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A novel minisequencing single-nucleotide polymorphism marker of the *lysozyme* gene detects high hatchability of Tsaiya ducks (*Anas platyrhynchos*)

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ABSTRACT

Lysozyme, one of the major albumen antimicrobials, can break down the polysaccharide walls of a broad spectrum of bacteria. This study presents a novel *lysozyme* marker of high hatchability in the form of minisequencing single-nucleotide polymorphisms (SNPs). Recently, *lysozyme* was identified by complementary DNA microarray analysis as one of several differentially expressed genes noted to influence hatchability and recognized as a marker candidate for animal marker-assisted selection. Higher levels ($P < 0.05$) of *lysozyme* mRNA (*via* real-time polymerase chain reaction analysis) and protein (in Western blotting results) were found to be associated with a high-hatchability phenotype. In the preliminary sequence analysis of this study, TsLy1-1 and TsLy1-2 primer pairs, designed according to the *lysozyme* sequence, were used to amplify small-scale genomic DNA samples from animals in two extreme groups of hatchability. Sequence analysis of the amplified 763-bp DNA products clearly showed that AA and GG genotypes of SNP g.390A > G were from the ducks of the low- and high-hatchability groups, respectively. The SNP g.390A > G also created a new specificity protein 1 transcription factor binding site in the *lysozyme* gene. Primer pairs of TsLy2-1 and TsLy2-2 then probed the amplified 763-bp DNA products to produce a shorter fragment for easier minisequencing analysis to divide 114 ducks into GG, GA, and AA genotypes. The GG ducks had the highest hatchability, representing that a new *lysozyme* SNP marker of good hatchability performance can be used for the purpose of marker-assisted selection in Tsaiya ducks.

1. Introduction

In avian reproductive systems, bacterial burdens present a challenge to animal health and reproductive performance [1–3]. Growing embryos in eggs are susceptible to microbial invaders that can be incorporated into the egg during its formation or can penetrate it after laying, ultimately affecting embryo viability. Antimicrobial proteins in albumen, such as lysozyme, avidin, and ovotransferrin, contribute to the defense of embryos from infection. Lysozyme is effective against bacterial invasion by means of direct bactericidal action [4] or stimulation of immunoglobulin formation [5]. Avidin [6] and ovotransferrin [7] create an environment unsuitable for bacterial proliferation.

Hatchability, the percentage of embryos surviving from fertilized eggs, is one of the determinants of reproductive ascendancy of animals. The proportion of successful hatching is generally considered only to be affected by the age or the egg sequence position of the avian [8], and by nutritional factors such as the concentrations of potassium or iron in the egg yolk [9]. Over the past few years, our

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research has suggested that factors that affect hatchability have a molecular basis: we identified some important candidate genes whose mRNA and protein expressions were reported to be associated with the percentage of duck offspring surviving from fertilized eggs; therefore, these genes may be involved in the process of hatching or influence hatchability [10,11]. Being one of the major components of egg white, lysozyme migrates with other proteins of albumen proteins into the amniotic fluid [12,13] and finally accumulates in the embryo [13–15]. Mutations in albumen proteins increase the incidence of embryonic death and decrease hatchability [16,17]. A study by Saino et al. [18] revealed that an increase in maternal lysozyme activity resulted in an increase in hatching success. As a result, the lysozyme content of albumen should be considered critical to avian hatchability.

Alteration of the nucleotide sequence of a gene may take the forms of base substitution, insertion, or deletion, and may lead to synonymous or nonsynonymous consequences in translation, protein function, and phenotype [11,19,20]. Therefore, DNA sequence variants are also responsible for the genetic components of individuality within different populations. Many genomic variations are attributed to single-nucleotide polymorphisms (SNPs), which are stable, inherited, and biallelic sequence variants, and are distributed throughout the genome [21,22]. Single-nucleotide polymorphisms are potentially useful for the mapping of molecular markers, phylogenetic relationship investigation, and disease diagnosis studies in different organisms [23–25]. Animal genetic polymorphism studies can also be applied in marker–trait association analysis, sexing, species identification, and paternity testing [26–28].

An expressed sequence tag HO188238 of the *lysozyme* gene was identified by our exploratory complementary DNA (cDNA) microarray study, which revealed differentially expressed transcripts between the high- and low-hatchability groups, and this expressed sequence tag presented a signal of upregulation in the former group [10]. Therefore, we hypothesized that *lysozyme* could be a marker associated with high hatchability. In this study, we used the major egg-laying duck in Taiwan, the Tsaiya duck (*Anas platyrhynchos*) [29,30], to analyze the association between the expressions of lysozyme mRNA and protein and the high-hatchability trait, and searched for DNA polymorphisms in the lysozyme gene to facilitate its use as a marker. The results shed light on the application of a novel SNP marker for marker-assisted selection to increase the hatchability performance in Tsaiya ducks.

2. Materials and methods

2.1. Animals and samples

The Livestock Research Institute, Council of Agriculture, Taiwan, provided a total of 114 laying brown Tsaiya ducks (*Anas platyrhynchos*). The care and use of these animals were in accordance with standard ethical guidelines. The reproduction phenotypes were recorded and used for marker–trait association analysis. Blood withdrawn from the veins of the ducks' wings was used for subsequent DNA extraction, and magnum tissues were collected from the genital tract for total RNA and protein extraction.

2.2. Preparation of magnum RNA and cell extracts

The RareRNA reagent (GenePure, Kaysville, UT, USA) was used to homogenize the magnum tissues from laying brown Tsaiya ducks of high hatchability (88.20 ± 1.21%, n = 5) and low hatchability (55.60 ± 1.04%, n = 5). Total RNA further extracted using chloroform and precipitated using isopropanol in accordance with the manufacturer's (GenePure) instructions was used for real-time polymerase chain reaction (PCR). For Western blot analysis, breaking buffer (10 mM Tris–HCl, 1.5 mM EDTA, 1 mM dithiothreitol, 1 × phenylmethanesulfonyl fluoride, pH7.4) was used for the preparation of cell extracts from the magnum of each extreme group as described previously. The tissues were homogenized using a Mini-Beadbeater (BioSpec Products Inc., Bartlesville, OK, USA) with glass beads for 30 seconds at 2500 rpm, four times. The supernatant was collected by centrifugation for 10 minutes at 1000 rpm at 4 °C.

2.3. Gene expression measurements

Complementary DNA was synthesized from 2 ng of total RNA using a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) at 25 °C for 10 minutes, 37 °C for 2 hours, and 85 °C for 5 minutes. The cDNA of isolated total RNA was subjected to real-time quantitative PCR in the presence of SYBR Green Mix (Applied Biosystems) along with *lysozyme*-specific primer pairs using an Applied Biosystems 7300 (Applied Biosystems) system as recommended by the manufacturer. For normalization, cDNA of *18s rRNA* was amplified and relative quantification was performed from curves generated for *lysozyme* and *18s rRNA*. Primers were designed using Primer Express 3.1 software (Applied Biosystems). The *lysozyme* and the *18s rRNA* primer sets used were *lys*-forward, *lys*-reverse, *18s*-forward, and *18s*-reverse (see Table 1). Details of the method used are given in the article by Huang et al. [31].

The protein concentrations of the magnum extracts of high- and low-hatchability animals were measured by Bradford assay with bovine serum albumin as the reference standard. Samples of equal amounts of total protein were resolved by 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA, USA) using SemiPhor semidry transfer

Table 1
The primer pairs used in the present study.

Primer name	Sequence (5′ to 3′)	Amplicon (bp)	Tm (°C)
lys-forward	TGCTGACCCTCGTCTTTTGC	130	61
lys-reverse	ACCCAGTTTCCCAGGCTGTA		62
18s-forward	GGACGCGTGCATTTATCAGA	100	58
18s-reverse	AGTTGATAGGGCAGACATTCGAA		59
TsLy1-1	GCCAAGTGAAGACTTCTTTCCTCC	763	61
TsLy1-2	AGCGGCTGTTGATCTGTAGGATTC		62
TsLy2-1	CACATGAGGGTCAGACGCAAT	345	60
TsLy2-2	TTTGAAGGGTAGAAATCCTGTGCT		59