SNP Discovery of Chicken Liver with Divergent Unsaturated Fatty Acid using Next Generation RNA Sequencing

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ABSTRAK

RNA sequencing memberikan peluang baru untuk mendeteksi variasi SNP (Single Nucleotide Polymorphism) pada perbedaan jaringan dengan perbedaan fenotipe. Tujuan dari penelitian ini adalah untuk mengkarakterisisasi penemuan SNP terbaru terkait perbedaan asam lemak tak jenuh pada ayam dengan menggunakan RNA sequencing. Sebanyak 6 sampel dipilih dari 62 sampel masing-masing 3 sampel tinggi dan 3 sampel rendah yang merepresentasikan perbedaan fenotip yang kontras terkait asam lemak tak jenuh dianalisis dengan menggunakan RNA Sequensing. Hasil identifikasi SNP memperlihatkan sebanyak 1208 SNP pada sampel tinggi dan rendah setelah disejajarkan dengan genom ayam Gallus gallus (GGA) v4.0. Sekitar 91% dari total SNP yang ditemukan memiliki tingkat polimorfisme yang tinggi pada 5 gen yang ditemukan terkait asam lemak yaitu gen SCD, COL6A2, CYP2J2L4, HSD17B4, dan SLC23A3. Gen SCD, HSD17B4, dan SLC23A3 memiliki jumlah titik mutasi dengan jumlah yang paling tinggi masing-masing berturut-turut 18, 13, dan 12 SNP. Tingkat level signifikan yang tinggi dan peranan dari ketiga gen tersebut yang sangat penting terkait komposisi asam lemak mengindikasikan bahwa gen SCD, HSD17B4, dan SLC23A3 merupakan tiga gen baru dan potensial untuk digunakan sebagai penanda seleksi kandungan asam lemak tak jenuh tinggi. Namun, hasil penelitian ini perlu divalidasi dan dikonfirmasi sebagai potensial kandidat gen dalam jumlah ayam yang lebih besar dan *breed* yang berbeda.

Kata kunci: asam lemak, ayam, RNA-Seq, variasi transkriptomik

ABSTRACT

RNA sequencing (RNA-Seq) reveals new opportunity for identification SNP discovery in different tissues with divergent phenotype. The objective of this study was to characterize SNP profile from divergent unsaturated fatty acids using RNA-Seq. Six liver samples were selected from 62 chicken which classified 3 high and 3 low unsaturated fatty acids were analyzed using RNA-Seq. The SNP identification showed 1208 SNPs in chicken samples and a large number of those corresponded to differences between high and low chicken genome assembly Gallus gallus (GGA) v4.0. Among them, about 91% of genes had multiple polymorphisms within 5 genes (SCD, COL6A2, CYP2J2L4, HSD17B4, and SLC23A3). The SCD, HSD17B4, and SLC23A3 contained the largest number of mutations with 18, 13, and 12 SNPs respectively. Combining the significant level of SNPs and gene function related with fatty acid composition allow us to suggest SCD, SLC23A3, HSD17B4 as the three novel and promising candidate genes for selecting unsaturated fatty acids. However, further validation is required to confirm the effect of these candidate genes in larger chicken populations.

Keywords: chicken, fatty acids, RNA-Seq, transcriptome variants

INTRODUCTION

Fatty acid composition plays an important role in meat quality, not only in the nutritional value, but also in the flavor quality of meat (Yu *et al.,* 2013). Fatty acids, especially unsaturated fatty acids (UFA) that consist of monounsaturated fatty acids (MUFAs) and some polyunsaturated fatty acids (PUFAs) are recognized as health benefits. The ability to genetically select for animal that better suit a consumers needs in term of high unsaturated fatty acids (UFA) that can be predicted would benefit the chicken industry. A candidate gene approach through single nucleotide polymorphisms (SNPs) analysis has been already successfully applied to identify several DNA markers (Van der Steen & Plastow 2005). SNPs are co-dominantly inherited, strong and enough marker to study diversity acceptable and adaptable for genome-scan association studies (Salem *et al.,* 2012; Wang *et al.,* 2008). The largest number of sources genetic variation and are connected with heritable differences among individuals could be detected by SNPs characterization (Aslam *et al.,* 2012; Suh *et al*., 2005). Additionally, SNPs are very important because they might cause a modification in protein function and associated with the phenotype. Detection of single nucleotide polymorphisms (SNPs) within genes affecting fatty acid compositions and their association, linkage analysis, and gene expression is the important and commonly applied tools to characterize candidate genes. Recent developments of the next-generation sequencing technologies playing an important role in fast discovery of next generation transcriptomes sequencing (RNA-Seq) and SNPs at accurate and affordable scale (Wang *et al.,* 2008).

RNA-Seq generates sequences on a very large scale at a fraction of the cost required for traditional Sanger sequencing, allowing the application of sequencing approaches to biological questions that would not have been economically or logistically practical before (Mortazavi *et al.* 2008). RNA-Seq platforms were applied in different studies to accomplish and fulfill highly inessential coverage of the genome, a necessity for high quality genome-wide SNP discovery in the complex genomes of animals and plants (Gunawan *et al.,* 2013a & 2013b; Kilian & Graner 2012; You *et al.* 2011). A few studies have been published that explored genetic variant in chicken genetic resources, especially that related to fatty acids effects in chicken. Genetic variation is responsible for variation in phenotypic traits, as a result, the determination of genetic variation in traits of economic importance such as fatty acid composition is considered as one of the main targets of livestock genomic research (Lin *et al.,* 2015). Taking this into account, we applied this novel approach to identify SNPs in the expressed coding regions of the chicken liver transcriptome with a divergent unsaturated fatty acid. The aim of this study was to characterize the SNPs discovery analysis in the unsaturated fatty acids transcriptome using RNA-Seq. The novel polymorphisms using RNA deep sequencing could reveal potential candidate genes affecting unsaturated fatty acids in chicken. It is presumed that in future these polymorphisms could be used as markers for chicken related to divergent unsaturated fatty acids.

MATERIAL AND METHODS

Animal and Phenotypic Data Collection

The carcass and meat quality data were collected according to guidelines of the Indonesian performance test. Sixty two Indonesian crossbred chickens (F2 Kampung × Broilers) were used in this study for fatty acid composition analysis. They were reared under the same feeding conditions until 12 weeks old and had approximately 1.6 kg of slaughter weight per chicken. Tissue samples from muscle and liver will be frozen in liquid nitrogen immediately after slaughter and stored at -80°C until used for RNA extraction. Total lipids in each sample were extracted from breast muscle (BM) using chloroform-methanol (2:1) according to the procedure of Folch *et al.* (1957). FA methyl esters were prepared from the extracted lipids with BF3-methanol (Sigma-Aldrich, St. Louis, MO, USA) and separated on a HP-6890N gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) as described previously (Jeong *et al.,* 2010).

RNA-Seq Library Construction

Six chicken with extreme high (n=3) and low (n=3) fatty acids level were selected from the 62 chickens. RNA was isolated from the liver tissues of 3 chicken (HUSFA, high unsaturated fatty acids group) with high PUFA and MUFA (57.06±2.16 %) and 3 chicken (LUSFA, low

unsaturated fatty acids group) with low levels of PUFA and MUFA (51.34 ± 1.25%) (Table 1). Total RNA was extracted using RNeasy Mini Kit according to manufacturer's recommendations (Qiagen). Total RNA was treated using on-column RNase-Free DNase set (Promega) and quantified using a spectrophotometer (NanoDrop, ND8000, Thermo Scientific). The RNA quality was assessed using an Agilent 2100 Bioanalyser and RNA Nano 6000 Labchip kit (Agilent Technologies).

Table 1. Fatty acid profile of F2 Kampung × Broilers

Parameter	Total (62)		Low (3)		High (3)	
	Mean	SD	Mean	SD	Mean	SD
Fat content (%)	4.61	1.56	2.29	0.94	5.41	1.38
Saturated fatty acid (%)	20.72	1.64	17.11	0.55	21.40	0.61
Unsaturated fatty acid (%)	55.05	2.18	51.34	1.25	57.06	2.16

RNA deep sequencing technology was used to obtain differential expression, polymorphism, and alternative splicing detection. For this purpose, a full-length cDNA library was constructed from 1 µg of RNA using the SMART cDNA Library Construction Kit (Clontech, USA), according to the manufacturer's instructions. Libraries of amplified RNA for each sample was prepared following the Illumina mRNA-Seq protocol. The library preparations were sequenced on an Illumina HiSeq 2500 as single-reads to 100 bp using 1 lane per sample on the same flow-cell (first sequencing run) at Macrogen, South Korea. All sequences are analyzed using the CASAVA v1.7 (Illumina, USA).

RNA-Seq Analysis and SNP Detection
Genome *Gallus gallus* 4.0 (http://www.ncbi.nlm.nih.gov/genome/guide/cow /index.html) were used as the annotated references to produce short sequence reads (36-40 bp) by mapping and assembly using CLC Genomics Workbench software (CLC Bio, Aarhus, Denmark). RNA-Seq and SNP discovery analyses were conducted from each sample sequencing reads (n=6 sample polled). Stringent criteria were implemented in order to reduce the rate of identification of false-positive SNPs. For the assembly step, the sequences were annotated to the consensus genome accounting for a maximum of two gaps or mismatches in each sequence. SNP identification was analyzed using the following quality and significance filters according to Abuzahraa *et al.* (2018).

Gene Variation Analysis
Gene variation analysis was analyzed on the mapping files generated by TopHat algorithm using same tools mpileup command and associated algorithms. Variants with criteria a minimum Root Mean Square (RMS), mapping quality of 20 and a minimum read depth of 100 were chosen for further analysis. The selected variants were cross-checked against dbSNP database to identify mutations that are already studied. To understand high and low unsaturated fatty acid sample or in both groups, we have calculated the coverage/quality depth of polymorphisms detected in highly polymorphic Differentially expressed genes (DEGs).

RESULTS AND DISCUSSION

Genetic Variation and SNP Detection
The variant discovery detection applied to sequence chicken was the pooled samples analyses and it revealed 1208 SNPs in 116 candidate genes. In the results, it was observed that most of the SNPs were in downstream gene variant with the maximum number of 1208 SNPs (Table 2). The RNA-seq data allowed to enhance SNP variants than whole-genome sequencing in coding exons (12%), intergenic (16%), downstream (30%), untranslated regions (3′UTRs; 9%), upstream (28%), and introns (2%). Specifically, in Illumina Genome Hiseq 2500 assigned 143 SNPs to exons. Only 47 of these SNPs in the exons were non-synonymous. The resulting SNPs were highly abundant in these eight categories (Table 2). Although the libraries for RNA-seq are supposed to be enriched for mRNAs via poly(A)+ selection,

a certain amount of immature pre-mRNA, which carries the introns, usually infiltrates into the libraries. Exonic compose a much larger fraction of the chicken genome than intron regions. Those two facts mainly elucidate why so many SNPs exist in exonic than in regions introns. Low coverage of short reads leads usually to low quality of intergenic and intronic regions. Only a small fraction, 16%, of SNPs fell into intergenic regions, which compose 84% of the chicken genome.

Table 2. Descriptive statistics results from the Variant Effect Predict (VEP) tool Ensembl of SNPs identified in chicken

Category	Count	Percent (%)
sample size	N=6	-
SNP	1208	-
intron_variant	26	2.15
3_prime_UTR_variant	110	9.11
downstream_gene_variant	366	30.30
missense_variant	47	3.89
intergenic_region	190	15.73
upstream_gene_variant	340	28.15
synonymous_variant	96	7.95
non_coding_exon_variant	33	2.73

The quantification process of the transcriptome, RNA-Seq approach supply beneficial information concerning gene polymorphisms that may directly correspond with the relevant phenotype (Gunawan *et al.* 2013a and 2013b). SNP discovery was achieved in chicken, a comparative analysis was carried out. In total, 1208 SNPs were concordant in chicken (Table 2). After SNP calling, a functional class was allocated to each SNP and provided some fields of information describing the affected transcripts and proteins were provided, if applicable. The explained SNPs identified in this study can serve as useful genetic tools and as candidates in searches for phenotype-altering DNA and transcript differences (Stothard *et al.* 2011). According to the present finding the location of SNPs should be within 3'UTR, or 5'UTR that have the potential to be causative mutations. Designing a focused SNP panel with causative mutations may be advantageous over SNPs selected to be of equal distance across the chromosomes on the current SNP-chip platforms and linkage disequilibrium could not affect such types of mutations, and can be helpful for selection in proceeding generations, and also in across breeds (Fortes *et al.* 2014). It should also be distinguished, that the *Gallus gallus* reference genome was used in the present study; consequently, the development of a *Gallus gallus* reference genome could greatly improve results from SNP discovery studies.

Variant Characterization and Candidate Genes

The distribution of SNPs number and selected SNPs as potential candidate gene for selecting unsaturated fatty acid shown in Figure 1 and 2, respectively. The results showed that about 91% of genes had multiple polymorphisms (Figure 1). Combining the significant level of SNPs and and gene function related with fatty acid composition allow us to suggest SCD, COL6A2, CYP2J2L4, HSD17B4 and SLC23A3 as the 5 novel and promising candidate genes for selecting unsaturated fatty acids (Figure 2). This study revealed 52 SNPs in 5 highly polymorphic DEGs from chicken liver tissues (Table 3). The average number of SNPs per gene was 10 SNPs (Figure 2). The fewest numbers of SNPs were COL6A2, with four polymorphism detection. Interestingly, the HSD17B4, SLC23A3, and SCD genes showed the highest polymorphic SNP count difference between high and low unsaturated fatty acid. The HSD17B4, SLC23A3, and SCD gene contained the largest number of mutations with 18, 13, and 12 SNPs respectively. A large amount of data was generated in this study, a detailed description of the SNPs is available from the authors upon request.

Table 3. . Polymorphisms detected in highly polymorphic DEGs

Gene_Name	Refseq ID	Position	Reference	Alternate	High Mean Coverage	High Mean Qual	Low Mean Coverage	Low Mean Qual	Sample Group	SNP Classification
HSD17B4	NC_006127.4	71547053	A	C	220	226	203	226	High and Low	3_prime_UTR_variant
HSD17B4	NC_006127.4	71547233	C	T	0	0	771	222	Low	3_prime_UTR_variant
Gene	**RefSeq ID**	**Position**	**Reference**	**Alternate**	**High Mean Covergae**	**High Mean Quality**	**Low Mean Coverage**	**Low Mean Quality**	**Sample group**	**SNP Clasification**
HSD17B4	NC_006127.4	71547258	T	C	0	0	1088	222	Low	3_prime_UTR_variant
HSD17B4	NC_006127.4	71556928	T	C	585	222	1344	223.5	High and Low	synonymous_variant
HSD17B4	NC_006127.4	71559009	T	C	0	0	960	222	Low	synonymous_variant
HSD17B4	NC_006127.4	71561440	G	A	409	225	879	224	High and Low	synonymous_variant
HSD17B4	NC_006127.4	71561479	T	C	446.5	225	991.66667	224	High and Low	synonymous_variant
HSD17B4	NC_006127.4	71561894	G	A	0	0	577	221	Low	synonymous_variant
HSD17B4	NC_006127.4	71561897	C	T	0	0	550	222	Low	synonymous_variant
HSD17B4	NC_006127.4	71561918	C	T	462.5	225	865.33333	224	High and Low	synonymous_variant
HSD17B4	NC_006127.4	71578047	T	C	573	228	0	0	High	synonymous_variant
HSD17B4	NC_006127.4	71579563	C	T	0	0	670.33333	225	Low	synonymous_variant
HSD17B4	NC_006127.4	71580587	G	A	258	228	0	0	High	synonymous_variant
HSD17B4	NC_006127.4	71580663	T	C	295	228	589	222	High and Low	synonymous_variant
HSD17B4	NC_006127.4	71580786	G	A	137	228	0	0	High	synonymous_variant
HSD17B4	NC_006127.4	71588060	G	A	264.5	225	764.5	223.5	High and Low	splice_region_variant
HSD17B4	NC_006127.4	71591056	G	A	226	225	486.66667	223	High and Low	synonymous_variant
HSD17B4	NC_006127.4	71595645	C	T	196	228	505.33333	226	High and Low	synonymous_variant
SCD	NC_006093.4	17491335	T	C	2194	222	0	0	High	intergenic_region
SCD	NC_006093.4	17491420	C	T	882	222	0	0	High	intergenic_region
SCD	NC_006093.4	17491496	T	A	374	222	0	0	High	intergenic_region
SCD	NC_006093.4	17491579	C	T	263.5	223.5	0	0	High	intergenic_region
SCD	NC_006093.4	17492385	G	A	336	222	0	0	High	intergenic_region
SCD	NC_006093.4	17492542	G	C	287	223.5	200	225	High and Low	intergenic_region
SCD	NC_006093.4	17493047	C	T	124	116	0	0	High	intergenic_region
SCD	NC_006093.4	17493192	C	T	0	0	360	222	Low	intergenic_region
SCD	NC_006093.4	17493398	A	G	165.5	223.5	214.5	223.5	High and Low	intergenic_region
SCD	NC_006093.4	17493468	G	C	151	225	191	225	High and Low	intergenic_region
SCD	NC_006093.4	17493578	A	G	0	0	192	222	Low	intergenic_region
SCD	NC_006093.4	17493606	A	T	0	0	196	222	Low	intergenic_region
SLC23A3	NC_006094.4	22383074	T	A	0	0	116	225	Low	upstream_gene_variant
SLC23A3	NC_006094.4	22383837	A	G	122.5	222	127.66667	225	High and Low	upstream_gene_variant
SLC23A3	NC_006094.4	22384526	A	T	100	222	0	0	High	upstream_gene_variant
SLC23A3	NC_006094.4	22384741	C	A	0	0	113	225	Low	upstream_gene_variant
SLC23A3	NC_006094.4	22384840	C	T	133.6666667	226	159	228	High and Low	upstream_gene_variant
SLC23A3	NC_006094.4	22384847	G	A	0	0	206	222	Low	upstream_gene_variant
SLC23A3	NC_006094.4	22385124	A	G	239	222	235	226.5	High and Low	upstream_gene_variant
SLC23A3	NC_006094.4	22385170	C	T	0	0	268.5	223.5	Low	upstream_gene_variant
SLC23A3	NC_006094.4	22385270	A	C	0	0	298.5	223.5	Low	upstream_gene_variant
SLC23A3	NC_006094.4	22385429	A	G	0	0	245	222	Low	upstream_gene_variant
SLC23A3	NC_006094.4	22385432	A	G	277	222	223	225	High and Low	upstream_gene_variant
SLC23A3	NC_006094.4	22385481	C	T	0	0	222	225	Low	Upstream gene variant
SLC23A3	NC_006094.4	22385690	A	C	223.5	222	165.5	225	High and Low	Upstream gene variant
COL6A2	NC_006094.4	6789335	C	T	111	222	102	225	High and Low	synonymous variant
COL6A2	NC_006094.4	6789794	C	T	116	222	0	0	High	3_prime_UTR_variant
COL6A2	NC_006094.4	6789919	T	C	0	0	141	225	Low	3_prime_UTR_variant
COL6A2	NC_006094.4	6790186	GCCC	GCC	204	222	198	222	High and Low	3_prime_UTR_variant
CYP2J2L4	NC_006095.4	26753228	A	G	0	0	132	222	Low	downstream_gene_variant
CYP2J2L4	NC_006095.4	26753715	A	G	273	222	0	0	High	downstream_gene_variant
CYP2J2L4	NC_006095.4	26763561	C	T	100	137	0	0	High	upstream_gene_variant
CYP2J2L4	NC_006095.4	26765487	A	G	109	92	0	0	High	upstream_gene_variant
CYP2J2L4	NC_006095.4	26765506	T	C	317	204	130.5	178	High and Low	upstream_gene_variant
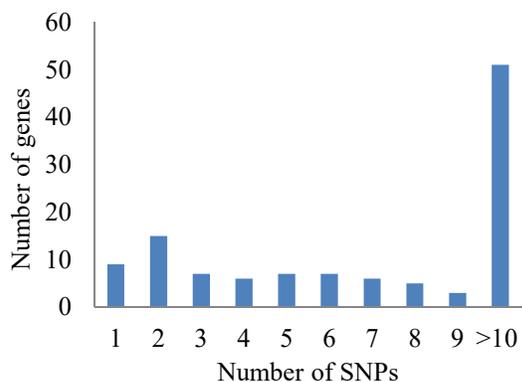
Figure 1. Distribution of the number of SNPs detected in the DEGs
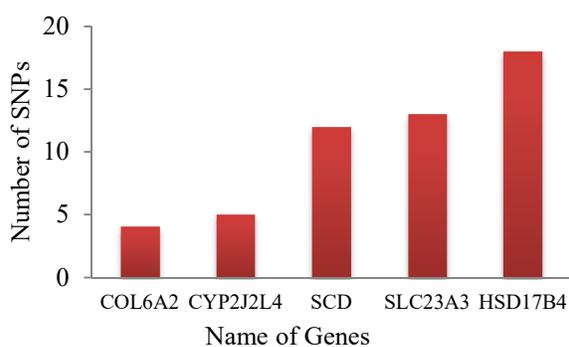
Figure 2. The distribution of the number of SNPs occurred in each gene

Combining the significant level of SNPs and gene function related with fatty acid composition allow us to suggest Stearoyl-CoA desaturase (SCD), Collagen VI member 2 (COL6A2), Cytochrome family 2J2L4 (CYP2J2L4), Hydroxysteroid 17-beta dehydro-genase 4 (HSD17B4), and Solute carrier family 23 member 3 (SLC23A3) as the 5 novel and promising candidate genes for selecting unsaturated fatty acids. SCD gene has been known to catalyze the unsaturated fatty acids metabolism. SCD is the rate limiting enzyme which catalysis the synthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA) (Dujková *et al.,* 2015; García-Fernández *et al.,* 2009). The composition in fatty acids kept in the fat depot's throwback the foregoing action of SCD on substrates such as palmitic acid and stearic acid

in chicken (Maharani *et al.,* 2013). Some SFA, ordinarily discovered in meat, particularly palmitic and mystric acids are one of the risk agent of heart diseases (Erkkila *et al.* 2008). Diet high in MUFA inclined to decrease blood cholesterol levels while Diet high in SFA inclined to raise blood cholesterol levels. Cholesterol is moved in the bloodstream as lipoproteins (Oh *et al.,* 2013). High-density lipoprotein (HDL) cholesterol is the good cholesterol since high HDL level related to less heart diseases, while low-density lipoprotein (LDL) is the bad cholesterol because high LDL levels are connected with a raise danger of heart disease (Jiang *et al.,* 2008).

Collagen VI (COL6) could be specific for adipocytes because it is the primary collagen expressed by adipocytes (Mariman & Wang 2010; Nakajima *et al.,* 2002). An in vivo study using BIP cells indicated that COL6 gene was able to raise and increase lipid synthesis (Nakajima *et al.* 2002). SLC (solute carrier family) is involved in infux transporters of various drugs, steroidal hormones, and some other substrates. SLC23A3 was extremely expressed in the ovary through growing follicle stage, dissimilarity, the expression of SLC23A3 was raised in granulosa cells at the growing follicle stage (Lee and Chang-Eun 2017). CYP2J2 is one of the major P450 enzymes to metabolize arachidonic acid (AA) predominantly via NADPH-dependent olefin epoxidation to 20-HETE and regioisomeric cis-epoxyeicosatrienoic acids. (Zangerand & Schwab, 2013; Xu *et al.,* 2011).

HSD17B4 was the gene with the largest number of SNPs in chicken in this study. HSD17B4 (hydroxysteroid (17-beta) dehydro-genase 4) is a bifunctional enzyme mediating anhydration and dehydrogenation through β-oxidation of long-chain fatty acids (Pierce *et al.* 2010). HSD17B4 might works as a possible regulator of muscle development, and its identification should help to select for improved economic traits of Berkshire pigs such as backfat thickness, drip loss, and carcass weight (Jo *et al.,* 2016). In the testicles, HSD17B4 leads to high levels of androstenone in Duroc pigs (Leung *et al.,* 2010; Moe *et al.,* 2007) and in the liver (Moe *et al.,* 2008) because liver contains many proteins that regulate androstenone and plays a role in steroid homeostasis. According to Jo *et al.* (2016) the liver is closely associated with meat traits and

male odor, accordingly, it could be concluded that HSD17B4, regulates steroid activity.

Most of these genes that involved SNPs in chicken in the present study were associated with unsaturated fatty acids in gene expression in a previous study (Maharani *et al.,* 2013). Biologically relevant polymorphisms are used to design a focused or low-density panel of selected SNPs (Fortes *et al.,* 2014). The present study is the first study to determine candidate genes to be determined as associated with unsaturated fatty acids in chicken and the SNPs discovered should be useful to increase the unsaturated fatty acids in chicken.

CONCLUSIONS

In the present study, RNA-Seq association study was carried out to identify candidate genes associated with unsaturated fatty acids in chicken. Several of the SNPs (SCD, COL6A2, CYP2J2L4, HSD17B4, and SLC23A3) founded in this study could be included as suitable markers in genotyping platforms to perform association analyses in commercial populations and apply genomic selection protocols in the chicken production. However, further investigation is required to confirm the effect of these genetic markers in other chicken populations.

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