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Selected tissues of two Polish goat breeds do not differ on genomic level*

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Majority of the Polish dairy goat population consists of two breeds: Polish White Improved (some 50% of the whole population) and Polish Fawn Improved (some 25% of the whole population). In a previous study no differences in basic performance parameters (milk yield, fat, protein, and lactose content) were found between these two breeds, though a difference in milk somatic cell score was noted. To examine the genomic differences, gene expression profiles of three types of biological material were investigated. Somatic cells isolated from milk, milk fat globules, and peripheral blood nuclear cells were subjected to bovine DNA microarrays. Hierarchical clustering of selected tissues' transcriptomic profiles revealed the presence of three distinct sub-clusters, representing each type

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of sample, but not the breed sampled. The only gene that was significantly different between the two breeds in terms of regulation was *Bos taurus* agouti signaling protein gene. In summary, no significant differences in the expression of 44,000 genes, including those involved in milk production and the immune system, were identified between the two studied Polish goat breeds, while, as expected, the differences in gene expression in the studied types of biological material were significant. These findings suggest that productively and immunologically these two main Polish dairy goat breeds consist one population.

KEY WORDS: genomics / goat / hierarchical clustering / microarray / milk fat globules / milk somatic cells/ peripheral blood nuclear cells

The entire Polish dairy goat population comprises approximately 80,000 does, and this number of animals has been stable for the last 10 years. The population is composed mainly of two breeds, Polish White Improved (PWI – ~50% of the population) and Polish Fawn Improved (PFI – ~25% of the whole population), followed by French Saanen and French Alpine. Detailed information on the history of goat breeding and the characteristics of the breeds maintained in Poland was presented in earlier papers [Kaba and Bagnicka, 2009, Bagnicka *et al.* 2015]. Briefly, goats of both main breeds are similar in size, fertility, and prolificacy, but they differ in coat color (white vs. red-brown coat, with a dark stripe along the back and darker legs). A study on the milk composition of these two breeds in the entire Polish dairy goat population, as well as in a herd in Central Poland, revealed differences only in somatic cell score (SCS) between them [Bagnicka *et al.* 2015, Bagnicka *et al.* 2016]. Genomic studies conducted on samples taken from experimental goats revealed no breed-specific differences in the expression of *GBD2*, *BAC5*, *BAC7.5*, *LZ*, or *HAMP* in milk somatic cells (MSCs) between the breeds [Jarczak *et al.* 2014]. Though genetic polymorphism studies in the Polish goat population have been limited, they likewise have not revealed differences between these two breeds [Bagnicka *et al.* 2002, Maj *et al.* 2010, Bagnicka *et al.* 2013].

Although these two Polish goat breeds visibly differ in the color of their coat, up-to-date published data suggest that the breeds are not different with regard to their genetic and genomic profiles. However, confirmed genetic or genomic differences could influence important pathophysiological mechanisms and processes like the response to caprine arthritis encephalitis virus (CAEV), which still remains one of the most serious problems in goat breeding. Since only few studies have been run on the genetic differences between these two goat breeds, we decided to evaluate their genomic differences, assuming that such differences should be a consequence of genetic differences. We hypothesized that if genomic differences do exist, they should be revealed in a hierarchical clustering of the transcriptomic profiles of select biological materials. If genomic differences exist, the resulting clusters would group goat breeds, as well as goat tissues. Thus, the aim of the present study was to investigate the differences in gene expression profiles of somatic cells isolated from milk (MSCs), milk fat globules (MFGs), and peripheral blood nuclear cells (PBNCs) in the two most popular goat breeds in Poland: PWI and PFI.

Material and methods

Ethics statement

Warsaw University of Life Sciences Institutional Animal Care and Use Committee (IACUC) is III Local Ethical Committee. All procedures were carried out in accordance with the guidelines of this committee. The III Local Ethical Committee approved this research, and the approval was presented as formal written decision No. 63/2012.

Animals

Transcriptomic study was conducted on eight goats at the peak of their third lactation (milk and whole blood sampling in May 2014; 4 PWI and 4 PFI), and seven 3.5-year-old dry does (whole blood sampling in November 2014; 4 PWI and 3 PFI) selected from a herd of 50 goats maintained in Central Poland. The goats at that age were chosen due to the completion of somatic development, and reaching full maturity of the mammary gland. For the preceding twenty years, goats from this herd had been tested serologically for small ruminant lentivirus (SRLV) twice a year (in November and June) since their birth, using ELISA (ID Screen® MVV-CAEV, Indirect Screening ELISA, IDvet Innovative Diagnostics, Grabels, France) as a part of a long-term study [Czopowicz *et al.* 2018]. All animals selected for this study were free from SRLV infection, as it could influence gene expression [Bagnicka *et al.* 2018, Reczyńska *et al.* in press]. The SRLV antibody examination was continued after the end of the experiment, and no goats had an elevated titre. Goats of both breeds were kept in groups, in pens, where water, a salt lick, and hay were given *ad libitum*. The goats were fed according to the system developed by the Institut National de la Recherche Agronomique (INRA) of France and adopted by the National Research Institute of Animal Production [IZ PIB-INRA, 2009], Poland. The basic diet consisted of maize silage, wilted grass silage, and concentrates. In the summer period, the diet was supplemented with green forage, by grazing on pasture. Goats were machine-milked twice a day. Animals were kept under constant care of a veterinarian.

To analyse the differences in productivity between the two breeds, the performance traits of 20 goats from this herd (10 goats of each breed) that were free from the SRLV infection, bring in their third lactation were collected across three subsequent years (2012-2014). Altogether, 117 milking records in monthly periods were used. The milk MSC count was measured using IBC apparatus (Bentley, USA), while fat, protein, and lactose content were assessed using MilkoScan FT2 (FOSS, Denmark).

Sampling and isolation of milk somatic cells, milk fat globules, and peripheral blood nuclear cells

Three distinct types of biological material were sampled: goat milk somatic cells (MSCs), milk fat globules (MFGs), and peripheral blood nuclear cells (PBNCs).

To begin, samples of "first milk" were collected for microbiological examination. One hundred μL of the milk as streaked on Columbia agar supplemented with 5%

sheep blood (bioMérieux, France) and were incubated at 37°C for 48 h. The isolated pathogens were identified using VITEK 2 equipment (bioMérieux, France). Only the does without pathogenic bacteria in their milk were used in our study.

For transcriptomic analysis, milk samples (400 ml) were collected during the morning milking 70 days after parturition. Samples were immediately refrigerated and transported on ice at 4°C, for further analyses.

MSCs were isolated according to the procedure described by Boutinaud and Jammes [2002], and by Boutinaud *et al.* [2002] with modifications as follows: the first milk centrifugation with EDTA (EDTA – Ethylenediaminetetraacetic acid solution, BioUltra, for molecular biology, pH 8.0, ~0.5 M in H_2O, Sigma-Aldrich, Poland) of a final concentration of 0.5 mM of EDTA was performed at 4°C at 1,700 x g for 25 min (Centrifuge 5810 R centrifuge, Eppendorf, Germany). The fat layer and the milk were removed, and the cell sediment was rinsed twice using PBS (pH 7.45 – PBS Gibco™, Invitrogen Corporation, UK) with EDTA (0.5 mM). The initial rinse was a cautious removal of sediment from the container bottom using 40 ml of PBS-EDTA, and the sediment was poured into a sterile 50 ml Falcon® (Becton Dickinson and Company, USA) test tube and centrifuged at 1,700 x g for 15 min. After the liquid over the sediment surface was removed, 20 ml of PBS-EDTA was added to the test tube. Following the last centrifugation, the cell sediment was rinsed with 5 ml of PBS and centrifuged at 2000 x g for 5 min. Then, the remaining cell sediment was secured by a reactant for cell lysis and RNA stabilization (500 µl of QIAzol, Qiagen, Germany). Collected samples were stored at -80°C for further analysis.

Milk fat globules were obtained according to Brenaut *et al.* [2012] with modifications as follows. Samples were centrifuged at 2,000 x g for 10 min at 4°C to isolate the milk fat. The fat layer was transferred to a new 15 mL Falcon® tube using a sterile spatula, then 500 µL of fat was transferred to a new tube, 1.5 mL of QIAzol reagent (Qiagen, Germany) was added, and the whole mixture was vortexed.

For total RNA isolation from goat MSCs and MFGs, the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) was used according to the manufacturer's procedure. Probes were stored at -80°C until further analysis.

Blood samples (5 ml) were taken aseptically from the jugular vein using disposable needles (0.9) and placed into S-Monovette® 9 mL test tubes containing EDTA (Sarstedt AG & Co., Germany). Two ml of blood was mixed with the erythrocyte lysis buffer RBCL (1:5 v/v), incubated on ice for 15 min, and then centrifuged at 3,000 x g for 10 min. After removal of the supernatant, the RNA was isolated using a Total RNA Mini Kit (A & A Biotechnology, Poland), and probes were stored at -80°C until the time of further analysis.

RNA quality assessment

RNA purity and concentration were rated by absorbance readings (A260/A230) and (A260/A280) using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNA quality and integrity were also determined based on capillary

electrophoresis technology using an Agilent 2100 Bioanalyzer (Agilent, USA). The overall quality of the RNA was rated by the RNA Integrity Number (RIN) generated by Agilent Software Expert.

Gene expression microarrays used for profiling

Since microarrays specific to goat were not available, we selected bovine ones for the transcriptomic analyses. The analysis of gene expression profiles was performed using the Bovine (V2) Gene Expression Microarray, 4x44K (Agilent, USA) and the Agilent Technologies Reagent Set, according to the manufacturer's procedures.

The whole experiment was performed using 24 transcriptomic microarrays (on MSCs, MFGs, and PBNCs from PWI and PFI goats) with a common reference (PBNCs pulled from PWI and PFI goats). The common reference comprised a pool of equal amounts of RNA isolated from the blood of seven dry, 3.5-year-old goats of both breeds (Fig. 1).

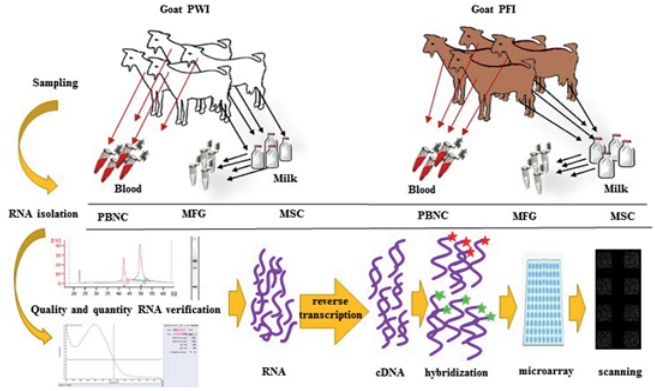

Fig. 1. The scheme of the transcriptomic analysis: PWI – Polish White Improved, PFI – Polish Fawn Improved, PBNC – peripheral blood nuclear cells, MSC – milk somatic cells, MFG – milk fat globules.

The complementary RNA (cRNA) of the common reference samples was labelled with Cy3, and the cRNA of each investigated goat was labeled with Cy5. On each of the two-colour microarrays, we hybridized 825 ng of labelled Cy3 cRNA and 825 ng of labelled Cy5 cRNA. An RNA Spike-In Kit was used as an internal control; Low Input Quick Amp Labelling Kits were applied to amplify and label (with Cy3 or Cy5) the target RNA and generate cRNA for the oligo microarrays; a Gene Expression Hybridization Kit was used for fragmentation and hybridization; and a Gene Expression Wash Buffer Kit was used for washing slides after hybridization (all kits – Agilent Technologies, USA). Hybridization intensities were acquired and analysed using an Agilent G2505C DNA Microarray Scanner. Microarray data were deposited in the Gene Expression Omnibus data repository under the number GSE89693.

A hierarchical sample-based clustering analysis was applied to cluster samples using all the genes as features. To reveal sets of samples in which the closest groups

were adjacent, hierarchical clustering analysis was performed on the conditions using the Euclidean distance approach.

The list of genes that differed in their regulation was generated by comparing the transcriptomic profiles obtained from the PFI goats with those of the PWI goats (each breed 12). In other words, all the transcriptomic profiles originating from the different tissues of one breed were compared with all the transcriptomic profiles from those of the second breed.

Statistical analysis

The statistical analysis of milk production traits was conducted using the SAS/STAT package v.9.4. The analysis of variance with test-day model, including fixed effect of goat breed (i=1,2), year (j=1,3), month (k=1,9) of milk recording, and animal (l=1,20), was conducted using the GLM procedure. Prior to the statistical analysis, the normality of the distribution of all traits was checked using a Univariate procedure (SAS/STAT), and values for the expression of genes at the mRNA level were transformed into natural logarithmic scale for SCS.

Data from the transcriptomic profiling were extracted, and the background subtracted using the standard procedures included in the Agilent Feature Extraction (FE) software version 10.7.3.1. FE performs the Lowess normalization. The statistical analysis was performed using GeneSpring 12 software (Agilent, USA). The transcriptomic profiles of both breeds were compared using an unpaired t-test ($p< 0.05$). A multiple testing correction was performed with the Benjamini and Hochberg False Discovery Rate (FDR) <5%.

Quantitative real-time RT-PCR

To verify the microarray results, the expression of two genes, *Bos taurus* agouti signaling protein mRNA (ASIP-B) and *Capra hircus* agouti signaling protein mRNA (ASIP), was analyzed using real-time PCR. The sequences of the chosen genes were obtained from the Ensembl or NCBI database. Primers were designed using Primer-BLAST software (NCBI database) and were verified using OligoCalc: Oligonucleotide Properties Calculator (free software from Northwestern University, available at http://biotools.nubic.northwestern.edu/OligoCalc.html) to exclude sequences showing self-complementarity. The secondary structures of the amplicons were examined using the Mfold Web Server (free online access at http://unafold.rna.albany.edu/?q=mfold). The reference genes Ribosomal Protein Large, P0 (RPLP0) and 18S ribosomal RNA (18S rRNA – protein synthesis) were chosen based on previously published results [Finot *et al.* 2011]. All primer sequences are listed in Table 1. Total RNA was reverse transcribed to first strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). All analyses were performed on individual samples of total RNA using SYBR Select Master Mix (Applied Biosystems, USA) on a Stratagene Mx3005P Quantitative PCR instrument (Agilent, USA) for RT-PCR, following the manufacturer's protocol. For all genes, the annealing temperature was

58°C. The relative expression of the target gene was quantified as the mean of triplicate measurements for each biological sample. Results were calculated using the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen 2001].

Validation of microarray data

To verify the microarray analysis results using RT-qPCR, *ASIP-B*, *ASIP, RPLP0*, and *18S rRNA* were chosen as reference genes. *ASIP-B* (*Bos taurus* agouti signaling protein) mRNA was not expressed in the analyzed samples, thus we used *Capra hircus* agouti signaling protein mRNA (*ASIP*), which confirmed the expression and direction of changes we determined via the microarray analysis. Due to the different origins of the samples used in the experiment, *RPLP0* and *18S rRNA* were selected as reference genes. For the pooled samples of PBNCs and MSCs, the *18S rRNA* gene was used, and for the MFGs, the *RPLP0* gene was applied. Common analysis of all samples using the GenEx/geNorm program showed the equivalence of both genes, and NormFinder software indicated that *18S rRNA* was the best reference (SD 0.4343) (MultiD Analyses & TATAA BIOCENTER, Sweden).

Table 1. Information about genes and primers used for quantitative real-time RT-PCR verification of microarray results, including gene name and function, NCBI accession numbers used for primer design, and forward and reverse primer sequences

Gene name	Forward primer (5'–3')	Reverse primer (5'–3')	NCBI accession number	
Bos taurus agouti signaling protein, mRNA (*ASIP-B*)	ACTTTCTCCCCCACAAATTC	CCTTTCATCTCTGGGCTTTT	NM_206843.2	target
Capra hircus agouti signaling protein mRNA (*ASIP*)	GCCCAGAGATGAAAGGAACC	TTCCGCTTCATTTCTGCTGA	transcript variant X1-X2: XM_018057735.1 XM_018057736.1	target
Ribosomal Protein Large, P0 (*RPLP0*)	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	NM_001012682.1	Reference genes Finot [2011]
18S ribosomal RNA *(18S rRNA)*	CAAATTACCCACTCCCGACCC	AATGGATCCTCGCGGAAGG	DQ_066896.1	Reference genes Finot [2011]

Results and discussion

Performance analyses confirmed lack of differences in milk fat, lactose content, and milk SCS between the breeds. However, the breeds differed significantly in milk yield and milk protein content (Tab. 2). Daily milk yield was significantly higher in the PWI breed, while protein content was higher in the PFI breed.

Hierarchical clustering of the transcriptomic profiles of selected tissues from the two goat breeds revealed three distinct sub-clusters, representing each sampled biological material (Fig. 2). Thus, the key driver of clustering was the origin of the sample and not the breed of the sampled goats.

Table 2. Least-squares means (±se) for daily milk parameters of two Polish goat breeds

Breed	Daily milk yield	Fat content	Protein content	Lactose content	SCS[1]
Polish White Improved	2.93^{A} ±0.08	3.59 ±0.11	2.96^{A} ±0.04	4.61 ±0.03	5.49 ±0.18
Polish Fawn Improved	2.13^{B} ±0.08	3.39 ±0.13	3.15^{B} ±0.04	4.53 ±0.04	5.48 ±0.22

[1]Somatic cell score – ln of SCS (somatic cell count).
^{AB}Within columns means bearing different superscripts differ significantly at P<0.01.

Table 3. Regulated gene differences-between Polish Fawn Improved and Polish White Improved goat breeds. Comparison was performed using 4 samples from MSCs, 4 from MFGs, and 4 from PBNCs per goat breed

Gene	Description	p (Corr)	Log FC	Entrez Gene ID	Genbank Accession
ASIP	*Bos taurus* agouti signaling protein (ASIP), mRNA	0.0001915	4.24	404192	NM_206843

Comparison between all transcriptomic profiles obtained from the PFI goats and all transcriptomic profiles obtained from PWI goats revealed that the expression of only one gene, coding the *Bos taurus* agouti signaling protein differs between the two goat breeds (Tab. 3).

The two dairy goat breeds are considered to be distinct. Our previous study, conducted on the entire active population of Poland (with data covering 18 years of milk records), showed no statistically significant differences in milk yield, milk fat, or protein content between the breeds (PWI, PFI, Saanen, Alpine) [Bagnicka *et al.* 2015], or in lactose content, though a higher SCS in the milk of PFI goats vs. PWI goats was noted [Bagnicka *et al.* 2016]. In the current study, differences in daily milk yield

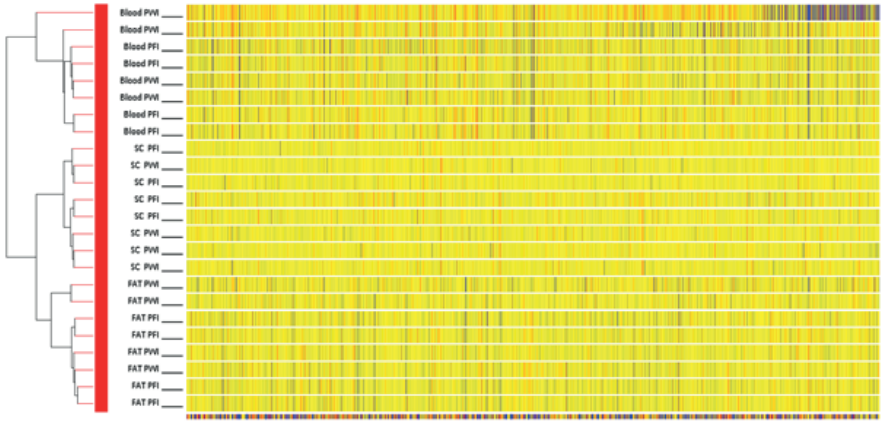

Fig. 2. Hierarchical clustering of gene expression profiles revealed three distinct sub-clusters of samples with similar gene expression, which were in accordance with the origin of the sampled tissues. *PWI – Polish White Improved, PFI – Polish Fawn Improved, Blood – peripheral blood nuclear cells, SC – milk somatic cells, and FAT – milk fat globules.

and protein content in these two breeds were apparent (Tab. 2). Our previous study was conducted on the whole Polish population and included a vast number of data (18,563 lactations of 8,938 goats maintained in 211 commercial herds in 12 breeding regions of Poland). In contrast, the present study was conducted in one experimental herd of a small number of animals, during the third lactation which is characterized (together with the fourth lactation) by the highest milk yield, for three consecutive years [Bagnicka *et al*., 2015]. Moreover, for many years, goats in this herd have been mated with Saanen or Alpine bucks, depending on the breed, which were introduced from different herds. Milk from the Saanen goat herd is marketed for liquid milk, while from the other herd for cheese processing, thus there are two distinct purposes of breeding: milk yield vs. protein content improvement. This distinction could provide the explanation for the differences between the breeds in terms of milk yield and protein content in the experimental herd.

However, the clustered group goat samples (MSCs, MFGs, and PBNCs – Fig. 2) did not show any relationship of the breeds. Consequently, the analytical tool did not recognize the goat breed as a driver of transcriptomic difference. Thus, we concluded that, in addition to the previously described lack of genetic differences between the PWI and PFI breeds [Bagnicka *et al.* 2013], that there was also a lack of genomic differences between the breeds.

This lack of genomic differences was also evident when the differences between the transcriptomic profiles of the two breeds were analysed. The use of bovine, instead of caprine, microarrays could be considered a limitation of the study. However, such an approach was described earlier by Ollier *et al.* [2007], who performed goat RNA hybridization on a bovine oligochip and obtained caprine gene expression. Magalhães-Padilha *et al.* [2013] also chose the Affymetrix Gene Chip Bovine Genome Array and

used the Affymetrix Gene Chip system for their goat transcriptome analyses. On this platform, they were able to investigate the gene expression of approximately 24,000 bovine transcripts, based on the International Goat Genome Consortium [Magalhães-Padilha *et al.* 2013]. It should also be noted that the aim of our study was not to identify specific regulated genes, and that is why the potential interspecies bias is not the key discriminative factor. One can assume that on bovine chips, some hybridizations were non-specific; however, they were non-specific in both goat breeds, and thus do not preclude the use of bovine chips in our study.

In the current study, we were able to find only one significantly regulated gene between the two breeds. Bearing in mind the level of statistical significance accepted for this study, this result may be considered a false-positive. The regulated gene was the bovine gene of the agouti signaling protein, which is involved in the determination of coat colour and is conserved in many species [Fontanesi *et al.* 2010]. In goats, mutations in the agouti signaling protein ASIP gene could also influence coat colour [Fontanesi *et al*. 2009, Adefenwa *et al.* 2013]. Therefore, this study confirmed that in fact, the PFI and PWI goats differed only in the expression of the gene involved in their coat pigmentation.

The results of our study support the idea that greater differences in gene expression occur between the types of obtained samples than the breed of dairy goat. Moreover, those results could aid in future research dedicated to e.g., the genes responsible for goat milk production. Hierarchical clustering showed that the expression of the MSC genes is different from that of the MFG genes, differing by more than 3,500 genes. Additional studies may answer questions about the possibility of testing the expression of genes affecting dairy and production yields, milk fat profile, and other parameters using non-invasive genetic material extraction methods.

Further research is necessary to identify the potential polymorphism of the agouti signalling protein gene between these two breeds, since the difference in gene expression could be the result of a polymorphism or a mutation'.

Considering that both breeds have been subjected to single-track selection for productivity traits for many years, we suggest that Polish dairy goat breeds do not differ in the genes involved in milk production traits or health status of the mammary gland. Thus, regardless of breed, the dairy goats selected as the parents of the next generation will exhibit similar expression levels of genes influencing their productivity and health.

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