

# GENETICS AND GENOMICS

## Catalog of genetic variants within mature microRNA seed regions in chicken

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**ABSTRACT** MicroRNA (miRNA) is a class of noncoding RNA important in posttranscriptional regulation of target genes. The regulation mechanism requires complementarity between target mRNA and the miRNA region responsible for their recognition and binding, also called the seed region. It has been estimated that each miRNA targets approximately 200 genes and genetic variability of miRNA genes has been associated with phenotypic variation and disease susceptibility in humans, livestock species, and model organisms. Polymorphisms in miRNA genes especially within the seed region could therefore represent biomarkers for phenotypic traits important in livestock animals. Using the updated Version 5.0 of our previously developed bioinformatics tool miRNA SNIPer we assembled polymorphic miRNA genes in chicken. Out of

740 miRNA genes 263 were polymorphic, among them 77 had SNPs located within the mature region, and 29 of them within the miRNA seed region. Because several polymorphisms in databases result from sequencing errors, we performed experimental validation of polymorphisms located within 4 selected miRNA genes in chicken (*gga-mir-1614*, *-1644*, *-1648*, and *-1657*). We confirmed the presence of nine polymorphisms and identified 3 additional novel polymorphisms within primary miRNA regions in chicken representing 3 layer-type breeds, one layer-type hybrid, and one meat-type intercrossed population. The developed catalog of mir-SNPs in chicken can serve researchers as a starting point for association studies dealing with poultry production traits and designing functional experiments.

**Key words:** microRNA, seed region, single-nucleotide polymorphisms, chicken

2015 Poultry Science 94:2037–2040  
<http://dx.doi.org/10.3382/ps/pev170>

## INTRODUCTION

MicroRNAs (**miRNA**) are noncoding RNAs that play an important role in regulation of target mRNA (Bartel, 2004). Approximately half of miRNA genes are resident within larger genes, which are mostly protein-coding, and these have been shown to have similar expression patterns as well as synergistic or antagonistic regulatory effects to the gene in which they reside (Baskerville and Bartel, 2005; Godnic et al., 2013; Lutter et al., 2010). Many miRNA have previously been revealed as species- and tissue-specific which suggests that they may have important roles in tissue development, immune response, and metabolism in livestock animals (Liu et al., 2010). Since single miRNA can have multiple targets, mir-SNP, especially when located

within seed regions (mir-seed-SNP), would be expected to exhibit a complex biological effect (Sun et al., 2009). Several chicken miRNA genes are expressed in adipose tissue and skeletal muscles (Wang et al., 2012). Chicken growth, particularly skeletal muscle growth and development, are regulated by miRNA (Xu et al., 2013). Additionally, miRNA *gga-mir-26a* and its direct target, *NEK6*, have been shown to be involved in Marek's disease virus infection (Li et al., 2014). Mature miRNA seed SNP located within miRNA genes *gga-mir-1657* and *gga-mir-1614-3p* have been associated with chicken production traits (Li et al., 2012; Li et al., 2013). Poultry species represent a major part of the food industry and are also important model organisms, but still little is known about miRNA SNP in chicken. In our previous study we collected SNP residing within mature miRNA regions in chicken (Jevsinek Skok et al., 2013); however, due to novel releases of genomic resources (Ensembl and miRBase) an updated study was needed. The aim of this study was to: 1) update the miRNA SNIPer tool with recent releases of source databases, 2) perform genome-wide *in silico* screening of the chicken genome for polymorphisms residing within miRNA regions, 3) develop a catalog of mature seed mir-SNP, and 4) validate selected polymorphisms located within mature miRNA seed regions experimentally.

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Received March 15, 2015.

Accepted April 27, 2015.

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**Table 1.** Twenty-nine miRNA genes in chicken containing polymorphism within mature miRNA seed regions.

Name of miRNA	Genomic Location of miRNA Gene	Polymorphism Identifier (rs Number)	Substitution
gga-mir-19a	Intergenic	rs313019541	C > A
gga-mir-34c	Intergenic	rs317487696	C > T
gga-mir-499	<i>MYH7B</i> , intron	rs314375017	T > A
gga-mir-1562	<i>OSBPL9</i> , intron	rs314758707	C > T
gga-mir-1568	Intergenic	rs14511527	A > G
gga-mir-1614	Intergenic	rs15172520	G > A
gga-mir-1644	<i>XYLT2</i> , intron	rs14076349	T > C
gga-mir-1648	Intergenic	rs14281065	T > –
gga-mir-1657	<i>RAB38</i> , intron	rs14934924	G > A
gga-mir-1658	<i>GMPS</i> , intron	rs16681031	C > G
		rs16681032	C > T
		rs16681033	– > G
gga-mir-1663	Intergenic	rs316833014	A > G
		rs317881467	C > T
gga-mir-1678	<i>TRAIP</i> , intron	rs316970596	T > C
gga-mir-1766	<i>MYO10</i> , intron	rs80672319	G > A
gga-mir-1800	<i>UNC79</i> , intron	rs314880301	C > T
gga-mir-1801	Intergenic	rs316917553	G > A
gga-mir-6545	Intergenic	rs312707438	C > T
gga-mir-6565	<i>ZNF462</i> , intron 2/exon3	rs317939537	A > G
gga-mir-6578	<i>HADHB</i> , intron 8/exon 9	rs313317647	C > G
gga-mir-6587	Intergenic	rs10731782	C > A
gga-mir-6604	<i>RRP12</i> , intron 29/exon 30	rs315812822	G > A
gga-mir-6612	Intergenic	rs315928758	T > C
gga-mir-6675	Intergenic	rs315355302	C > T
gga-mir-6680	Intergenic	rs313253631	T > A
gga-mir-6689	<i>KLHL13</i> , intron	rs16351803	T > C
gga-mir-7438	<i>TSTD2</i> , intron	rs16777695	G > C
gga-mir-7458–1	<i>PQLC1</i> , intron	rs313155456	C > G
gga-mir-7464	<i>CHGB</i> , intron	rs15278991	T > A
gga-mir-7465	<i>KCNQ5</i> , intron	rs15408995	CT > –
gga-mir-7468	Intergenic	rs16414597	G > A

## MATERIALS AND METHODS

Our previously developed bioinformatics tool miRNA SNIPer has been updated and upgraded to the Version 5.0 using data from 6 source databases: Ensembl Variation database Release 78, miRBase 21, TargetScan 6.2, mirTarBase 4.5, Human MicroRNA Disease Database, and SNPchiMp v. 3 (<http://www.integromics-time.com/miRNA-SNIPer/>). The seed region was defined as the area including Nucleotides 2 to 8 from the 5' end in the mature miRNA region (Friedman et al., 2009). Chicken blood samples were obtained from an intercross population generated by crossing 2 lines of chickens divergently selected for BW at 8 wk age (Terčič et al., 2009), layer hybrid Lohmann Brown classic, and 3 Slovenian layer-type breeds: Slovenian Brown hen, Slovenian Barred hen, and Styrian hen. DNA samples were isolated using a standard phenol–chloroform extraction protocol. Four miRNA genes were amplified by PCR using the primers and cycling conditions described in the Appendix.

## RESULTS AND DISCUSSION

Genome-wide screening of the chicken genome using the updated version of the miRNA SNIPer tool revealed a high number of polymorphic miRNA genes, several of them residing within protein-coding genes. Polymor-

phisms of 4 miRNA genes were selected for experimental validation in 5 chicken populations with different genetic backgrounds.

The updated version of the miRNA SNIPer Tool 5.0 enables a search of genetic variability of miRNA genes in 22 genomes, including chicken. Among 740 known miRNA precursors (994 mature) genes in chicken, 263 (35.5%) were found to contain polymorphisms, including 77 with polymorphisms within the mature miRNA region. Among these, 29 genes displayed polymorphisms within the miRNA seed region (Table 1). Out of 29 miRNA genes with polymorphic miRNA seed regions, 13 were intergenic and 16 miRNAs were located within protein-coding genes. Three out of 29 miRNA seed polymorphisms are also present on commercial whole-genome SNP arrays (rs316970596, rs317939537, rs313317647). Four miRNA genes (*gga-mir-1614*, *–1644*, *–1648*, and *–1657*) and their flanking regions in total comprising 1890 bp and 10 polymorphisms deposited in the Ensembl database were selected for experimental validation using DNA sequencing. Out of 10 analyzed polymorphisms 9 were polymorphic in animals belonging to 5 tested chicken populations (Supplementary Figures S1 to S4, Supplementary Table S2). Additionally, we identified 3 novel polymorphisms within regions flanking 3 miRNA genes. We found that mir-seed-SNP rs15172520 within *gga-mir-1614* was polymorphic in

Slovenian Brown and Styrian hen. A nucleotide substitution rs315267283 C>T, located 5 bp upstream of the pre-miRNA was polymorphic in the Slovenian Brown hen. Similarly, the mir-seed-SNP rs14076349, located within the *gga-mir-1644* and the protein-coding gene *xylosyltransferase 1* (*XYLT1*), was validated in the Slovenian Brown and Styrian hen, whereas 2 polymorphisms downstream of the pre-miRNA, one of them a newly found substitution (C > G), were confirmed in the Slovenian Brown hen. An indel residing within the mature miRNA seed region of *gga-mir-1648* has been identified in Lohmann Brown hen and polymorphism G > C residing 1 bp upstream of pre-miRNA G > C is polymorphic in Styrian hen. Gene *gga-mir-1657* residing within host gene *RAB38*, member Rat sarcoma oncogene family (*RAB38*) contained 2 SNPs; rs14934923 within the pre-miRNA region and rs14934924 within the mature seed region. Three SNPs were present in the upstream region of this miRNA gene and were all polymorphic in the Lohmann Brown layer hybrid. Seven seed SNPs from 5 miRNA genes (rs14076349, rs14281065, rs14934924, rs16681031, rs16681032, rs16681033, and rs15172520) have been experimentally validated to date (Geng et al., 2011; Jevsinek Skok et al., 2013; Li et al., 2012; Li et al., 2013; Zhang et al., 2011). Validated mir-SNPs could be further applied for discovering their associations with different trait phenotypes in chicken.

Developing and maintaining novel bioinformatics tools is of special importance in the field of chicken genomics, which has fewer bioinformatics tools available in comparison to other model organisms. Our previous study using miRNA SNIper Version 3.0 revealed 18.4% (92/499) polymorphic miRNA genes including 9 with polymorphisms within the seed region (Jevsinek Skok et al., 2013). Version 3.0 of the miRNA SNIper tool was based on the Ensembl Release 68 and miRBase v. 19 and miRNA SNIper Tool Version 5.0 includes Ensembl Release 78 and miRBase v. 21. The number of assembled miR-polymorphisms is not final and will change with time as all miRNAs have not yet been systematically sequenced and screened for polymorphisms. Our recent genome-wide in silico screening of 15 genomes revealed that based on the latest database releases miRNA genes are most polymorphic in cattle, followed by human, fruitfly, mouse, chicken, and pig (Zorc et al., 2015). These results clearly demonstrate the relationship among genetic variation discovered in miRNA genes and the number of individual genomes available for different species. Therefore we can expect that the number of polymorphic sites within the miRNA genes in poultry will increase with the growing number of sequenced individual genomes. Additionally, 2 of the miR-seed-SNPs have also been found to be associated with poultry production traits: rs14934924 has been associated with chicken growth and meat traits in the Chinese Gushi-Anka F2 resource population (Li et al., 2012), and rs15172520 with breast muscle shear force and leg muscle water loss rate, wing weight, heart weight, and

weight of the abdominal fat (Li et al., 2013). Polymorphisms found in miRNA genes could therefore be potential biomarkers for phenotypic traits in chicken (Geng et al., 2011). However, because not all miRNA genes have systematically been sequenced yet, many polymorphisms remain unvalidated, while on the other hand some SNPs in the databases may be results of sequencing errors.

In the future it will be necessary to explore if polymorphisms within seed regions cause formation of seed region belonging to other miRNA genes. Additionally, miRNA/Quantitative trait locus overlap analysis will enable functional characterization of miRNAs. Identification of miRNA downstream targets will reveal miRNA regulatory networks. Results of this research are the first step which enables further functional SNP analysis in chicken.

## ACKNOWLEDGMENTS

This work was supported by the Slovenian Research Agency through the Research Program (P4-0220).

## SUPPLEMENTARY DATA

**Supplementary Table S1.** Primers used for PCR and sequencing of 4 chicken miRNA genes.

**Supplementary Table S2.** Genotyping results of miRNA polymorphisms using DNA sequencing in chicken.

**Supplementary Figure S1:** Experimental validation of polymorphisms located within miRNA gene *gga-mir-1614*. Intergenic *gga-mir-1614* containing miR-seed-SNP rs15172520 and rs315267283 located upstream of the pre-miRNA.

**Supplementary Figure S2:** Experimental validation of polymorphisms located within miRNA gene *gga-mir-1644*. Protein-coding host gene *XYLT1* with resident *gga-mir-1644* containing miR-seed-SNP rs14076349 and 2 additional SNPs located downstream of the pre-miRNA region.

**Supplementary Figure S3:** Experimental validation of polymorphisms located within miRNA gene *gga-mir-1648*. Intergenic *gga-mir-1648* containing one SNP within the mature region, indel within seed region, and novel substitution G > C located one nucleotide upstream of the pre-miRNA region.

**Supplementary Figure S4:** Experimental validation of polymorphisms located within miRNA gene *gga-mir-1657*. Protein-coding host gene *RAB38A* with resident *gga-mir-1657* containing a miR-seed-SNP (rs14934924) and SNP located within pre-miRNA (rs14934923). An additional 3 SNPs were validated in the upstream region.

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## APPENDIX

### MATERIALS AND METHODS

Four miRNA genes containing miR-seed polymorphisms were selected for experimental validation using PCR using primers and conditions described in Supplementary [Table S1](#). Conditions for the PCR were 94°C for 10 min, 30 cycles of 94°C for 1 min, 58 to 65°C for 1 min, 72°C for 1 min, followed by 10 min extension at 72°C. PCR products were purified using Exonuclease I and shrimp alkaline phosphatase (both from Fermentas, Vilnius, Lithuania) and following sequencing reaction, prepared for capillary electrophoresis on ABI3130xl.