could be responsible for the

effect - any gene involved in fat metabolism or storage, for example. We could thus identify "comparative positional candidates" and more quickly home in on the gene of interest.

Chromosome recombination due to meiotic crossover

Linkage mapping is quite different from physical mapping although the ends result will be ordered positions of markers on chromosome maps. However the relative spacings between markers will differ between the two types of maps, since in linkage maps probability of recombination rather than physical distance determines the spacing.

Figure 2. - Chromosome recombination due to meiotic cross-ver



To make a linkage map, we must have families (two or preferably three generation pedigrees), in which we detect the products of crossing-over which occur during meiosis (the important process leading to formation of eggs or sperm). Crossing over leads to recombination, as illustrated above in Figure 2. Genes which are close together on a chromosome are unlikely to have a crossover between them and alleles at the different will co-segregate (stay together), whereas genes which are far apart are very likely to have a crossover and the alleles at the two loci will be randomised. By working out the proportion of recombinants in a group of progeny, the recombination frequency can be determined and this will then allow ordering of the gene along the chromosome.

Consequences of Recombination

By systematically looking at pairwise combinations of genes, it is possible to allocate genes to chromosomes (linkage groups). Using a slightly more sophisticated technique called multipoint mapping, the order of genes can be worked out. In some cases, there is insufficient information available to unambiguously order all genes on a chromosome and it would be uneconomic to breed pedigrees large enough to obtain the necessary information, so only the best guess order is presented for some genes, particularly genes which are very close together.

This process of mapping has been used by research groups in Europe (the PiGMaP consortium with which my lab is affiliated) and the United States (the USDA) and as a result the linkage map of the pig now has several thousand highly informative markers on it. The human and mouse maps have over 6,000 markers each.

Mapping quantitative trait loci

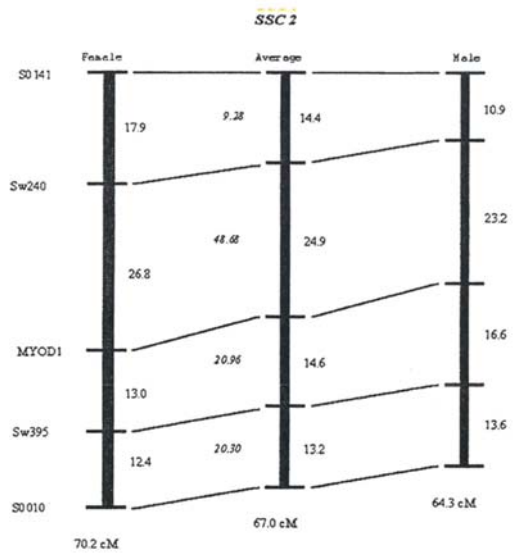
The main practical reason for constructing linkage maps is to detect genes which cause variation in economically important quantitative traits (QTL). In general, it is impossible to recognise QTL on their own and they can only be detected in relation to closely linked genetic markers with which they co-segregate. Presented are some early results from a study performed by Seung Soo Lee as part of a collaboration between my laboratory and the University of Hohenheim in Germany. Dr Gerhard Moser from Hohenheim is responsible for the QTL analyses to be presented here.

The German group has bred a three generation pedigree consisting of all pairwise combinations of Pietrain, Wild Boar and Meishan, with over 300 F2 progeny being produced from each of the three crosses. The F2 progeny have been measured for 43 performance traits. My laboratory has agreed to genotype the pedigree for markers on chromosomes 2 and 5, with laboratories in Germany and the Czech Republic doing the other chromosomes.

For the Pietrain by Meishan pedigree, four microsatellites have been genotyped by Mr Lee and the MyoDI locus has been genotyped by the Czech group and the data have been analysed. Work is still under way for the other families.

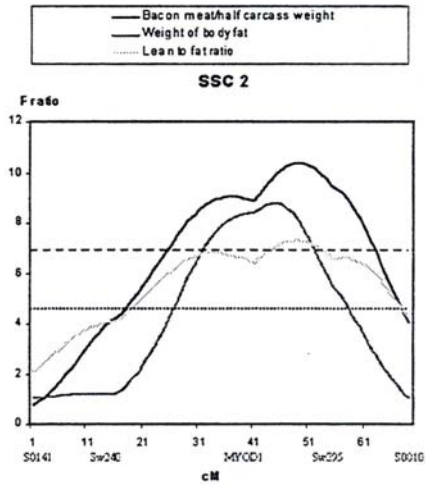
The linkage map shown below in Figure 3, covers about half of chromosome 2. It is worth highlighting some interesting features. First the female map is longer than the male map. This is true for most chromosomes in the pig and for most species of mammal. The frequency and distribution of crossovers is different between the sexes, generally being higher in females and also tending to be concentrated towards the ends of chromosomes. Secondly the order of these markers is unambiguously established in this map with any other order being extremely unlikely.

Figure 3. - Linkage Map of a region of porcine chromosome 2



The next step in QTL mapping is to "scan" across the chromosome looking for points where the data provide evidence for the existence of a QTL. Certain statistics are calculated for each point in this scan and if this statistic exceeds a threshold, then it provides evidence for the presence of a QTL. The simplest way of presenting this information is as a plot along the chromosome map (Figure 4). The highest point in the profile which exceeds the significance threshold is taken to be the most likely position of the QTL. It is possible to choose extremely stringent thresholds (genome wide threshold - higher dashed line) or less stringent thresholds (chromosome wide threshold - lower dotted line) depending on whether you want to be more certain of the existence of the QTL or whether you want to avoid missing the detection of QTL, perhaps due to their smaller effect.

Figure 4. - Plot for evidence for a QTL



In any case, this scan of only about half of chromosome 2 detected 12 potential QTL, half of which exceeded the stringent genome wide significance test. Eight of the performance measures are related to fatness or leanness measures and may be detecting the same QTL. However three other traits, teat number, liver weight and early growth are unrelated traits and the QTL appear to map to different positions from the fatness QTL.

As can be seen, the position of the QTL is not accurately mapped. There is generally a quite broad region of the chromosome in which the QTL could lie. This is a chronic problem of QTL mapping which has troubled workers in all species. Breeding larger F2 families or adding more markers to the map does not provide substantial improvements in accuracy, as there is a rapidly diminishing return on genotyping effort. Some research groups, such as the Swedish group which discovered the QTL for backfat on chromosome 4 in the pig, are breeding deeper pedigrees, extending for 5-6 generations. This approach permits a gradual paring away at the edges of the QTL map and a refinement of QTL position.

For marker assisted selection, extremely accurate estimation of the QTL position is not necessary. Although it would be ideal to actually identify the gene responsible for the

QTL and to be able to genotype the allelic variants causing the performance effect, a bracket of markers whose relationship to the QTL has been established, will be very beneficial. Research is about to begin in Prof Mike Goddard's laboratory in Melbourne to determine just how big the QTL effects should be, how close the markers and the cost of genotyping, performance testing and analysis for MAS to provide an overall economic benefit for the pig industry. I am confident that these studies will find a benefit in the application of molecular marker technology in pig breeding and hopefully it will not be too many years before PigBLUP will be assisting pig breeders in reaping the rewards.

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