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Short communication: Identification of variation in the ovine prolactin gene of Chios sheep with a cost-effective sequence-based typing assay

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ABSTRACT

The present study identified single nucleotide polymorphisms (SNP) in the coding and untranslated regions of the ovine prolactin gene of Chios sheep. By developing a cost-effective direct sequence-based typing assay, around 600 bp of reliable sequencing data and clear identification of heterozygous positions was achieved. Five SNP were found, located in exons 2 (KC764410:g.567G > A, g.625C > T, g.683C > A) and 3 (KC764410:g.2015C > A, g.2101G > A), whereas the remaining exons were monomorphic. The identified SNP were synonymous, with the exception of the g.567G > A SNP, which results in an Arg to His amino acid change. As the sequencing cost of the sequence-based typing assay was 20 orders of magnitude lower compared with a standard Sanger method, the assay was also used as a genotyping tool. The identified polymorphism was genotyped for 247 ewes and was subsequently used in mixed model association analyses of milk yield, milk fat content, and litter size at birth. The association analysis revealed a significant dominance effect of 0.17 ± 0.07 of the g.2015C > A SNP on milk fat percentage, whereas a dominance effect of −21.33 ± 10.51 of the same SNP on total lactation milk yield was also estimated. The g.2015C > A SNP explained 2.47 and 3.68% of the total phenotypic variance of milk yield and milk fat percentage, respectively, whereas the corresponding values for the animal variance were 7.14 and 11.75%. A suggestive association of the nonsynonymous g.567G > A SNP with litter size at birth was also detected.
Key words: sheep, prolactin association, SNP identification, sequence-based typing (SBT)

Short Communication

Prolactin (*PRL*) is essential for milk production in mammals, as its suppression inhibits lactation. In dairy ruminants, a good body of evidence has shown that *PRL* is galactopoietic and increases feed intake to provide the nutrients necessary to support lactation (Lacasse and Ollier, 2015). Therefore, *PRL* could be regarded as a functional candidate gene associated with milk production and composition.

Whereas SNP identified within the bovine *PRL* gene have been associated with milk production traits (Dong et al., 2013; Lü et al., 2010; Raven et al., 2014), the polymorphism of the ovine *PRL* gene has only received limited attention. Among the polymorphisms identified, 2 variants (A and B), differing by a 23-bp deletion within intron 2 (Orford et al., 2010), have been associated with milk traits (Ramos et al., 2009; Staiger et al., 2010). These *PRL* intron 2 genotypes significantly affected milk yield and fat and protein content in the Serra da Estrela breed (Ramos et al., 2009). Staiger et al. (2010) also suggested that the *PRL* intron 2 polymorphism significantly affected milk yield in East Friesian sheep, and could therefore be used as a potential marker in breeding programs. In addition, an SNP within the 5′ flanking region of the ovine *PRL* gene has been associated with litter size in Small Tail Han sheep (Chu et al., 2009).

The objectives of the present study were to (1) develop a cost effective sequence-based typing (**SBT**) assay to identify and genotype variations in all 5 exons of the *PRL* gene, including the regulatory untranslated regions, and (2) conduct a preliminary association analysis of the identified polymorphism in (1) with sheep traits.

Genomic DNA was isolated from 247 randomly selected Chios sheep from a commercial farm in Cyprus, using the Genomic DNA Blood kit (Macherey-Nagel, Düren, Germany) according to the manufacturers' instructions. For SNP identification and genotyping of *PRL* exons, PCR primers were designed based on the sheep genome sequence Oar v3.0 (https://isgcdata.agresearch.co.nz/), using the program Primer3 (http://frodo.wi.mit.edu/primer3/), and PCR reactions were set up in 25-μL volumes containing 25 ng of genomic DNA, 0.5 U of Taq DNA polymerase (Qiagen Inc., Valencia, CA), 0.5 μ*M* each primer (Table 1), and 1.5

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Table 1. PCR primers used to amplify prolactin (*PRL*) exons

Gene fragment[1]	Forward primer	Reverse primer	Size (bp)
5′UTR–exon 1-partial intron 1	GCCTTATAAAGCCAACATCTGG	TGATACCCCCATTGGAACAT	171
Partial intron 1-exon2-partial intron 2	ATGACAAACTCCTACAAGCTG	CCACATCTTATGAGCTAATGTCTTA	697
Partial intron2-exon3-partial intron3	GCCCAAACAACCCTAATGAA	CGTGAAGCCAGGTAACATCA	219
Partial intron3-exon4-partial intron4	TTTAATGAGATTGTTTCCTGTGG	TCATGAGAACAGCAAGGAAGA	250
Partial intron4-exon5–3′UTR	TCTTTCCTGTATCTTCCCCAAT	GAAACATTGACAAAATTGCCATC	468

[1]UTR = untranslated region.

m*M* $MgCl_2$ (2.5 m*M* $MgCl_2$ for *PRL* exon 2). After an initial 5-min denaturation step, the PCR reactions were subjected to 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, followed by a final elongation step for 5 min at 72°C.

To reduce the sequencing cost, different cleaning procedures of the PCR products (agarose gel extraction and isopropanol precipitation) and different volumes of the fluorescent terminator mix were tested (8, 4, 2, 1, 0.5, and 0.25 μL) for direct sequencing of the amplified fragments. A comparative experiment was set up to ensure the accuracy and specificity of the sequencing results. Sequencing of all 5 exons of the *PRL* gene from 20 animals was performed with both a standard and the modified sequencing protocol for SBT, as described below. To ensure that the SBT assay works efficiently, a similar comparison experiment was performed for a second gene, the *ACAA2* gene, with primers and conditions described in Orford et al. (2012).

For standard sequencing, PCR products, generated with the primers shown in Table 1, were resolved on 2% agarose gels and the excised bands were gel purified using the Qiagen gel extraction kit (Qiagen Inc.). The purified templates were then subjected to cycle sequencing, using the Big Dye Terminator Reagent (Applied Biosystems, Foster City, CA) according to manufacturer's recommendations.

For the SBT assay, the same PCR products described above were generated in 96-well plates and precipitated by the addition of 5 μL of 3 *M* sodium acetate, pH 5.3, plus 25 μL of isopropanol. The reactions were then incubated at −70°C for 20 min and centrifuged at 4°C for 45 min at 2,720 × *g*. The supernatants were removed by inverting the plates on paper towels and briefly spinning them upside down for 2 s at 500 *x g*. The precipitated products were then washed by the addition of 100 μL of 70% ethanol without mixing, recentrifuged for 10 min at 2,720 × *g* at 4°C, and the supernatants were removed as before. Following a 5-min drying step at 50°C, 40 μL of deionized water was added to each well to redissolve the purified DNA.

Cycle sequencing was then performed in 10-μL reaction volumes using 2 μL of the purified templates, 0.5 μL of Big Dye Terminator Reagent v.3.1 (Applied Biosystems), 0.5 μL of the reverse primer (5 μ*M*), 1.75 μL of 5× sequencing buffer, and 5.25 μL of distilled water. The sequencing reactions were subjected to an initial 2-min denaturation step at 96°C, 30 cycles at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. After cycling, 10 μL of water was added, followed by 5 μL of 125 m*M* EDTA and 60 μL of 100% ethanol to precipitate the termination products. The plates were then incubated at room temperature for 15 min in the dark, centrifuged at 2,720 × *g* at 4°C for 45 min, and the supernatants were removed as before. Following a wash with 250 μL of 70% ethanol, the plates were dried for 5 min at 50°C and the termination products were dissolved in 10 μL of formamide before reading on an ABI 3130 genetic analyzer (Applied Biosystems).

The SBT protocol described above was used for identification of SNP in the coding and untranslated regions of the *PRL* gene from 20 additional ewes to those used for the validation experiment and for genotyping exons 2 and 3 of the *PRL* gene, which were found to be polymorphic. Individual records were collected for all animals, including lactation number, lambing date, and age of lambing. Phenotypic data included repeated records for total lactation milk yield, milk fat percentage, and litter size at birth. A total of 621 records were available for the 247 ewes of the study.

The effect of each genotype on the phenotypic traits was assessed with the following mixed linear model; each trait was analyzed separately:

$$Y_{jklmn} = \mu + YS_j + L_k + b_1 age + b_2 dur + G_l + A_m + e_{ijklmn},$$

where Y = total lactation milk yield, milk fat percentage, or litter size at birth record n of animal m, μ = overall population mean for the trait, YS = fixed effect of year (2009–2015) by season (1–2) of lambing interaction j, L = fixed effect of lactation k (1–4), b_1 = linear regression on age at lambing (age), b_2 = linear regression on lactation duration (dur; for milk yield only), G = fixed effect of genotype l at each locus (1–3, denoting