—Original Article—

A new DNA marker of the *TMIGD1* gene used to identify high fertilization rates in Tsaiya ducks (*Anas platyrhynchos*)

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Abstract. In a prior study, comparisons of individuals of *Anas platyrhynchos* with higher/lower reproductive performances showed that the expression of the transmembrane and immunoglobulin domain containing 1 (*TMIGD1*) gene significantly differed between the two groups. Here, we demonstrate that ducks with the *TMIGD1* GG genotype have a significantly higher fertilization rate than other *TMIGD1* genotypes. Primers designed based on the *TMIGD1* sequence of Pekin duck were able to successfully amplify a *TMIGD1* fragment from Tsaiya ducks, and sequencing results indicated that a single nucleotide polymorphism (SNP) of the *TMIGD1* gene existed. We also developed a cost-effective method of restriction fragment length polymorphism. Using the above methods, ducks were classified into three genotypes. To identify the relationships between genotypes and traits, we recorded the ducks' performance; to ensure the coverage of the entire duration of the fertile period, the egg collection period was extended to 18 days, and therefore, lower than usual fertilization rates were observed. Further assessment using a high-throughput system showed that the ducks with the GG genotype exhibited the highest fertilization rates among genotypes (P < 0.05). We suggest that TMIGD1 may affect the release of sperm protection factors from the female genital tract, and thus alter fertilization rate. In conclusion, the results of this study demonstrate that the *TMIGD1* GG genotype can be used as a new DNA marker to identify animals with high fertilization rates at a young age, a process which could improve farming efficiency.
Key words: *Anas platyrhynchos*, DNA marker, GG genotype, *TMIGD1*

Fertilization rate is one of the most important reproductive traits in poultry science, as a high fertilization rate results in better returns on investment, regardless of meat production or profit from egg-laying ducks. Many studies have reported on the possible factors influencing fertilization rate in poultry, including: supplementation with vitamin E and selenium, which can improve fertilization rate [1, 2]; female age, which is often negatively correlated with fertilization rate [3]; time of artificial insemination (AI), which should be performed in the morning to improve fertilization rate [4]; and heat stress, which can reduce sperm viability and thus reduces fertilization rate [5, 6]. However, very few studies have examined the influence of genetics on fertilization rates.

A continuation of our previous study of the link between reproductive performance and genome-wide transcript expression in the uterovaginal junction of female birds [7], the present study aimed to further understand the relationships between changes at the level of the gene and fertilization rate. The connection between reproductive performance and the uterovaginal junction is important, as the uterovaginal junction is where sperm is stored after it enters the vagina [8]. The interaction of sperm with epithelial cells in this

sperm reservoir has a significant effect on sperm motility [9–11]. Prolonged sperm lifespan in the female sperm reservoir indicates an extended fertile period, which not only reduces the number of required AIs and thus, manpower, but also increases fertilization rate [12, 13].

Tsaiya duck (*Anas platyrhynchos*) is the major egg-laying duck breed in Taiwan, and is also the maternal line of the major meat duck breed, the Mule duck [14, 15]. Previously, we have identified that the *TMIGD1* gene is differentially expressed among ducks with good and poor reproductive performances [7], but we did not investigate the possible applications of this discovery. In the present study, we demonstrate that nucleotide polymorphisms are present in the *TMIGD1* sequence, and we explore the possible implications and applications of these polymorphisms.

Materials and Methods

Preparation of Animals and genomic DNA

One hundred and sixty-three female Tsaiya ducks were used in this study and were provided by the Livestock Research Institute, Council of Agriculture, Taiwan. The care of animals was provided according to standard ethical guidelines, and all experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Livestock Research Institute, Council of Agriculture, Taiwan. The pooled semen of 10 to 15 Muscovy drakes (*Cairina moschata*) was used in AI. Female reproductive performance data was recorded once every three weeks after AI, including: fertilization rate, which was represented by the ratio of fertilized to collected

eggs (fertilized eggs as determined by candling for the presence of embryos); fertile period, i.e., the duration during which fertilized eggs were continuously observed; and hatchability, which was the number of successful hatchings from the fertilized eggs described above. In order to record the length of the fertile period, eggs were collected for 18 days, a period longer than the usual eight-day collection period. As the fertile period is not indefinite, fewer fertilized eggs were observed in the later part of the 18 day collection period in some ducks, which led to reduced fertilization rate values than would be observed during a normal egg collection period [12].

DNA samples were prepared from the blood of ducks: 80 µl of blood was slowly mixed with 1.0 mL of TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0), and the supernatant was removed after centrifugation at $2862 \times g$ for 5 min. After adding 1.0 ml of TNE buffer again to wash away any impurities, followed by centrifugation at $716 \times g$ for 5 min, the supernatant was removed and 3.0 ml of TNE buffer was added to re-suspend the blood cells. The blood cells and TNE buffer were then chilled at -20 °C for at least 20 min and were thawed in warm water to lyse the red blood cells. Following mixing with 375 µl of 10% NH_4Cl, the samples were incubated in a 42°C water bath and shaken at 110 rpm for 20 min. Proteinase K (Merck, Darmstadt, Germany), collagenase (Merck, Germany), and SDS (Merck, Germany) were added to the samples, which were then mixed and incubated in a 42°C water bath shaken at 110 rpm for least 12 h. After adding the same volume of phenol and gently mixing, the samples were centrifuged at $1948 \times g$ for 20 min. The samples were then subjected to phenol:chloroform:isoamyl alcohol (25:24:1) cleanup, isopropanol precipitation, and ethanol washing. The DNA samples were vacuum-dried, and then reconstituted at 55°C in sterile deionized water. The concentration of DNA was measured using a spectrophotometer, and this concentration was then adjusted to 25 ng/µl with sterile deionized water for further analysis.

PCR primer design for the preliminary comparison of sequences

Primers were designed using the primer design tool Primer3 [16, 17]. The primers for sequencing were i2F1 (5′-CAGCATCCCAAGTTTTCG-3′) and i2R1 (5′-GCCCAACCTTCTGCTACAAA-3′). PCR was then carried out with a mixture volume of 25 µl (containing 1 × DyNAzyme buffer, 200 µM dNTPs, 300 nM of each primer, 1 unit of DyNAzyme II polymerase (Thermo Scientific, Waltham, MA, USA), and 25 ng of DNA) using a PCR thermocycler (GeneAmp 2700; Applied Biosystems, Thermo Scientific, Waltham, MA, USA). The reaction conditions were: 95°C, 2 min; 28 cycles of 30 sec at 94°C, 30 sec at 55°C, and 22 sec at 72°C, followed by 1 min at 25°C. Five µl of the amplified PCR product were then electrophoresed on 2% agar gel (Aresco, Cleveland, OH, USA) and visualized using ethidium bromide staining to validate the PCR product as 451 bp (base pair) in length using a gel imaging system.

Single nucleotide polymorphism (SNP) identification

The PCR products were sequenced using an ABI 3730 automated sequencer (Applied Biosystems, Thermo Scientific) according to the manufacturer's protocol, and the resulting sequences were compared against database entries using the Nucleotide BLAST program of The National Center for Biotechnology Information (NCBI). For those

products which matched the *TMIGD1* sequence, their sequences were further aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (https://www.ebi.ac.uk/Tools/msa/muscle/) to identify the position of the single nucleotide polymorphism (SNP). A g.175 G>A SNP was identified, which was a polymorphic site differing by guanine (G) and adenine (A) at nucleotide 175 in the *TMIGD1* gene from the duck genome.

Restriction fragment length polymorphism

Using the NEBcutter program, version 2.0 (http://nc2.neb.com/NEBcutter2/, New England Biolabs, Ipswich, MA, USA), we found that restriction enzyme Rsa I (New England Biolabs) cuts GTAC sequences (GT/AC blunt end), but not GTGC. Because the primer pair i2F1 and i2R1 used in the sequencing described above generated a longer PCR product that could not be used to clearly determine genotypic differences between results of restriction fragment length polymorphism, another primer pair, i2F2 (5′-CCTGAGGATTGCCTAAAAGC-3′) and i2R2 (5′-TGGGAAAACAGTATGGGAA-3′), was used to amplify a fragment of 218 base pairs. The following reaction conditions were used: 95°C, 2 min; 30 cycles of 30 sec at 94°C, 15 sec at 53°C, and 11 sec at 72°C, followed by 1 min at 25°C. The amplified PCR product was digested using Rsa I according to the manufacturer's recommendation, electrophoresed, and visualized using the afore-mentioned method.

Combination with PCR and probe hybridization for genotyping

A primer pair (forward: 5′-GCCTAAAAGCTGGCCTCTGATTT-3′, and reverse: 5′-TTTTCTATCAAGTTAAAACTCACTAGACT-3′, ordered from Applied Biosystems) and probes (5′-CTGCAATGTGCTTTTAG-3′, labeled with VIC presenting the guanine polymorphism, and 5′- CTGCAATGTACTTTTAG-3′, labeled with FAM presenting the adenine polymorphism, ordered from Applied Biosystems) were used to amplify a 185-bp fragment and in genotyping using a StepOne real-time PCR system (Applied Biosystems). The 10-µl reaction mixture consisted of 1 × real-time PCR master mix (Applied Biosystems), 1 × SNP assay mix (containing primers and probes, Applied Biosystems), and 5 ng DNA. The reaction was performed as follows: 30 sec at 60°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C, and was ended at 60°C for 30 sec. The results were classified using the StepOne software v2.3 (Applied Biosystems), which was able to distinguish between the three genotypes, GG, GA, and AA. A group plot was analyzed using TaqMan Genotyper software (Applied Biosystems).

Data analysis and statistics

Differences in reproductive performance between the three geno-types were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). All data from the different groups were compared using one-way analysis of variance (ANOVA) and Duncan's multiple comparison tests. A value of $P < 0.05$ indicated a statistically significant difference, and the results were expressed as means $\pm$ SE.

i2F1R1_025   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_032   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_110   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_123   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_016   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_095   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_021   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_057   TTTATATTCTGCAATGT<u>A</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_056   TTTATATTCTGCAATGT<u>A</u>CTTTTAGCTCATTAATCCATGA

i2F1R1_074   TTTATATTCTGCAATGT<u>G</u>CTTTTAGCTCATTAATCCATGA

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Fig. 1. Preliminary comparison of the PCR products of *TMIGD1* fragments from 10 Tsaiya ducks. The codes on the left represent different ducks and the name of the primer pair. The positions in the sequences marked with asterisks at the bottom are those with identical nucleotides, and the underlined positions are the single nucleotide polymorphism. It is worth noting that only the duck i2F1R1_074 was heterozygous. This figure is different to Fig. 2, and each sequence position only represents one nucleotide. Therefore, at the site of the nucleotide polymorphism for the duck i2F1R1_074, only the stronger signal of guanine (G) is shown. Moreover, only ducks i2F1R1_057 and i2F1R1_056 exhibited the nucleotide substitution of adenine.

Results

TMIGD1 sequences of Tsaiya duck and Pekin duck are highly similar

As the *TMIGD1* DNA sequence of Tsaiya duck has not been sequenced, we used the *TMIGD1* genomic sequence from Pekin duck (NW_004677361, range: 776929–782216) as the reference sequence to design the primers. Using the DNA of Tsaiya duck as the template, the PCR reaction amplified an expected 451-bp product that was later confirmed by sequencing. Using nucleotide BLAST from NCBI, it was found that the 451-bp sequence *TMIGD1* PCR product from Tsaiya duck was 99% identical to the sequence found in Pekin duck.

Genetic variations in the TMIGD1 gene exist among ducks

We next examined genomic DNA from ten randomly-selected ducks. After PCR amplification and product size validation, the samples were bi-directionally sequenced and aligned using MUSCLE. The results are shown in Fig. 1, with the underlined position denoting the single nucleotide variation. After verifying the sequencing chromatograms (Fig. 2), it was clear that the SNP, named g.175 G>A, was present in the sequences. Alternatively, the three genotypes can be distinguished using restriction fragment length polymorphism
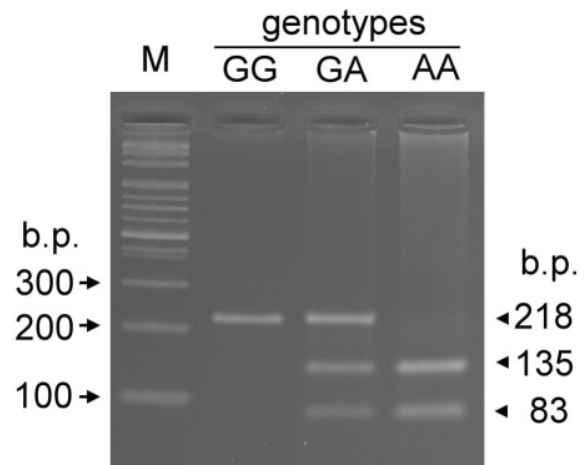

Fig. 3. Representative results of PCR restriction fragment length polymorphism for the three genotypes. Arrowheads on the right indicate the fragment sizes of different genotypes after Rsa I digestion. M is a 100-bp DNA ladder marker.

(Fig. 3). The 218-bp PCR product amplified from ducks with the GG genotype duck cannot be digested by the Rsa I restriction enzyme, while the product from ducks of the AA genotype resulted in two fragments of lengths 135 and 83 bp, and the digestion products of sequences from the GA genotype generated three fragments of lengths 218, 135, and 83 bp. We have thus demonstrated that restriction fragment length polymorphism can be used to identify *TMIGD1* genotypes, and that this method is also a good technique to use when laboratory funding is limited. By sequencing and performing restriction fragment length polymorphism, ducks were classified into the GG, GA, and AA genotypes.

To quickly screen and further analyze an increasing number of ducks, we next designed new primers and probes for use in the real-time PCR-based screening of SNPs, allowing the amplification of a shorter product using PCR with higher reaction efficiency. A primer pair which generated a 185 bp fragment was designed. We also used probes labelled with the fluorescent dyes VIC and FAM, facilitating artificial hybridization and allowing the GG, GA, and AA genotypes to be easily distinguished. Figure 4 shows multicomponent plots created using the StepOne software which clearly present the results of the no-DNA template control (NTC) (Fig. 4A), GG genotype (Fig. 4B), GA genotype (Fig. 4C), and AA genotype (Fig. 4D). In other words, the accumulation of VIC and FAM fluorescences as PCR cycles progressed (Fig. 4) indicated that individual VIC or FAM fluorescence corresponded to genetic variation, that the hybridization of the probes to polymorphic sites was successful, and that the separation of the fluorescence reporter and the probe's quencher was completed following polymerization Taq DNA polymerase in the chain reaction. Using this genotyping method and the StepOne PCR system, 48 individual DNA samples can be analyzed within two hours, which is much faster than the typical method which uses PCR and agar gel electrophoresis, although the former method requires more expensive equipment. This approach is suitable for high-throughput SNP genotyping once the position of
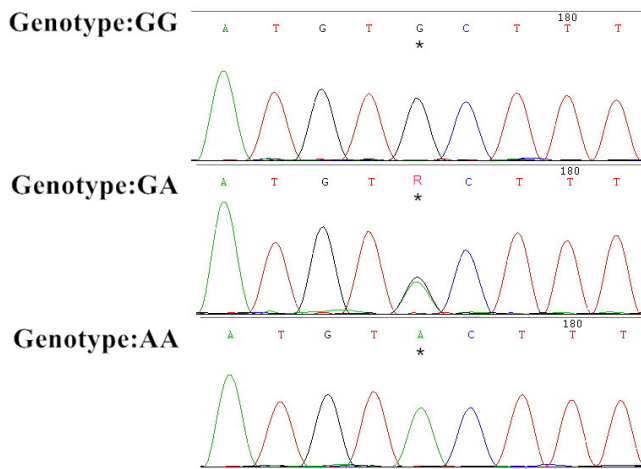
Fig. 2. Representative sequencing chromatograms of ducks with the three *TMIGD1* genotypes, two homozygotes and one heterozygote. The four different peak colors, green, red, blue, and black, represent nucleotides A, T, C, and G, respectively. The positions marked with asterisks are the sites of the polymorphism, and R is the heterozygote of GA.
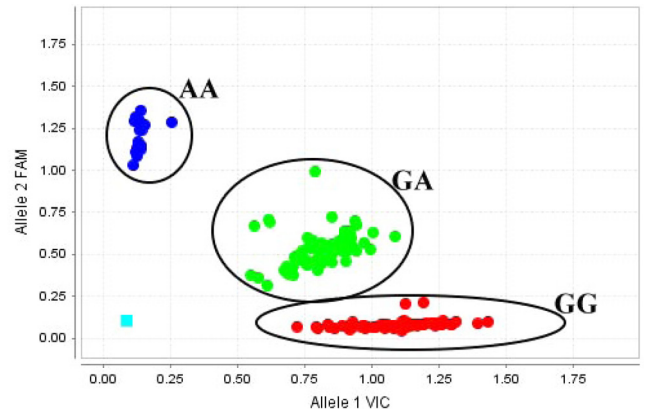

Fig. 5. Comprehensive results of the genotype screen from 163 ducks. The data obtained using the StepOne PCR system shown in Fig. 4 were analyzed using TaqMan Genotyper software, and each genotype was grouped and expressed in a different color. The light blue square on the lower left corner indicates the no-DNA template control (NTC).
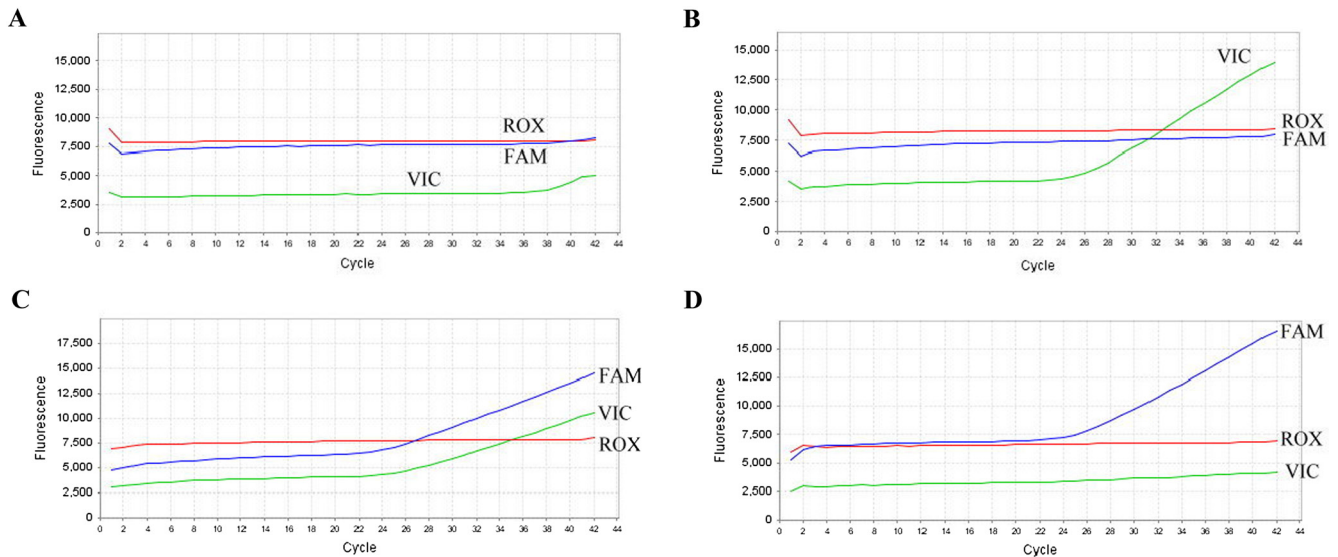

Fig. 4. Representative multicomponent plots collected by the StepOne real-time PCR system to perform high-throughput screening. The red line shows ROX, which was used as an internal control of sample volume loaded, and its fluorescence intensity should not increase from the beginning to the end of the reaction. The green line represents the status of the hybridization between the VIC probe and the guanine polymorphic site. The blue line represents the FAM probe hybridization status to adenine. (A) No-DNA template control (NTC), (B) homozygous guanine nucleotide, (C) heterozygous guanine and adenine nucleotides, and (D) homozygous adenine nucleotide at the site of polymorphism.

the SNP has been located, as demonstrated in our study. Using this method, we tested the genotypes of 163 ducks, and the results of this genotyping found that 76 ducks were heterozygotes (Fig. 5, green) and 87 ducks were homozygotes consisting of 70 GG individuals (Fig. 5, red) and 17 AA individuals (Fig. 5, blue).

GG genotype ducks have the highest fertilization rate compared to the other studied genotypes

　　Figure 6 presents an analysis of the relationships between genotypes and traits. The fertilization rates of ducks with GG, GA, and AA genotypes were $47.64 \pm 1.73\%$, $44.38 \pm 1.67\%$, and $39.32 \pm 2.50\%$, respectively. Statistical analysis showed that the fertilization rates were significantly different ($P < 0.05$) among the three genotypes.
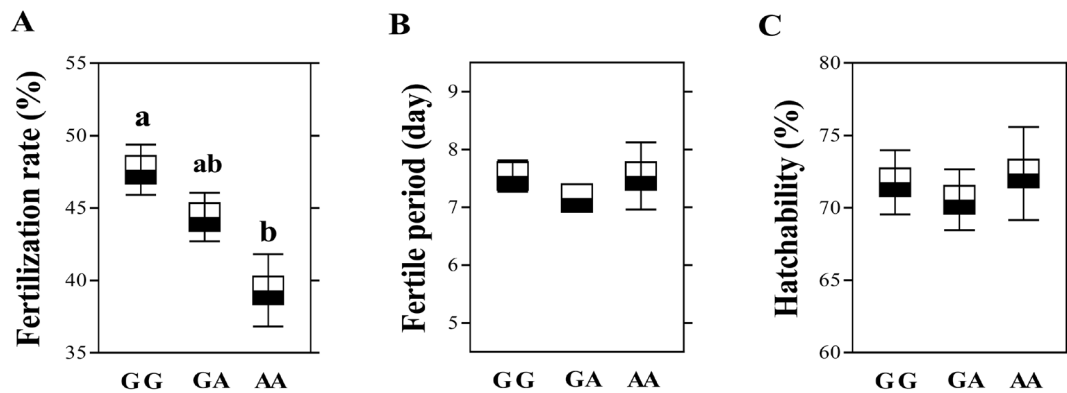
A

B

C

Fig. 6. Correlation between reproductive traits and genotypes. (A) *TMIGD1* GG genotype showed a significantly high fertilization rate (P < 0.05) among genotypes. Other parameters of reproductive performance, fertile period (B) and hatchability (C), did not show correlations with genotype. The GG, GA, and AA genotypes were recorded in our study of 70, 76, and 17 ducks, respectively. The line between black and white colors in each box represents the mean value, and the lower and upper bars represent ± SE. The lack of an upper and lower bar in (B) was due to the SE being less than the upper and lower bounds of the box. Different lowercase letters denote significant differences (P < 0.05).

No significant differences in fertile period and hatchability were observed among the three genotypes.

Discussion

Transcriptome study assists the identification of DNA markers

In order to find meaningful SNPs (i.e., SNPs that have significant associations with traits), we first compared two groups of animals whose specific reproductive performance was different and analyzed their transcriptomes to discover candidate genes with significant levels of gene expression. We hypothesized that significantly-expressed genes were more likely to contain differences in their DNA sequences which would cause alterations in transcription factor binding sites. These alterations would further affect the binding of transcription factors to the gene. We assumed that differences in the DNA sequences of the candidate genes analyzed in our transcriptome experiments would result in the alteration of the gene's expression at the transcription or protein level, which may lead to differences in phenotype and/or performance. In the past, we have used this hypothesis to identify several meaningful SNP markers associated with hatchability [18–20].

Novel approach to the genotyping of the TMIGD1 gene to predict fertilization rate

The *TMIGD1* gene was also identified as a candidate gene in our previous study, in which we used microarray analysis to compare the transcriptomes of ducks with long and short fertile periods. We found that *TMIGD1* mRNA was differentially regulated in the uterovaginal junction of ducks between two fertile period groups [7]. However, in the present study, we demonstrated that different genotypes of the *TMIGD1* gene were significantly correlated with fertilization rate (P < 0.05) but not fertile period (Fig. 6). This result suggests that other SNPs or factors were responsible for the results of our previous study. It is likely that these factors would have caused the differential regulation of the transcript expression level of the *TMIGD1* gene in the two groups of ducks with different fertile periods [7]. Importantly, based on the results of the present study, duck farms

can utilize *TMIGD1* genotyping methods to select specific genotypes using either the low-cost restriction fragment length polymorphism method or the high-throughput rapid detection method using equipment such as the StepOne PCR system. Both approaches can screen the genotypes of ducks at a young age before large investments in their feeding and management are required, allowing the prediction of their future fertilization rates without harming the animals. Because we needed to complete records of the fertilization rate, fertile period, and hatchability of individuals to analyze and find the new DNA marker, we did not use fertilized eggs or duck hatchlings which are too young to yield information about their reproductive performance. To analyze fertilized eggs or young hatching ducks is a future aim and an application of our experiments.

Cell permeability adjustment by TMIGD1 may affect the release of factors that maintain sperm activity

After being encoded from the gene, the TMIGD1 protein is located on the cell surface and functions as an adhesion molecule [21]. The identification of TMIGD1 as an adhesion molecule is based on the extracellular domain of the TMIGD1 protein which contains two putative immunoglobulin domains [21, 22]. TMIGD1 likely contributes to the aggregation of cells and enhances cell–cell adhesion by increasing the transepithelial electrical resistance [21]. It has been suggested that TMIGD1 and its barrier function [21, 23] adjusts the release of factors that maintain the activity of sperm and therefore it may have a direct effect on fertilization rate.

Many factors from the female reproductive tract contribute to maintaining sperm activity

Many characteristics of the female reproductive tract contribute to the maintenance of sperm activity. For example, the expressions of TGFβ isomers in the uterovaginal junction and TGFβ receptors in lymphocytes in the uterovaginal junction, which inhibit the proliferation of B cells and T cells [24], are all increased following AI [25]. This suggests that TGFβs and their receptors play important roles in protecting sperm from the immunoreactions of anti-sperm

or anti-exogenous substances during storage in the uterovaginal junction. Additionally, sperm viability is prolonged by the storing of sperm in a quiescent state in the uterovaginal junction of female birds as a result of flagellar dynein ATPase inactivation of sperm which is induced by lactic acid [26, 27]. Fatty acids [28] and heat shock proteins [29–31] in the female reproductive tract have been shown to reactivate sperm from the quiescent state, and also trigger the release of sperm from the sperm storage tubule located in the uterovaginal junction. With regards to the *TMIGD1* g.175 G>A SNP that we identified in this study, it is located on the second intron. Studies have shown that the first or third intron can either negatively or positively regulate gene expression [32, 33]. Further study is required to confirm whether the *TMIGD1* g.175 G>A SNP affects gene transcription and translation, and to reveal the nature of its association with epithelial cell permeability in the uterovaginal junction.

In this study, we identified that ducks with the *TMIGD1* GG genotype have the highest fertilization rate among the three *TMIGD1* genotypes. Our results can be applied to selection techniques used on duck farms, and minimally-invasive screening can be carried out in young birds to identify those with higher fertilization rates.

Acknowledgements

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