Applied genomics: The Brazilian experience

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Introduction

Embrapa Swine and Poultry Research Center and ESALQ/USP have started the Poultry Genomic s_tudies in Brazil in 1999, with the objective of mapping QTL for traits of economic importance for the poultry industry. The projects in this area led to the establishment of the Brazilian Poultry Genomics Network, which adds efforts of EMBRAPA and Universities, in alignment with the Industry directions. Besides the QTL mapping research, candidate gene and gene expression studies have also been conducted. The integration of these results will contribute to the identification of genes responsible for the control of complex traits and its use in poultry breeding programs, aiming at the improvement of production efficiency and product quality. With the development of this research, Embrapa Swine and Poultry and its partners have been effectively engaged to form a critical mass in strategic areas for the Brazilian agribusiness and to contribute to the advance of our knowledge in poultry science. In a previous review, Jorge *et al*. (2007) reported the Brazilian initiatives in chicken genomics and its contribution to the field, from biological model to export commodity. Here, we report our trajectory in Poultry Genomics and the main results achieved by the National Poultry Genomics Network to date.

Experimental populations

The genetic stock from the EMBRAPA Poultry Breeding Program has been maintained as an *in Situ* Conservation Nucleus since 2000. This flock comprehends meat type chickens (male and female lines) and laying hens (white and brown eggs), and it is kept under multi-trait selection and rigorous sanitary control and biosecurity. This genetic material allows the generation of the industrial and colonial products of EMBRAPA. Two control lines are also maintained without selection for research purposes. This genetic stock is free from salmonella, mycoplasma and it is monitored for the main avian diseases in a rigorous biosecurity program. The high sanitary status and the variability of the pure lines make this genetic material unique in Brazil. Pure lines are not available in the market, so Embrapa Swine and Poultry is the only Brazilian official Institute able to provide this kind of genetic stock for research purposes.

In 2008, the Conservation Nucleus integrated the Animal Genetic Resources Network. This network aims to genetically and phenotypically characterize genetic resources (in our case, chickens for egg and meat production) to assist the maintenance of the maximum genetic variability and the conservation of these populations as a reservoir to search for favorable alleles related to productive traits.

The Conservation Nucleus provided the genetic stocks for the poultry genomic studies carried out in Brazil. This genetic material was used to create two reference populations: **1)** the EMBRAPA F2 Chicken Resource Population, derived from the cross of a broiler (TT) and a layer (CC) line, and was used to map important QTL regions in the chicken genome, to identify potential candidate genes and potential markers associated to productive traits under Brazilian climatic conditions and production practices (Ledur *et al*., 2000b) and **2)** the TT Reference Population, formed from the expansion of the paternal broiler pure line TT, was developed to validate potential markers uncovered in the previous resource population and for gene discovery (Peixoto *et al*., 2011). Furthermore, those genetic stocks will serve as a model for incorporating genomic information into the EMBRAPA Poultry Breeding Program and to analyze its impact in the genetic

gain of economically important traits. This genetic material is also important for other investigations in genetics, such as disease resistance, animal behavior and welfare. As these genetic resources will continue to play an important role in the present and future research in Brazil, the future directions are the maintenance of the high sanitary status and the increase of actions for conservation and characterization of those lines.

Reference populations:

1) EMBRAPA F2 Chicken Resource Population: this segregating population was derived from crosses between a broiler and a layer line. The TT broiler line is a paternal line, selected since 1992 for body weight, feed conversion, carcass and yield parts, chick viability, fertility, hatchability of fertile eggs, reduced abdominal fat and metabolic disorders (Figueiredo *et al*., 2003a). The CC layer line has been selected since 1989 for egg production, egg weight, feed conversion, chick viability, sexual maturity, fertility, hatchability of fertile eggs, egg quality and decreased body weight (Figueiredo *et al*., 2003b). The TT body weight (2,395g) exceeded in almost five-fold the CC body weight (513g) when reared as broilers. The breast yield for TT was 20.4% while for CC was 14.2%, and abdominal fat percentage related to body weight was 2.41% for TT and 0.16% for CC (Ledur *et al*., 2000a, b). Divergence between TT and CC growth and carcass traits makes this F2 resource population powerful for QTL mapping in poultry. The resource population was created from reciprocal crosses between both lines, in the proportion of one male for each female. The F1 generation was obtained from crosses between seven males and seven females from each line, resulting in seven families from crosses between broiler males and layer females (TC), and seven families from reciprocal crosses between layer males and broiler females (CT). The F2 generation was derived from crosses between one male and three females from different F1 families, by randomly mating non-related animals. A total of seven males and 21 F1 females from each cross (CT and TC) generated around 100 F2 chickens per F1 family, in 17 hatches, in a total of approximately 4,000 F2 chickens, being half of each sex and cross (TC and CT). About 51 phenotypic traits, mainly associated with performance (body weight at 1, 35, 41 and 42 days of age, feed consumption and feed efficiency), carcass yield (breast, drums and thighs, and wings weight), carcass chemical composition (water, crude protein, fat and ash contents) and physiology (organs weight, triglyceride and cholesterol levels) were evaluated and recorded in the F2 chickens (Nones *et al*., 2006; Jorge *et al*., 2007).

2) TT Reference Population: this population was generated by the expansion of the paternal TT broiler line, developed by the EMBRAPA Poultry Breeding Program, to validate potential genetic markers and for gene discovery. Twenty males were mated to 92 females (1:5) to produce 1600 day-old chicks from 5 hatches, half of each sex. For phenotypic evaluation, chickens were kept in collective pens till 35 days of age and then housed in individual cages for feed conversion evaluation. At 42 days of age, 1465 chickens were slaughtered and evaluated for approximately 60 traits: performance (birth weight and weights at 21, 35, 41 and 42 days of age), carcass and cuts (body weight without feathers and blood, carcass weight, abdominal fat weight, weights of head, feet, neck, wings, middle joint wings, wing sticks, drumstick, drumstick muscle, thigh, thigh muscle, breast, breast muscle, breast fillet and back weight), internal organs (heart, gizzard, liver and lungs weight), skin (drumstick, thigh and breast skin weight), and bone integrity traits (tibia, femur and breast bone weight). Feed intake, weight gain and feed conversion from 35 to 41 days were also evaluated, as well as the yield of carcass cuts and percentage of bones related to body weight. The tibia and femur bones were collected for posterior analysis of bone integrity related traits (weight, length, width, strength, ash, Ca and P). The TT Reference Population has a DNA bank and a data base with pedigree information and phenotypic data from about 85 important traits for the poultry industry. The phenotypic observations and their standard deviations, as well as the existence of moderate heritability for the traits, suggest that there is considerable phenotypic variability among individuals of the TT Reference Population. This demonstrates that variability remains within lines, even in highly selected populations in breeding programs. The phenotypic variability in this population allowed the identification of genes and markers associated with traits of interest in poultry production, since the existence of variability indicates the segregation of alleles in populations (Peixoto *et al*., 2010a; Cruz *et al*., 2011). The allelic segregation of markers in some candidate genes was investigated in this population. The markers analyzed showed satisfactory allelic segregation among TT chickens, indicating the potential use of this population to validate results from genomics research applied to broiler production (Peixoto *et al*., 2011).

In chickens, reference populations for genetic studies are scarce. There is no other population in Brazil made up from a pure line. The data from the TT Reference Population and from the EMBRAPA

F2 Chicken Resource Population might be used by national and international institutions, through agreements with Embrapa Swine and Poultry, for QTL mapping, validation of markers and gene discovery.

QTL mapping

Using the TC cross from the EMBRAPA F2 Chicken Resource Population, linkage maps for 22 chromosomes of the chicken genome were constructed and described by Nones *et al.*, (2005) and Ambo *et al.*, (2008). QTLs for performance, carcass yield, fat, and carcass chemical composition traits were mapped in the chicken genome (Nones *et al.*, 2006; Ambo *et al.*, 2009; Campos *et al.*, 2009, Baron *et al.*, 2010 and Nones *et al.*, 2012). Novel QTLs were mapped on GGA5, GGA23 and GG27 for serum triglyceride concentration; on GGA3 for shank% and wings%, on GGA14 for carcass and breast percentages, on GGA15 for drums and thighs percentage, and on GGA 1, 10, 15 and 27 for protein, ash and water contents in the carcass (**Figure 1**). On GGA1, novel QTLs were mapped for liver, gizzard, lungs, heart and feet weight, intestine length, and feed conversion. In addition, many QTLs for body weight, abdominal fat and carcass yield were mapped using this population, confirming regions associated with QTL previously described in other populations, reinforcing possible candidate regions for further studies.

Multiple trait analysis was also used to map QTL. This methodology allowed mapping new regions of the genome associated with traits of interest, as well as discerning linked from pleiotropic QTLs, which is not possible with the single trait analysis (Pinto *et al.*, 2006a). The interaction of sex and QTL was also investigated, allowing the identification of QTLs significant only in males and others with greater effect in males than in females (Pinto *et al.*, 2006b).

The reciprocal CT cross from the EMBRAPA F2 Chicken Resource Population was created to allow the study of effects such as maternal, cytoplasmic, imprinting and those from the sexual chromosomes (Ledur *et al.*, 2000a, b). In addition, this cross is used to validate QTLs found in the TC cross and to investigate other methodologies of analysis (Rosário, 2007).

QTLs mapped in the Brazilian population such as the QTLs for feed conversion, abdominal fat, carcass protein content, and for lung and heart weights are of great interest to the poultry industry, since these traits are difficult to measure and to select independently from body weight. QTLs mapped for breast, and drums and thighs yields are also relevant due to their great economic importance. Therefore, these QTLs should be further explored to better understand their genetic correlation with other traits and in the future, assist the selection processes to produce meat more efficiently and to meet the consumers demand for leaner carcasses.

Candidate genes

The study of candidate genes is a well-known strategy to associate genes with traits of interest in animal production. Normally, genes selected for this approach are genes of known biological activity and are involved in the development or physiology of traits of interest (Bryne & McMullen, 1996). Some examples of successful implementation of this strategy can be found in Dekkers (2004).

Candidate genes were selected according to their functions described in the literature. The polymorphisms identified in those genes have been analyzed for their association with traits evaluated in

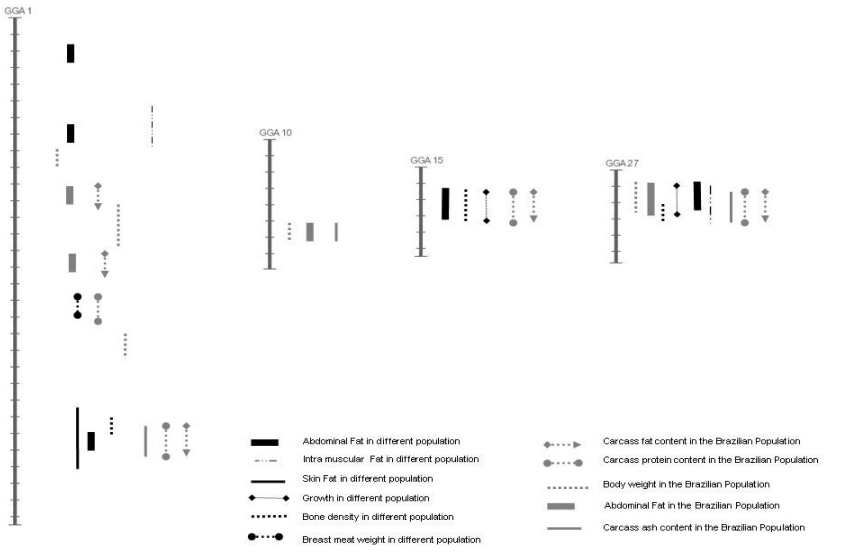

Figure 1 - QTLs associated with carcass composition traits using the EMBRAPA F2 Chicken Resource Population and other QTLs mapped in those regions (QTLdb; http://www.animalgenome.org/QTLdb/) (Nones *et al.*, 2012).

our reference populations. Gene polymorphisms and their association with various groups of traits, such as fat metabolism, muscle development and bone integrity are under investigation. In these studies, the association analyses have been performed using SAS (SAS, 2008), and lately the QxPak software (Perez-Enciso & Misztal, 2004), with a mixed model including the fixed effects of sex, hatch and SNP, and the infinitesimal (polygenic) and residual effects as random. The additive and dominance effects of the SNP and its interaction with sex are also being investigated. After the association analysis, to integrate the approaches of candidate genes and the classical QTL mapping, searches for QTL regions mapped near the gene under study were made.

SNPs in genes involved in muscle growth (*myostatin, MyoD, MRF4, Myf-5, myogenin*) were characterized in the parental lines from the EMBRAPA F2 Chicken Resource Population. Associations of the myogenin gene with body weight at 42 days, weight gain and weights of carcass, wings, abdominal fat, liver and lungs were found in the F2 population (Souza, 2004).

Genes associated with fat metabolism in chickens (*ghrelin, leptin receptor, ADIPOR2, HNF4α and others*) were also investigated. Ninov *et al*., (2006) identified SNPs in the leptin receptor gene (*LEPR*); two SNPs segregating only in the TT line (broiler) and four were more frequent in the CC line (layer). Two SNPs were chosen for association analysis in the F2 population. The C352T SNP was associated with crude protein and ash contents in the carcass, and liver, breast and carcass yield. The G915A SNP was associated with feed intake, percentage of lungs and legs (drums and thighs) yield. With this approach, potential genetic markers in the leptin receptor gene were identified to improve the chicken carcass, breast, and legs yield (Ninov, 2007). Previously, Wang *et al*., (2004) correlated *LEPR* polymorphisms with abdominal fat and liver weight in a population divergently selected for fat deposition. Although the leptin receptor gene is well studied in cattle and pigs, there are few association studies of this gene with traits of interest in chickens.

The previous associations between *LEPR* and production traits were detected in segregating populations and the effect of this gene should to be tested in pure lines. To further explore these findings, the association between another polymorphism in the leptin receptor gene (A286G) with growth (Peixoto *et al*., 2010b), carcass (Peixoto *et al*., 2012) and skeletal traits (Marchesi *et al*., 2011) were investigated in the TT Reference Population. The growth traits analyzed were: birth weight, weight at 21, 35, 41, 42 days, body weight without feathers and blood, and carcass weight. The SNP in the *LEPR* was associated with weight at 35, 41, 42 days, body weight without feathers and blood, and carcass weight (Peixoto *et al*., 2010b). These traits are genetically highly correlated and the results show that the SNP is associated with late growth (35 days up). Significant associations of the SNP *LEPR* A286G with several carcass traits were found, including carcass weight, weights of wings, thigh, thigh muscle, breast, breast muscle, breast fillet, thigh and drumstick muscle, and drumstick, breast muscle, and thigh and drumstick yields (Peixoto *et al*., 2012). Marchesi *et al*., (2011) studied the same SNP (A286G) and observed associations with bone integrity related traits. The SNP had a significant additive effect for the tibia weight ($0.10x10^{-13}$), tibia length ($0.21x10^{-2}$), tibia width ($0.24x10^{-3}$), femur weight ($0.97x10^{-6}$), and femur length ($0.30\ x10^{-3}$). These results indicate a possible role of this gene also in the chicken bone metabolism.

The leptin receptor gene was associated with a wide variety of traits of economic interest, indicating that markers in this gene can potentially be used in poultry breeding programs. The *LEPR* is located at approximately 29.1Mb of the chicken chromosome 8, in a region where several QTLs have been reported, such as body weight, breast muscle weight, drumstick muscle weight, tibia weight, tibia width, etc. (Hu *et al*., 2010: QTLdb; http://www.animalgenome.org/ QTLdb/). The biological action of the *LEPR* gene together with its location in the genome show evidences that this gene might be directly responsible for the significant associations found. The fact that the leptin receptor gene has a consistent effect in several growth, carcass and bone traits is quite interesting since the leptin gene in chickens has not been mapped yet. These findings suggest a molecular evidence for the existence of a leptin-mediated control mechanism in poultry.

An important finding is that the *ADIPOR2* gene, involved in the fat metabolism, was associated with several traits in our TT population (unpublished results). This gene is located at approximately 63Mb in GGA1, in a region where several QTLs have been mapped (QTLdb). For instance, in our F2 population, this gene is in a QTL region between markers *Lei 0146* and *Lei 0174*, associated with residual carcass weight, adjusted abdominal fat, drums and thighs weight, and heart weight (Nones *et al*., 2006). The *ADIPOR2* might be a positional candidate gene for some of the QTLs mapped.

Another candidate gene related to fat deposition is the *HNF4* gene (hepatocyte nuclear factor 4, alpha). This gene is a hepatic transcription factor related to the lipid and insulin metabolism in humans. Silva *et al*., (2012) investigated an *HNF4* SNP in the EMBRAPA TT Reference Population. The SNP additive effect was strongly influenced by sex, being significant only in males, for the following traits: abdominal fat, wings yield, and wing sticks yield. Thus, this association analysis indicates great potential of the *HNF4* gene in reducing abdominal fat, which is a relevant issue in poultry.

Other candidate genes related to fat metabolism have been investigated by our team showing promising results for possible use as markers in selection, and to better understand the genetic control of the evaluated traits.

In broilers, the rapid growth rate and carcass yield have not been accompanied by the proper bone tissue development, increasing the incidence of leg problems and bone fragility. These problems represent significant losses for the poultry production and industry. Efforts have been made to reduce the occurrence of these abnormalities in the chicken skeleton. A promising alternative to reduce this problem is the application of genomics (Burt, 2002).

Genes biologically related to bone integrity traits and located in QTL regions previously detected are under investigation. For instance, a SNP in the bone sialoprotein gene, located in GGA4, was significantly associated with body weight at 41 days, wings weight, breast bone weight and width of the tibia in the TT Reference Population (Fornari *et al*., 2012). This SNP might be a potential marker to be used in selection to improve skeletal structure.

A limitation to apply the candidate gene strategy is that only a small proportion of genes that control quantitative traits are known. Difficulties to define the candidate gene effect also exist, because the identification of the causal variation for genes of small effect is not easily determined. Nevertheless, these studies are of great importance to better understand the function of genes involved in the development of the different tissues.

Simulation study

Hundreds of QTLs for several traits have been mapped using linkage analysis with F2 resource populations (http://www.animalgenome.org/QTLdb/). However, a problem with linkage QTL analysis is that confidence intervals for QTL positioning are very large, in the order of several Mb, making it difficult to select candidate genes. Genome-wide association studies (GWAS) have emerged as the method of choice for fine mapping complex trait genes. This is because microarray have made large-scale genotyping affordable and due to the advantages of association versus linkage in terms of accuracy for QTL positioning. It is well-known that there is no advantage of applying GWAS in crosses between inbred lines. However, many crosses in domestic species are actually made up of divergent, yet outbred, populations, as our F2 resource population.

Given that many of these crosses have been already generated, with dozens of traits measured in hundreds of individuals, a simulation study was carried out to verify how useful GWAS would be if applied in such crosses (Ledur *et al*., 2010). The influence of marker density, QTL effect and QTL allele frequency on power, false discovery rate (FDR) and accuracy were investigated. Our results suggest that GWAS in outbred F2 crosses is useful, especially in large populations. Under these circumstances, accuracy increased and FDR decreased as compared with classical linkage analysis. Another important finding was that SNP ascertainment had an important effect; the best option was to select SNPs as uniformly distributed as possible without setting any restriction on allele frequency.

Gene expression

The approaches used by our group for the functional genomic studies were the ESTs generation, microarrays, qRT-PCR and the investigation of miRNAs. Important insights on point and global gene expression in several tissues of interest have been reported, especially in the skeletal muscle development.

An EST database was constructed by Alves (2004) from the pectoral muscle of the two chicken lines TT and CC in two developmental stages (embryonic and post-hatching). A total of 8928 ESTs were sequenced from the 5' end of cDNA inserts. After the analysis of quality, 6247 sequences were validated. Cluster analysis revealed a novelty index of 55.7% for this database. In addition, RT-PCR analysis of five genes related to the skeletal muscle development in chickens (*MyoD, MRF4, Pax-3, myogenin and myostatin*) revealed significantly lower levels of *MyoD, Myf5* and *myostatin* in the broiler line. The

demonstration that genes encoding myogenic factors were differentially expressed between the lines suggested that *MyoD, Myf5, myogenin, MRF4* and *myostatin* could be used to select animals with higher skeletal muscle deposition potential.

Two pituitary and hypothalamus cDNA libraries from 21 day broiler (TT) and layer (CC) chickens lines were constructed allowing the identification of 3,074 unique sequences and 77 line-specific SNPs. Out of those, 52 SNPs were TT-specific and 25 CC-specific. Most SNPs found in these ESTs libraries were related to the mitochondrial genome, to structural proteins, neuronal constituents, ribosomal proteins and iron binding proteins. The collection of expressed sequence tags (ESTs) and SNPs identified in this study represents an important resource for future studies aimed at identifying genes responsible for growth in chicken (Cassoli *et al*., 2007). Sequences from the CC line library were deposited in dbEST division of GenBank (NCBI, http://www.Ncbi.nlm. nih.gov) with accession numbers ranging from CO419474 to CO421626, and those from the TT line library received numbers ranging from CO421627 to CO423759.

A chicken skeletal muscle-associated array was constructed based on a muscle-specific EST database from TT and CC lines, which was used to generate a tissue expression dataset of ~4500 chicken genes across 5 adult tissues (skeletal muscle, heart, liver, brain, and skin). The skeletal muscle macroarray platform was first used to search for evidence of tissue-specific expression, focusing on the biological function of genes/transcripts, since gene expression profiles generated across tissues were found to be reliable and consistent. Screening the skeletal-muscle platform using 5 chicken adult tissues allowed the identification of 43 'tissue-specific' transcripts, and 112 co-expressed uncharacterized transcripts with 62 putative motifs. This platform represents an important tool for functional investigation of novel genes; to determine expression pattern according to developmental stages; to evaluate differences in muscular growth potential between chicken lines, and to identify tissue-specific genes (Jorge *et al*., 2010).

Aiming to understand the molecular mechanisms involved in determining the development and growth of muscle tissue, gene expression of myogenic factors (*MyoD, Myf5, Myogenin* and *MRF4*), *Pax7, Myostatin*, and *Shh pathway (Shh, Ptch1, Smo, Pka, Sufu, Gli2* and *Gli3*) was monitored in the pectoralis muscle of two EMBRAPA lines: TT (broiler line) and CC (layer line). Gene expression was measured by qRT-PCR in breast tissue of 90 animals divided into two lines and five stages of development: 9 and 17 days of embryo and 1, 21 and 42 days post-hatching (n=9/time point/line). The genes *Myf5, Myogenin, MRF4, Pax7, Shh* and *Ptch1* were differentially expressed in ontogeny and among strains. The genes *MyoD, myostatin, Smo, Pka, Sufu, Gli2* and *Gli3* were differentially expressed only in ontogeny. The genes *MyoD, Myf5* and *Pax7* were more expressed in the embryo, where there is greater cell proliferation. The genes of the Shh pathway were more expressed in embryonic ages, where this pathway acts as a survival factor and cellular proliferation, and less expressed in later times after hatching, probably to allow for the differentiation of myoblasts (Ninov, 2010).

MicroRNAs (miRNAs) encompass a class of small and noncoding RNAs that regulates gene expression. In chickens, the full set of miRNAs and their expression patterns during development are still poorly understood when compared to other vertebrates. The profile of chicken skeletal muscle miRNAs was described in the Embrapa chicken lines by miRNAs clone library construction and quantitative expression analysis. Clone library sequence analysis revealed 47 small RNAs presenting significant similarities with already described miRNAs. Of this total, seven miRNAs showed high homology with miRNAs described in other species and not yet described in chickens. In addition, six sequence clusters were identified as putative novel miRNAs. Furthermore, a quantitative RT-PCR was used to measure the expression of some cloned miRNAs at embryonic and post-embryonic stages.

The expression patterns of three miRNAs allowed assessing the action of these molecules in the control of physiologic and morphologic conditions in the muscles cells, concerning the balance between cell proliferation and cell differentiation. These data may support subsequent functional studies aimed at understanding the function performed by each of the identified miRNAs in chicken skeletal muscle. (Andreote, 2009).

The TT and CC lines from Embrapa are also being phenotypically characterized for resistance and susceptibility to coccidiosis to study the host-pathogen interaction. The differential expression of genes from the immune system has been investigated in these lines using microarrays. In addition, genes involved in innate immune response are being studied by real-time PCR (Bertani *et al*., 2009). Recently, Novaes *et al*., (2012) described the transcriptome of three species of *Eimeria*, which

causes coccidiosis, one of the most important poultry production diseases. This information will contribute to improve our knowledge on the host-pathogen interaction.

Perspectives

The perspective of the poultry genomics research in Brazil is to advance in the identification and use of genes of interest in production systems. Thus, we expect to improve our understanding on the genetic control of economic important traits by identifying genes and understanding their functions, aiming to apply this knowledge in the production system. Identification and functional characterization of genes that influence production traits are extremely important to the advancement of knowledge needed to develop innovative biotechnology tools, and might be also important to improve Genomic Selection. Therefore, projects on GWAS, re-sequencing, RNAseq and metagenomics are under way. This will ensure our contribution to science, at the same time as preparing tomorrow's human resources, in such strategic area for the country´s development.

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