Identification of candidate genes for paratuberculosis resistance in the native Italian Garfagnina goat breed

Francesca Cecchi[a*], Claudia Russo[a], Daniela Iamartino[b], Alessia Galiero[a], Barbara Turchi[a], Filippo Fratini[a], Sara Degl'Innocenti[a], Raffaele Mazza[b], Stefano Biffani[c], Giovanna Preziuso[a], Carlo Cantile[a]

[a]Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, Pisa, Italy. [b]Laboratorio Genetica e Servizi (LGS), Associazione Italiana Allevatori (AIA), via Bergamo, 292, Cremona, Italy. [c]Istituto di Biologia e Biotecnologia Agraria (IBBA-CNR), Consiglio Nazionale Delle Ricerche, Via Einstein Cascina Codazza, Lodi, Italy

*Corresponding Author: Dr Francesca Cecchi, email: francesca.cecchi@unipi.it Tel. +39-050-2216879 - Fax +39-050-2210655

Abstract Paratuberculosis disease is a chronic bacterial disease infection of ruminants of global relevance, caused by Mycobacterium avium subsp. paratuberculosis (MAP). The present study was conducted on the Garfagnina goat breed that is an Italian native goat population registered on the Tuscan regional repertory of genetic resources at risk of extinction. Forty-eight adult goats (27 serologically positive to MAP-positive and 21 serologically negative to MAP-negative) belonging to a single flock that had experienced annual mortalities due to MAP infection were identified and genotyped with the Illumina GoatSNP60 BeadChip. Diagnosis was achieved by serological tests, as well as post-mortem examination of affected animals. A genome-wide scan was then performed on the individual marker genotypes, in the attempt to identify genomic regions associated with MAP infection disease. Nine significant markers were highlighted and they were located within, or nearby to annotated genes. Two genes found in this study encode or are linked to protein kinases that are among the most important enzymes involved in the immune response to Johne's disease and four genes are involved in the functions of the Golgi complex.

Keywords: Garfagnina goat breed; disease susceptibility; granulomatous enteritis; GoatSNP60 BeadChip; GWAS paratubercolosis

Introduction

Paratuberculosis (Johne's disease) is a chronic infection of ruminants, caused by Mycobacterium avium subsp. paratubercolosis (MAP). This disease causes reduction in milk production and diarrhea, although in sheep and goats it is often asymptomatic. Economic losses experienced by farmers, as well as the possible role of MAP in Crohn's disease of humans (Sechi and Dow 2015) are the major reasons for interventions against MAP infection. The infection in sheep and goats is also worldwide distributed. Ovine paratuberculosis has been detected in many countries as well as in para-Mediterranean countries (Windsor 2015), while caprine paratuberculosis has been detected in many extra-European and in many European countries (Angelidou et al. 2014; Liapi et al. 2015). In Italy, although there are no large-scale investigations on the spread of the infection in sheep (Attili et al. 2011; Galiero et al. 2015; Galiero et al. 2016) and goats (Galiero et al. 2017), a high incidence is estimated, with a prevalence at farm level ranging from 0.4-1.5% to 29-39% and at seropositive animals level in infected herds from 0.3 to 15.4%. Indeed, Attili et al. (2011) reported that in the Italian Marche region 73.7% of the dairy flocks were infected.

Genetic susceptibility to MAP infections in ruminants (cattle, sheep, goat) has been investigated using quantitative and/or molecular genetics. Resistance to MAP infection has been found to be heritable with heritability estimates ranging from 0.06 to 0.27 (Zare et al. 2014). Despite low heritability estimates, all studies confirm genetic influence on paratuberculosis susceptibility. Several attempts to locate loci associated with resistance to paratuberculosis have been made during the last 15 years. Reddacliff et al. (2005) found an association of one microsatellite allele in SLC11A1 gene (formerly NRAMP1) with MAP resistance in sheep. Studies based on either candidate genes or genome-wide association studies (GWAS) can be found in the literature mainly in cattle (Minozzi et al. 2010, 2012;

Kirkpatrick et al. 2011; Zanella et al. 2011; Finlay et al. 2012; Küpper et al. 2014; Zare et al. 2014; Richardson et al. 2016), and more recently in sheep (Moioli et al. 2016). The bovine SLC11A1, NOD2, SPP110, TLR2 and TLR4 genes were described as MAP susceptibility loci (Ruiz-Larrańaga et al. 2017; Várquez et al. 2014). TLR6 may be a potential marker of exposure to MAP and could be used to identify sheep resistant to MAP infection (Plain et al. 2010). Recent studies suggested that miRNA expression is affected by MAP infection and play a key role in turning the host response to infection (Malvisi et al. 2016).

Garfagnina breed is important for livestock biodiversity preservation, being a key animal for specialized cheese market in Tuscan Region. Garfagnina is an Italian native goat population registered on the Tuscan regional repertory of genetic resources at risk of extinction, with about 745 animals belonging to 17 flocks. Local breeders report that the population was reared for generations for its milk and meat production (www.assonapa.it).

Given the importance of MAP as pathogen in animals and as a potential risk factor for human diseases, our study is one of the first attempts to identify genomic regions associated with MAP infection disease in goat.

Materials and methods

Diagnostic assessment and selection of animals for genotyping

The study was performed on a Garfagnina goat flock consisting of 269 females and 20 males; the animals age ranged from 2 to 9 years. The flock was located in the Garfagnana district (Lucca, Italy).

77 The goats grazed during the morning (feed supplements are given mainly during winter), and were
78 housed overnight, when they received a forage and feed integration..

79 Serum samples were analyzed by ELISA ID screen® Paratuberculosis Indirect screening test
80 (ID.VET, Montpellier, France) and the positive samples were subsequently tested with ELISA ID
81 screen® Paratuberculosis Indirect confirmation test (ID.VET, Montpellier, France).

82

83 **Pathological examination**

84

85 Three seropositive and two seronegative goats (control tissues) were slaughtered and examined post-
86 mortem. Samples for histopathological examination included duodenum, jejunum, ileum, caecum and
87 colon; mesenteric lymph nodes (duodenal, colon, ileocaecal), submandibular, retropharyngeal and
88 mediastinal lymph nodes, liver and hepatic lymph node, pancreas, kidney, spleen, lungs and tonsils.
89 Tissue samples were fixed in 10% neutral buffered formalin and dehydrated through graded alcohols
90 before being embedded in paraffin wax.
91 The fixed tissues were routinely processed for histology and 5 µm thick sections were stained with
92 hematoxylin and eosin (HE). Sections from all tissues, including intestines and lymph nodes, were
93 also stained with Ziehl-Neelsen (ZN) for acid-fast bacteria (AFB) demonstration.
94 Immunohistochemistry was also performed using antibodies anti-CD68 (monoclonal mouse anti-
95 human CD68, clone EBM11, 1:100, Dako, USA), anti-CD79α (monoclonal mouse anti-human
96 CD79α, clone JCB117, 1:100, Dako, USA), anti-CD3 (monoclonal mouse anti-human CD3, clone
97 F7.2.38, 1:100, Dako, USA) for the characterization of the leucocyte subset.
98 Sections were examined for the presence of granulomatous inflammation and by AFB.
99 Paratuberculosis lesions were classified on the basis of presence of granulomatous lesions,
100 distribution of granulomata in the different tracts and layers of the intestine, severity of lesions, cell
101 types present in the infiltrate, and presence and number of intralesional AFB (Corpa et al. 2000). An

AFB grade was assigned to each evaluated tissue, as proposed by Dennis et al. (2011) for sheep, where grades 0 to 2 were considered paucibacillary and 3 to 4 grades were interpreted as multibacillary lesions, similar to the method of Clarke and Little (1996). In this study Paratubeculosis lesions were classified as focal, diffuse multibacillary, diffuse lymphocytic or diffuse mixed (Corpa et al. 2000).

Genotyping and SNP quality control

Forty-eight Garfagnina goats, 27 positive and 21 negative to MAP test screening, were genotyped using the Illumina GoatSNP60 BeadChip (Illumina Inc., San Diego, CA), containing 53347 SNP, designed by the International Goat Genome Consortium (IGGC). The available phenotype was a binary character, 0 = MAP-Negative and 1 = MAP-Positive.

Blood samples of the 48 goats were collected according to the recommendations of the European Council (1986) concerning animal care. Whole blood was collected in Vacutainer tubes with K-EDTA as anticoagulant and stored at −20 °C until genomic DNA was extracted using Qiagen QIAamp DNA blood mini/midi kit (Qiagen, San Diego, CA, USA).

The association SNP/Chromosome was performed using the information available on the site http://bioinformatics.tecnoparco.org/SNPchimp/ (Nicolazzi et al., 2015). The number of SNP for chromosome was reported in Table 1. SNP genotyping was outsourced at the Associazione Italiana Allevatori - Laboratorio di Genetica e Servizi facility (http://www.lgscr.it). Raw signal intensities of the 53347 SNPs were converted into genotype calls with GenomeStudio software V2011.1, Genotyping Module v1.9.4 (Illumina). The markers not satisfying the following filtering parameters were excluded: SNP call rate < 99%; SNP minor allele frequency<5%; out of Hardy-Weinberg

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equilibrium at P<0.01. The fitted model included both the first 3 principal components to adjust for possible stratification and the age of the animal at sampling.

Data analysis, identification of SNP location and gene enrichment

Before proceeding with the genome scan, the selected SNPs were used to verify the population structure. A matrix of genomic relationships was then calculated and a matrix of genetic distances was built. This latter matrix was used to graphically display the relationships between the sampled individuals. A genome scan using the *qt-score* function of the GenABEL package (Aulchenko et al., 2007) by R software (http://www.genabel.org/GenABEL/qtscore.html) was then fitted in order to test associations between Negative/Positive (0/1) phenotype and the 49417 SNP.

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It was verified if the significant SNPs were near a gene and determined the distance using the goat genome map and annotation reference from NCBI (http://www.ncbi.nlm.nih.gov/genome?term=capra%20hircus), assembly CHIR_1.0 (International Goat Genome Consortium). Using the corresponding genes on bovine species (*Bos taurus*, assembly UMD 3.1), a gene ontology (GO) enrichment analysis was carried out using InnateDB (www.innatedb.com) and a network of interaction between genes was investigated to aid interpretation of GWAS results.

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Results

146 27 MAP-positive goats have been identified and twenty-one serologically negative goats were
147 considered as control group.

148 Generally, no changes were found in all examined organs. No lesions were observed in seronegative
149 goats and in control tissues. Microscopically lesions associated with MAP were restricted to the
150 intestines and related lymph nodes of seropositive goats, and to the liver in one seropositive goat.
151 Lesions associated with MAP infection were not found in all duodenum samples. Diffuse lesions
152 were found in all samples collected from other selected intestinal tracts, mainly in the ileum and
153 caecum of seropositive goats. Lesions were characterized by diffuse granulomatous enteritis, with a
154 high number of macrophages in the intestinal mucosa, not always associated with Peyer's patches.
155 Macrophages were the main inflammatory cells, confirmed by immunohistochemical labeling with
156 anti-CD68 antibody, and the inflammation caused a severe change of the intestinal villi, that appeared
157 thickened with flat apices. Macrophages were found both in the basal area of the lamina propria and
158 at the apices of the intestinal villi. Scattered macrophages containing AFB were also found in
159 lymphoid tissue associated with the intestinal mucosa (Fig. 1A). The inflammatory infiltrate
160 contained also lymphocytes, mainly with a sub-epithelial localization, clearly visible with
161 immunohistochemical labeling, using anti-CD79α and anti-CD3 antibodies (Fig. 1B). A mild to
162 moderate degree of lymphangiectasis was also observed. AFB was demonstrated by ZN staining in
163 the cytoplasm of macrophages. The number of AFB was usually more than 10 per macrophage and
164 many macrophages were distended by AFB, so the lesions were classified as multibacillary in all
165 cases.

166 The ileocecal and colon lymph nodes of two seropositive goats showed inflammatory infiltrates.
167 Macrophages and epithelioid macrophages were the main inflammatory cells. Macrophages were
168 scattered in the lymph nodes interfollicular cortex but were also found in clusters at the base of
169 follicles with a moderate amount of epithelioid macrophages. Rare macrophages were observed in
170 the paracortex and near the medullary sinus. The number of intracytoplasmic AFB within

171 macrophages was variable in the positive lymph nodes, but was consistently less than in granulomata
172 located in the intestinal wall (Fig. 1C). A high number of granulomata were found in the liver of all
173 seropositive goats, but only in one goat the ZN stain revealed the presence of AFB in the inflammatory
174 cell infiltrate. The hepatic granulomata consisted of epithelioid macrophages with large, clear nuclei,
175 prominent nucleoli and lightly foamy cytoplasm (Fig. 1D).

176 Concerning molecular analysis the edited dataset included 48 animals and 49417 (93.27%) SNPs
177 because after quality control 2466 (4.66%) markers were excluded as having low (<5%) minor allele
178 frequency, and 1218 (2.29%) markers were excluded because of low (<99%) call rate. All markers
179 were in HWE (P<0.01).

180 In Figure 2 genomic relationships among the animals can be observed and 3 main clusters can be
181 identified. Even if it was not possible to trace back the ancestors of any individual, we can postulate
182 that each cluster refers to daughters of related individuals. Group 2 are mainly MAP-positive (5-
183 positive vs 1-negative); Group 3 are mainly MAP-negative (8-negative vs 1-positive), while Group 1
184 are 21 MAP-positive and 12 MAP-negative. Nevertheless, the principal component analysis explains
185 only a small proportion of the observed variance (Figure 3). Figure 4 represents the Manhattan Plot
186 of the F-test values obtained. The horizontal red line separates the 8 significant markers (p-value
187 <0.0005). In Table 2 the significant SNPs with relative positions and nearby genes are displayed
188 according their own degree of statistical significance.

189

190 **Discussion**

191 The results of this study, the first performed on goat, suggest that 13 genes might have a role in disease
192 resistance but none of these have been previously highlighted by other authors. The main genes found
193 nearby the significant SNP are: DGKB, CCNA2, PSMA7, BCas3, GOLGA3, LOC102187381,
194 PCSK5, BBS7, MANEA, PSD3, ANKLE2, TRNAS-AGA and TRNAC-ACA.

195 The DGKB gene, encodes for the diacylglycerol kinase beta molecule. Kinases are between the most
196 important molecules involved in the immune response to the Johne's disease. In bovine, responses of
197 monocytes to infection with different MAP genotypes are used as an indicator of bacterial phenotypic
198 variability.

199 Another gene whose function is related to kinase activity is CCNA2 (Cycline A), located at ~103 Kb
200 from the significant snp4243-scaffold1131-586101 on Chromosome 6. The protein encoded by
201 CCNA2 belongs to the highly conserved cyclin family, which function as regulators of CDK kinases.
202 This cyclin binds and activates CDC2 or CDK2 kinases, and thus promotes both cell cycle G1/S and
203 G2/M transitions (Dash and El-Deiry 2005).

204 PSMA7 (proteasome subunit alpha 7, Chromosome 13) gene, is at a distance of 621bp from the
205 significant snp59142-scaffold97-1302272. PSMA7 is involved in many different cellular processes,
206 ranging from the cell cycle process to antigen processing and tumorigenesis.

207 BCAS3 gene plays a role in angiogenesis, it participates in the regulation of cell polarity and
208 directional endothelial cell migration by mediating both the activation and recruitment of CDC42
209 (Jain et al. 2012) and the reorganization of the actin cytoskeleton at the cell leading edge. In bovine,
210 BCAS3 interacts with VIM gene (Vimentin) that encodes a member of the intermediate filament
211 family helping to making up the cytoskeleton (Fig 5).

212 BBS7 gene, located at ~63kb from the significant snp4243-scaffold1131-586101, encodes one of
213 eight proteins that form the BBSome complex. The BBSome complex is believed to recruit Rab8
214 (GTP) to the primary cilium and promote ciliogenesis. ANKLE2 gene encodes a member of the LEM
215 family of inner nuclear membrane proteins that function as a mitotic regulator through post-mitotic
216 formation of the nuclear envelope. TRNAC-ACA, (transfer RNA cysteine-anticodon ACA,
217 Chromosome 8) and TRNAS-AGA (transfer RNA serine -anticodon AGA, chromosome 9) genes

encode for cysteine transfer RNAs which function is to translate UGU and UGC codons in mRNAs to cysteine residues during cytoplasmic protein synthesis.

GOLGA3, LOC102187381 MANEA and PCSK5 are related to the Golgi apparatus:. It is well know that Toll-Like Receptor 4 (TLR4) resides in the Golgi apparatus with a widespread distribution in the Golgi cisternae and the trans-Golgi network and studies on macrophages indicated an important role of TLR2 and TLR4 for MAP recognition. The Golgi apparatus resides at the intersection of the secretory, lysosomal, and endocytic pathways. It is of particular importance in processing proteins for secretion, containing a set of glycosylation enzymes that attach various sugar monomers to proteins as the proteins move through the apparatus. The Golgi apparatus also protects against the apoptosis

GOLGA3 gene encodes a member of the golgin family of proteins, which has been postulated to play a role in nuclear transport and Golgi apparatus localization. In bovine, GOLGA3 interacts with 12 other genes but we can't observe any interaction with BCAS3 gene (Fig 5). LOC102187381 gene, (alpha-N-acetylneuraminide alpha-2,8-sialyltransferase), on Chromosome 5 at ~46kb from the significant snp8910-scaffold1319-1157713, encoded for a protein belonging to the process of the synthesis of polysialic acid, a modulator of the adhesive properties of neural cell adhesion molecule (NCAM1). MANEA gene (glycoprotein endo-alpha-1,2-mannosidase), on Chromosome 9, encodes for an endomannosidase and is involved in the transport to the Golgi. PCSK5 gene, located more distant from the same SNP (442kb), encodes a protein involved in the activities of Golgi apparatus sorting to the *trans*-Golgi network where a second autocatalytic event takes place and the catalytic activity is acquired.

As previously reported, many genome-wide association studies (GWAS) were found in literature. In particular, Moioli et al. (2016) performed GWAS in 100 Sarda sheep breed with the Illumina Ovine SNP50K BeadChip, and found 30 putative candidate genes, five of which had been previously reported to play a direct role in the immune system: SEMA3D, CD109, PCP4, PRDM2 and ITFG2.

243 Many studies were carried on cattle: Settles et al. (2009) published the first study relating to MAP
244 susceptibility and utilizing SNP assays (BovineSNP50BeadChip) on 245 Holstein dairy cattle.
245 Although sixteen individual SNP were identified, not all were statistically significant in all four
246 diagnostic variables (presence of Map in the tissue, in faeces, in both tissue and faeces and in tissue
247 but not faeces). Also Zanella et al. (2011) performed a GWAS in with the Illumina Bovine SNP50
248 BeadChip, and found only the GNA12 gene as a positional candidate associated to tolerance to
249 Johne's disease. Kirkpatrick et al. (2011) performed a GWAS with the SNP50 BeadChip and they
250 found that only the PRGER4 gene was in proximity of one of the SNP of the proposed set. Minozzi
251 et al. (2010) found that the PRDM1 gene on the chromosome 9 was a good positional and functional
252 candidate. In Jersey cattle, Zare et al. (2014) found that the significant markers were in proximity of
253 the following candidate genes: major histocompatibility complex, TCF19, FAT10, HIVEP1,
254 CCDC17, ZNF684, UBE2 L3, UBE2K, FAM5C, and FAM109A. The rs208222804 C allele (CD209
255 gene) was found to be associated with latent paratubercolosis in Holstein-Friesian cattle (Vázquez et
256 al 2014).

257 With the exception of the PRDM members, which were proposed as potential candidate of MAP
258 resistance in sheep (Moioli et al. 2016) and in cattle (Minozzi et al. 2010) and with the exception of
259 the significant SNP found on chromosome 12 in cattle (Gonda et al. 2007; Minozzi et al., 2012), each
260 GWAS has identified different genes. As reported by Moioli et al. (2016), it is evident that the
261 complexity of the immune system cannot be summarized in few genes with major effect. Moreover
262 Kirkpatrick et al. (2011) reported that the results of two genome scans did not correspond due to
263 differences in statistical power and differences in the methods of classification of the infection status
264 of animals. In fact, one of the main problems for genetic studies regarding paratuberculosis is the
265 definition of infection and disease status, resulting in difficult acquisition of material (Reddacliff et
266 al. 2005). Minozzi et al. (2012) found that the different genomic genes and candidate genes involved
267 with specific and general immune response to paratubercolosis depend on the specific measures of

infection used (MAP tissue infection or humoral immune response). In this study, diagnosis was achieved by serological tests, as well as by post-mortem examination of affected animals.

In conclusion, histopathological examination confirmed the serological diagnosis of paratuberculosis infection in the analyzed group of Garfagnina goats. The localization and type of lesions resembled those previously reported in goats, with diffuse multibacillary lesions as the commonest lesion type. Despite our study was performed on a limited number of individuals, and our results must be verified, it describes the first genome-wide characterization of selective sweeps in goat addressing disease resistance. The results of the Genome-Wide Association analysis show that the most part of the genes found are related to the Golgi apparatus. Future work may include replication of this study with a larger number of animals ad fine mapping of candidate regions.

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Compliance with ethical standards "Committee on the Ethics of Animal Experiments of Minimally Invasive Surgery Centre" (Italian laws).

Conflict of interest The authors declare that they have no conflicts of interest

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383

384 **Table 1** Distribution of the goat chromosomes of the markers used in the present study.

Chromosome	Total markers	% markers
0	1420	2.66
1	3256	6.10
2	2829	5.30
3	2380	4.46
4	2415	4.53
5	2243	4.20
6	2437	4.57
7	2191	4.11
8	2351	4.41
9	1894	3.55
10	2098	3.93
11	2138	4.01
12	1749	3.28
13	1649	3.09
14	1911	3.58
15	1639	3.07
16	1592	2.98
17	1469	2.75
18	1291	2.42
19	1227	2.30
20	1495	2.80
21	1430	2.68
22	1169	2.19
23	1047	1.96
24	1323	2.48
25	855	1.60
26	1044	1.96
27	928	1.74
28	914	1.71
29	977	1.83
X	1986	3.72
Total	53347	

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387 **Table 2** Genes near to the most 9 significant SNP.

SNP name	P	Chr	Position	NCBI RefSeq	Within gene	<1000bp
snp39529-scaffold502-698462	<0.0005	19	11287052	NC_022311.1	BCas3	
snp49505-scaffold706-1364074	<0.0005	4	20570314	NC_022296.1	DGKB	
snp35452-scaffold427-1738556	<0.0005	9	42054390	NC_022301.1		TRNAS-AGA
						MANEA
snp26891-scaffold2837-184265	<0.0005	27	37488332	NC_022319.1		PSD3
snp59142-scaffold97-1302272	<0.0005	13	53040550	NC_022305.1		PSMA7
	<0.0005					LOC1021873
snp8910-scaffold1319-1157713		5	85352445	NC_022297.1		81
snp44572-scaffold606-2014619	<0.0005	8	50112108	NC_022300.1		TRNAC-ACA
						PCSK5
snp4243-scaffold1131-586101	0.00061	6	2759590	NC_022298.1		BBS7
						CCNA2
snp16281-scaffold1714-362140	0.00062	17	44009868	NC_022309.1	GOLGA3	ANKLE2

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Fig. 1. Fig.1A Goat. Ileum. Severe enteritis with marked infiltration of macrophages laden with AFB. Ziehl-Neelsen stain, x125. Fig.1B Goat. Ileum. Large number of CD68-positive macrophages are evident in the apices of intestinal villi. Anti-CD68 IHC, x125. Inset: Subepithelial distribution of T lymphocytes. Anti-CD3 IHC, x500. Fig. 1C Goat. Aggregates of macrophages containing AFB in the colic lymph node. Ziehl-Neelsen stain, x320. Fig. 1D Goat. Liver. Multifocal perivascular inflammatory cell aggregates with macrophages laden with AFB. Ziehl-Neelsen stain, x320.

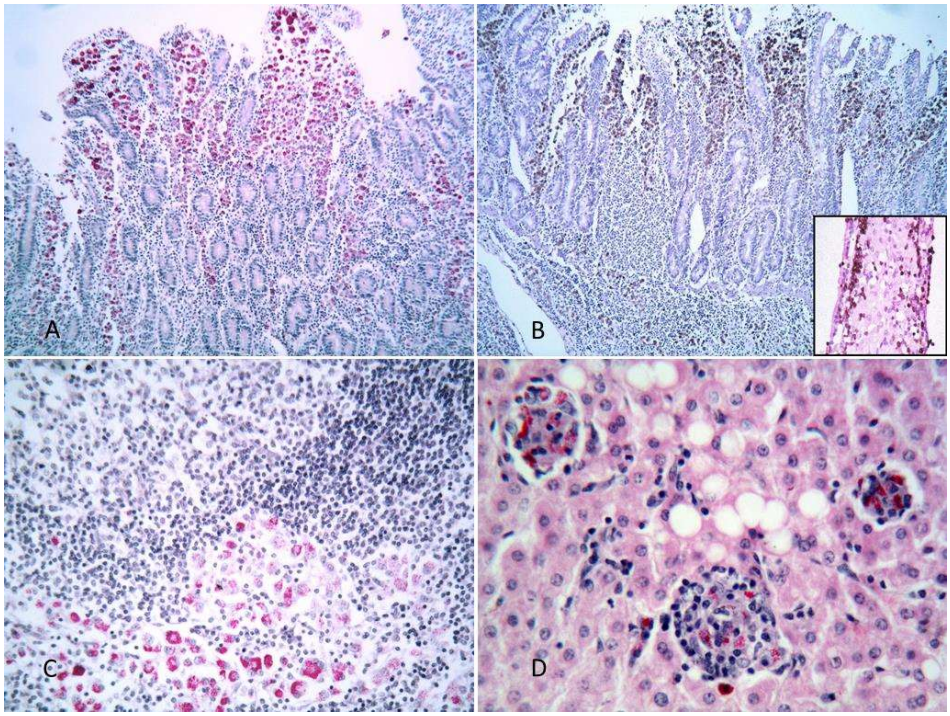

Fig. 2. Relationships between the animals of the sample

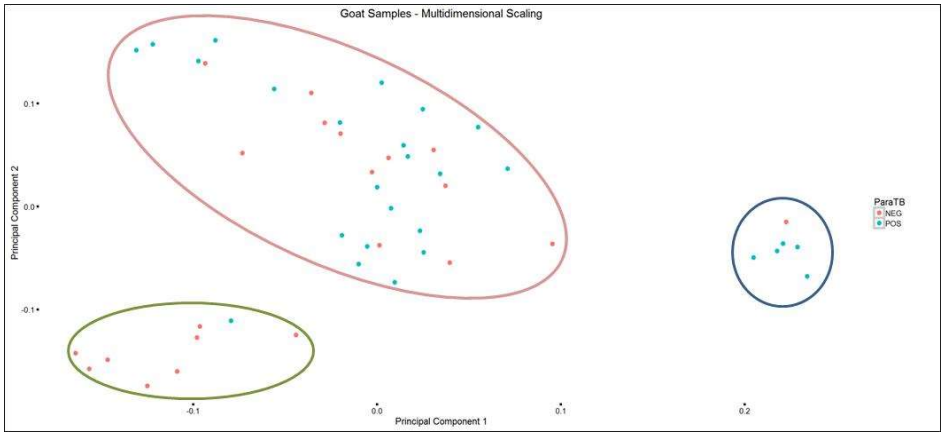

Fig. 3. Multidimensional scaling **(**MDS) analysis screen plot.

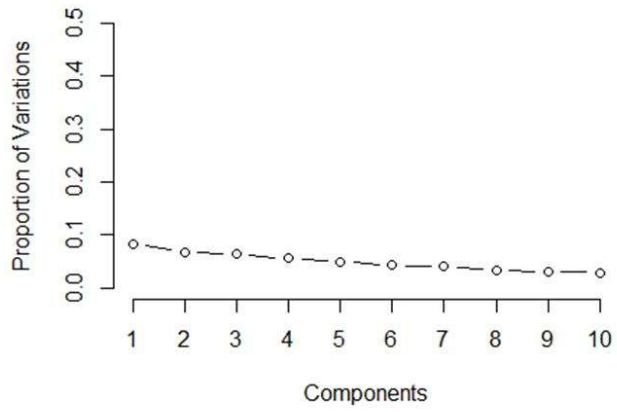

Fig. 4. Manhattan plot of the test values obtained, for each marker. The horizon red line separates the 7 most significant markers (P<0.0005).

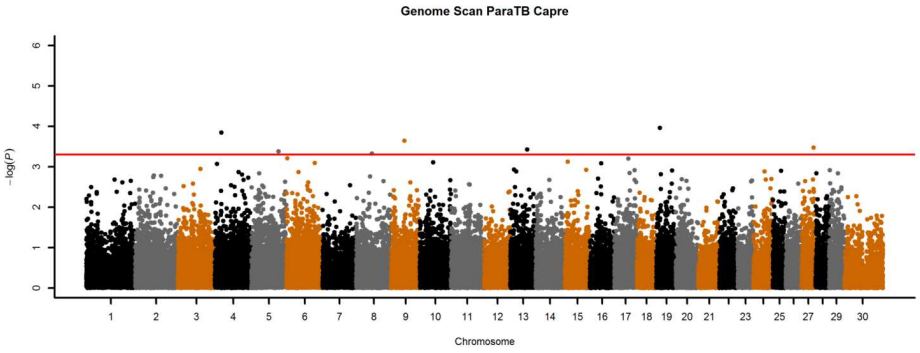

Fig. 5. Network based on GOLGA3 and BCAs3 genes interaction differentiated by cellular components. Include interaction predicted by othology.