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RNA-seq based SNP discovery in liver transcriptome of Polish Landrace pigs

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Abstract

Background: RNA-seq technology is most commonly used in quantitative measurement of gene expression levels and identification of non-annotated transcripts. It is also used for the coding SNPs (cSNPs) discoveries in an efficient and cost-effective way. The aim of this study was to identify the putative genetic cSNPs variants in liver transcriptome of Polish Landrace pigs fed with high and low (normal) omega-6 and omega-3 polyunsaturated fatty acids (PUFAs) diets.

Methods. RNA-seq based NGS experiment was performed on Polish Landrace pigs fed with high and low diets. Total RNA were isolated from liver tissues ofPUFAs dietary of Polish Landrace pigs. The RNA-seq libraries preparations were performed by mRNA enrichment, mRNA fragmentation, second strand cDNA synthesis, adaptor ligation, size selection and PCR amplification using the illumina TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego CA, USA), followed by NGS sequencing on MiSeq illumina platform. The quality control (QC) of raw RNA-seq data of liver transcriptome was performed using the Trimmomatic and FastQC tools. The paired-end mapping of the liver transcriptome RNA-seq data (n=12) was performed on the reference genome *Sus scrofa* v.10.2, followed by cSNPs discovery using GATK and SAMtools bioinformatics SNPs caller tools.

Results: Two pooled paired-end libraries of 151bp liver transcriptome of Polish Landrace pigs were generated from MiSeq instrument and subsequent Fastq RNA-seq data were submitted to NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra). Our study identified 25.3 million paired-end reads: representing 13,509,248 paired-end reads of high PUFAs dietary group and 11,815,696 paired-end reads of low PUFAs dietary group liver transcriptome. The SNP discovery results revealed identification of 25909 homozygous and 23290 heterozygous cSNPs in the liver transcriptome of both dietary groups of Polish Landrace pigs. With regards to same or alternative SNPs alleles encoding amino acids regions, a total of 27141 synonymous cSNP and 5989 non-synonymous cSNPs were identified in liver transcriptome representing high PUFAs dietary group. However, a total of 15128 synonymous cSNPs and 3900 non-synonymous cSNPs were identified in liver transcriptome representing low PUFAs dietary groups of Polish Landrace pigs. The identification of single nucleotide variations (SNVs) representing substitutions of all four possibilities (A,T,G,C) were ranged 2872 to 6868 SNVs (high PUFAs) and 2574 to 3654 SNVs (low PUFAs) in the homozygous cSNPs and 2452 to 2678 SNVs (high PUFAs) and 2094 to 2230 SNVs (low PUFAs) in the heterozygous cSNPs of liver transcriptomes of Polish Landrace pigs, respectively.

Conclusions. Study concluded that identification of cSNPs dataset representing the liver transcriptome of Polish Landrace pigs fed with a control diet (low) and pigs fed with a PUFAs diet (high) may be helpful to develop a new set of genetic markers for trait-associated studies (*viz.*, growth and metabolic traits) specific to Polish Landrace pig breed. Such cSNP markers eventually can be utilized in the genetic improvement of the pig production traits using the genome-wide association studies (GWAS) and to finally implement on marker assisted selection (MAS) and genomics selection (GS) program in active breeding population of Polish Landrace pigs in Poland.

Keywords: Single nucleotide variations; SNPs; genetic markers; RNA-seq; illumina; paired-end read; GATK; SAMtools; mapping; bioinformatics; NGS; transcriptome; liver; Landrace; pig; Omega-6 and omega-3 polyunsaturated fatty acids.

Background

High-throughput next-generation genome sequencing (HT-NGS) based RNA-seq studies in past decade, allow us to detect numerous *de novo* and reference-based single nucleotide polymorphisms (SNPs) discoveries in cattle and pig domesticated animals [1–9]. The SNPs detection in any transcriptome are the single nucleotide variations (SNVs) caused by transitions (C/T or G/A) or transversions (C/G, C/A, or T/A, T/G) in the genome [10–11]. In RNA-seq studies, SNPs and differentially expressed genes (DEGs) markers are the most common type of DNA markers of genetic variation observed in the intergenic genes, coding (exon) genes, or non-coding introns, 5'UTR, 3'UTR, or exon-intron splicing sites genes [12–14]. SNPs in the coding region are known as cSNPs. There are two types cSNPs: i) Synonymous cSNPs are those SNPs that have different alleles that encode for the same amino acid; ii) Non-synonymous cSNPs are SNPs that have different alleles that encode different amino acids. In mammals, synonymous cSNPs (affecting coding region) and nonsynonymous cSNPs (affecting protein sequence) have considerable effects on protein function and gene expression, particularly at the regulatory coding regions. Therefore, cSNP as a genetic marker has great impact and significance in genetics and breeding studies in veterinary sciences [3–4]. Due to the high density, scalability in the genome and genome-wide distribution, SNPs are the best genetic

markers to characterize the genetic resources and functional genes associated with economic traits [15] and to identify the trait-associated variation within the domesticated animals [7–9], as well as, to discover genes linked to complex genetic traits in human [16–17]. The availability of wide range of NGS bioinformatics tools for RNA-seq technique provides a convenient approach to discover all SNPs and obtain relevant information on genomic position and genotyping in a single step. RNA-seq data from reliable large-scale sequencing, especially in domesticated animals could improve the cost-effectiveness and efficiency of detection of abundant SNPs. Several bioinformatics tools have been used for initial SNPs discovery in a high-throughput manner, such as whole-genome sequencing, exome capture, RNA sequencing, methylated DNA sequencing, and restriction enzyme (RE) digestion [18–19]. In the presented study, we performed the transcriptome sequencing of porcine liver using one of the most effective HT-NGS based RNA-seq method to discover wide range of cSNPs variations. The potential advantages of SNP discoveries using RNA-seq data are to identify of thousands of cSNPs and its expression levels of functional genes with sequence variations at a reasonable cost; to identify genetic variations in coding regions associated with the economic trait of domesticated animals [20]; as well as, to perform the transcriptome studies for the gene characterization, gene expression quantification and post translational process analysis [21]. In this study, we have performed the transcriptome sequencing of the liver of Polish Landrace pigs fed with high and low PUFAs diets using MiSeq illumina NGS platform for the SNPs discoveries.

Results

Raw RNA-seq data of high PUFAs and low PUFAs dietary group: Based on the high PUFAs and low PUFAs dietary groups of pooled total RNA samples of Polish Landrace pigs' liver tissues, TruSeq-type libraries were paired-end sequenced using the Illumina MiSeq genomic sequencing platform. The obtained raw RNA-seq data in the form of two *.fastq* format representing high PUFAs and low PUFAs dietary group of Polish

Landrace pigs liver transcriptome were submitted to NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra).

Transcriptome mapping of the Polish Landrace pig liver to the reference genome: Transcriptome sequencing of two pooled liver samples of Polish Landrace pigs were performed by Illumina MiSeq™ NGS device and paired-end read lengths of 151bp were sequenced. For each pooled liver sample of high PUFAs and low PUFAs dietary group of Polish Landrace pigs, the processed *fastq* data were mapped to the Sus scrofa genome in version 10.2 (https://may2017.archive.ensembl.org/Sus_scrofa/Info/Index). A summary of the paired-end fragments counts of liver transcriptome of Polish Landrace pigs is shown in Table 1. A total of 25.3 million paired-end reads were identified in the liver transcriptome of Polish Landrace pigs. Obtained results revealed a total of 13,509,248 paired-end reads in the high PUFAs dietary group and a total of 11,815,696 paired-end reads in the low PUFAs dietary group of Polish Landrace pig. However, proportion of counted (approximately 73%) and uncounted (approximately 27%) fragments were equally distributed in both high and low PUFAs dietary groups of Polish Landrace pigs (Table 1).

Table 1. Paired-end fragments count and read count of liver transcriptome of Polish Landrace pigs

Mapping parameters	High PUFAs group		Low PUFAs group	
	Paired-end fragments	%	Paired-end fragments	%
Counted fragments	4,906,123	72.63	4,319,199	73.11
Uncounted fragments	1,848,501	27.37	1,588,649	26.89
Total fragments	6,754,624	100.00	5,907,848	100.00
Reads mapped in pairs	9,812,246	72.63	8,638,398	73.11
Reads mapped in broken pairs	381,668	2.83	339,171	2.87
Reads not mapped	3,315,334	24.54	2,838,127	24.02
Total	13,509,248	100.00	11,815,696	100.00

Mapping statistics of liver transcriptome of Polish Landrace pig is analyzed and presented in Table 2. Results revealed that liver transcriptome of Polish Landrace pig was successfully mapped to the Exon-exon and Exon-intron boundaries, total exon, total intron and total genes of the *sus scrofa* reference genome (Table 2).

Table 2. Detailed mapping statistics of the liver transcriptome of Polish Landrace pig

RNA pools groups	High PUFAs group				Low PUFAs group			
	Mapped	% of total mapped	Uniquely mapped	Non-specifically mapped	Mapped	% of total mapped	Uniquely mapped	Non-specifically mapped
Exon-exon	2,605,211	53.10	2,396,880	0.92	2,383,713	55.19	2,222,284	0.93
Exon-intron	68,811	1.40	63,095	0.92	36,443	0.84	34,067	0.93
Total exon	4,638,432	94.54	4,185,515	0.90	4,139,989	95.85	3,758,643	0.91
Total intron	267,691	5.46	215,861	0.81	179,210	4.15	140,398	0.78
Total genes	4,906,123	100.00	4,401,376	0.90	4,319,199	100.00	3,899,041	0.90

Data filtration using Microsoft Excel and SNP discoveries: The processed paired-end reads of 151bp were assembled into contigs to the reference sus scrofa genome using the GATK [30] and SAMtools [31] pipelines to detect the novel cSNP in liver transcriptome of Polish Landrace pigs. The detailed obtained results are presented in two supplementary tables (sTable S1 and sTable S2) and further summarized in Table 3. According to Table 3, a total of 25909 and 23290 cSNP were observed in the high and low PUFAs dietary group liver transcriptome of Polish Landrace pigs, respectively. The number of heterozygous cSNPs were lower (11109 and 9256, respectively) than homozygous cSNPs (14800 and 14034, respec-

tively) in liver transcriptome of both dietary groups of Polish Landrace pig. With regards to the same or alternative SNPs alleles coding amino acids regions, a total of 27141 synonymous cSNPs and 5989 non-synonymous cSNPs were identified in liver transcriptome representing high PUFAs dietary group. Whereas, a total of 15128 synonymous cSNPs and 3900 non-synonymous cSNPs were identified in liver transcriptome representing low PUFAs dietary groups of Polish Landrace pigs. Lastly, the SNVs representing substitutions of all four possibilities (A,T,G,C) were ranged 2872 to 6868 SNVs (high PUFAs) and 2574 to 3654 SNVs (low PUFAs) in the homozygous cSNPs and 2452 to 2678 SNVs (high PUFAs) and 2094 to 2230 SNVs (low PUFAs) in the heterozygous cSNPs of liver transcriptomes of Polish Landrace pigs, respectively (Table 3).

Table 3. Summary of SNP discoveries statistics in liver transcriptome of Polish Landrace pigs, based on the large dataset results illustrated in supplementary files sTable S1 and sTable S2

cSNPs category	cSNPs statistics	Identified cSNPs in high PUFAs group (sTable S1)	Identified cSNPs in low PUFAs group (sTable S2)
Homozygous cSNPs	Insertion	822	798
	Deletion	634	583
	SNV	13344	12653
	Total homozygous cSNP	14800	14034
	cSNP changing coding region (synonymous SNPs)	10317	9611
	cSNP changing amino acid (nonsynonymous SNPs)	2078	2176
	SNV: T,G,C>A	3098	2788
	SNV: A,T,G>C	4015	3654
	SNV: A,T,C>G	6868	3637
	SNV: A,G,C>T	2872	2574

Table 3. Summary of SNP discoveries statistics (continuation)

cSNPs category	cSNPs statistics	Identified cSNPs in high PUFAs group (sTable S1)	Identified cSNPs in low PUFAs group (sTable S2)
Heterozygous cSNPs	Insertion	403	313
	Deletion	419	302
	SNV	10287	8641
	Total heterozygous cSNP	11109	9256
	cSNP changing coding region (synonymous SNPs)	16824	5517
	cSNP changing amino acid (nonsynonymous SNPs)	3911	1724
	SNV: T,G,C>A	2621	2230
	SNV: A,T,G>C	2536	2094
	SNV: A,T,C>G	2452	2116
	SNV: A,G,C>T	2678	2201

Discussions

Aim of the presented study was to investigate the liver transcriptome and to explore the genetic variability in terms of SNPs/SNVs in the Polish Landrace pigs fed with PUFAs diets. Our results provide novel cSNP transcriptomic data (https://www.ncbi.nlm.nih.gov/sra) of Polish Landrace breed to explain the regulatory mechanisms of the liver tissue's biological response to increased concentration of PUFAs diets [22]. Related studies showed that consumption of PUFAs and other fatty acids diets were genetically regulated the growth performance and immune system in pigs [23]. Our mapping results identified a total of 25.3 million paired-end reads (13.5 million in high PUFAs and 11.8 million low PUFAs group) in the liver transcriptome of Polish Landrace pig, in comparison to 50.5 million paired-end reads (18 million in high PUFAs and 32.5 million low PUFAs group) in *gluteus medius* muscle transcriptome of Polish

Landrace pig [24]. In an another study on the domestic Nero Siciliano male pig in Brazil [25], 346.8 million paired reads were identified and after QC check 99.03% of the reads were successfully mapped to the reference genome. Moreover, they mapped the RNA-seq data a male domestic Nero Siciliano pig on pig reference genome *Sus scrofa11.1* and identified over 11 million SNPs, including 6,747 genetic variants at a rate of 1 variant every ~276 bases, and among these variants 1,132 were novel to the dbSNP151 database [25]. Our SNP detection results identified 25909 and 23290 cSNPs in the high and low PUFAs dietary group of liver transcriptome, in comparison to 27404 and 36676 cSNPs in the high and low PUFAs dietary group of *gluteus medius* muscle transcriptome [24]. Finally, with regards to the same or alternative SNPs alleles encoding amino acids regions, 27141 synonymous cSNPs and 5989 non-synonymous cSNPs were identified in high PUFAs dietary group of liver transcriptome, in comparison to 18701 synonymous and 4389 non-synonymous cSNPs, respectively in *gluteus medius* muscle transcriptome [24]. Whereas, 15128 synonymous cSNPs and 3900 non-synonymous cSNPs were identified in the low PUFAs dietary groups liver transcriptome in comparison to 24491 synonymous and 5879 non-synonymous cSNPs, respectively in *gluteus medius* muscle transcriptome of Polish Landrace pigs [24].

Conclusions

In our study, we have identified two cSNPs dataset of liver transcriptome of Polish Landrace pigs fed with a control diet (low) and fed with a PUFAs enriched diet (high). Identification of cSNPs in liver may be helpful to develop a set of candidate genetic markers for growth traits specific to Polish Landrace pig breed, which eventually can be utilized in genome-wide association studies (GWAS), and to finally implement on marker assisted selection (MAS) and genomics selection (GS) program in active breeding population of Polish Landrace pigs in Poland.

Materials and Methods

Research materials: The liver tissues samples of Polish Landrace pigs for RNA-seq based NGS experiment were selected on the basis of fatty acid profiles in order to compare the liver transcriptomes characterized by low (control groups) and high (PUFAs enriched diet groups) omega-6/omega-3 fatty acids ratio in Polish Landrace pigs. The threshold value of < 7 for low and > 7 for high omega-6/omega-3 fatty acids ratio was used in accordance to previous study [22]. After slaughtering, the liver tissues were collected at the Institute IGHZ, Jastrzębiec and were frozen in liquid nitrogen and stored at −80°C for further RNA extraction. The number of samples used in RNA-seq based NGS experiment were 6 pigs each for high and low PUFAs group of Polish Landrace purebred.

Research methods: The detailed laboratory procedures of RNA extractions [26], library preparations, and MiSeq sequencing, as well as detailed bioinformatics analysis procedures [27-31], including analysis of the RNA-seq data [27-29], transcriptome mapping, and SNP detection [30-31] were described in the Pierzchala *et al.* (2019) paper of this issue [24].

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files (sTable S1 and sTa-

ble S2). The transcriptome sequencing raw Fastq data has been deposited in NCBI SRA web resources (https://www.ncbi.nlm.nih.gov/sra).

Supplement file 1 (sTable S1):
https://box.pionier.net.pl/f/ccc835ea242d4fb8b754/?dl=1
Supplement file 2 (sTable S2):
https://box.pionier.net.pl/f/fff40eabf53a4f0b9c42/?dl=1

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