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ORIGINAL ARTICLE

Searching for DNA markers for milk production and composition on chromosome 6 in sheep

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Summary

Several milk protein polymorphisms are potential tools for selection in dairy ruminants. However, research results for dairy sheep are not as conclusive as those for goats or cattle and are often controversial. The main objective of this study was to find and later use molecular genetic markers in selection to improve milk production and milk composition in Awassi ewes. Chromosome 6 was chosen because several studies have reported the presence of significant quantitative trait loci (QTL) affecting milk production traits on ovine and bovine chromosome 6. Altogether, genotypes for 13 microsatellite loci were determined for 258 ewes, which were purebred Awassi or Awassi-Merino crosses. Phenotypic data were lactation yield of milk, milk fat, protein and lactose (kg), average milk protein and fat percentage and average somatic cell count. Five out of the 13 microsatellites showed significant association with at least one of the examined traits.

Introduction

Hungary is the fifth largest sheep milk-producing country in Central and Eastern Europe. Awassi sheep are considered to be the most productive dairy breed in Hungary (Ugarte & Gabina 2004), normally producing 600–700 kg of milk per lactation in intensive production systems. In order to keep up with genetic advancements in other dairy breeds, a research consortium (FVM 46040/2003 project) was formed to support an Awassi breeding programme. Its main objective, to which this study will contribute, is to find and later use molecular genetic markers in selection to improve milk production and composition.

Polymorphisms at several milk protein loci are potential tools for selection in dairy ruminants. However, research results on genetic polymorphisms of caseins and milk whey proteins for dairy sheep are not as conclusive as they are for goat or cattle and are often controversial (Barillet *et al.* 2003). The rapid development of DNA markers and linkage maps for livestock species provides a tool for detection of quantitative trait loci (QTL) and their practical implementation in marker-assisted selection (MAS). Some years ago, the European project, 'genesheepsafety' was initiated to investigate both milk production and functional traits in sheep (Barillet *et al.* 2005). Preliminary results reported significant QTL for milk production traits on ovine chromosomes OAR1, OAR3, OAR5, OAR6, OAR9, OAR16 and OAR20 (Diez-Tascón *et al.* 2001; Carta *et al.* 2002, 2003; El Zarei *et al.* 2002; Schibler *et al.* 2002).

Chromosome 6 is highly conserved between bovine and ovine species (Kappes *et al.* 1997; De Gortari *et al.* 1998); thus, any information on QTL location obtained in cattle is of interest as a possible reference for sheep. Several studies identified significant QTL affecting milk production traits on ovine

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chromosome 6 (Diez-Tascón *et al.* 2001; Schibler *et al.* 2002) and also on bovine chromosome 6 (Georges *et al.* 1995; Velmala *et al.* 1999; Nadesalingam *et al.* 2001; Ron *et al.* 2001; Schrooten *et al.* 2004; Olsen *et al.* 2005; Reinecke *et al.* 2005; de Koning 2006; Kucerova *et al.* 2006), so it was chosen for our initial investigation.

Materials and methods

Animals and phenotypic data

Animals used in this study were from a population initiated by crossing Awassi rams with Hungarian Merino ewes and then mating females in each subsequent generation back to new groups of purebred Awassi rams. Altogether 258 ewes were included in this study, of which 8 were purebred Awassi (PA), 53 were 87.5% Awassi, 12.5% Merino (R2) and 197 were 75% Awassi, 25% Merino (R1) crosses. The relationships between the animals were not known. The ewes produced first, second and third lactations during the years between 2001 and 2005. Ewes were inseminated in autumn, resulting in a lambing season from February to May. The lambs were reared on milk replacer starting 2 days after birth. Feeding was indoor and minimum grazing was practiced. Milk production and composition were measured at monthly intervals. Total lactation yield was calculated by summing the product of the average daily milk yield (evening plus morning) for two consecutive records times the number of days between recordings. If lactation had begun prior to the first recording or continued after the last recording, then milk yield for that period was calculated by multiplying the daily milk yield at recording times the number of days in milk before and after the recording. Milk composition (%) and somatic cell count were determined on every test day in the laboratory, separately from the morning and evening milk samples. The lactation fat, protein and lactose (in kg) were calculated by multiplying the kilogram of milk for the given test period with the respective percentage and were then summed up. All previous performance data were updated, when the next test day data was available. Lactations of ewes in which milk production was recorded at least four times were included in the analysis.

Phenotypic data were lactation milk yield (LMY), lactation milk fat (LMF), lactation milk protein (LMP) and lactation lactose (LL) in kilograms, lactation milk protein% (LP%), lactation milk fat% (LF%) and average somatic cell count (SCC). The average milk yield of the sample population was 227.2 kg during 170 days in milk. These records were retrieved from the database of the farm, where the flock was kept.

Microsatellite data

Thirteen microsatellite markers on ovine chromosome 6 (OAR6) were chosen from the Australian Sheep Gene Mapping website, using the Sheep Best Position Linkage Map (v4.7) with an average distance of 12.38 cM between the markers (http://rubens.its.unimelb.edu.au/~jillm/jill.htm; Figure 1). The authors tried to involve those microsatellites in the investigation which previously were used for QTL mapping in sheep by other research groups.

DNA from each animal was extracted from frozen blood samples using the procedure of Zsolnai & Orbán (1999). After polymerase chain reaction (PCR) amplification, fragment lengths were determined with capillary electrophoresis on an ABI3730 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) using LIZ-500 size standard. GeneMapper 3.7 software (ABI; Perkin Elmer, Foster City, CA, USA) was used for genotype scoring.

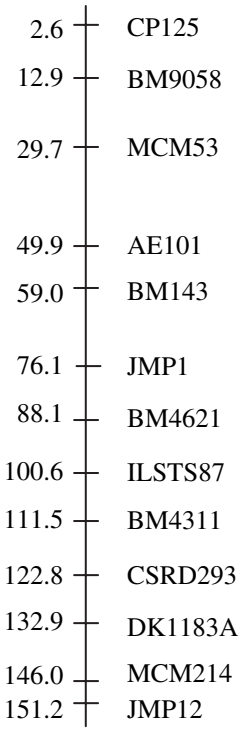

Figure 1 Linkage map for the ovine chromosome 6 containing the examined 13 microsatellites and their position (in cM) based on the Sheep Best Position Linkage Map (v4.7).

Statistical analysis

Differences in production traits among breed types (PA, R1, R2) were not significant in preliminary analyses, so the breed type effect was omitted from subsequent analyses.

Each response variable was analysed separately, and marker associations were analysed one at a time. First, a model was fitted which included fixed effects of year, lactation number and test day code. This model was considered to be the reduced model. Additional models were then fitted that included one marker locus effect in addition to the effects considered in the reduced model. The repeated effect of the ewe, assuming a heterogeneous error covariance structure, was also included in all the models.

The reduced and full models were compared by the log likelihood ratio test (LRT) as proposed by Shaw (1987). Twice the difference between likelihood functions of alternative models is assumed to follow a chi-squared distribution. If in the full model the impact of the marker locus was shown to be significant at $p < 0.05$, allele effects were then calculated. The effect of each allele at one locus at a time on each performance trait was treated as a regression using the procedure of Ostergard *et al.* (1989), and is shown next. Data were coded as 0, 1 or 2 for number of copies of a given allele in each individual ewe's genotype, and the partial covariate coefficient b estimated the substitution effect of that allele. To avoid dependencies among equations, the effect of the allele with the highest frequency was set to zero. This allele was assumed to be replaced, and the effects of other alleles were expressed as deviations from the effect of the most frequent allele. All alleles with frequency less than 5% were pooled into one 'rare' category, which was treated as a single allele.

The statistical model containing the multiple regression on the number of alleles was as follows:

$$y_{ijklmn} = \mu + year_j + lactation_k + lactation\_length_l + \sum_{m=1}^{M-1} b_n x_{imn} + e_{ijklmn}$$

where y_{ijklmn} is the response of ewe i in year j in lactation k at test day code l; μ is the population mean of the trait; $year_j$ is the effect of year j from 2001 to 2005; lactation_k is the effect of lactation k from 1 to 3; $\text{lactation\_length}_l$ is the effect of length of lactation (inferred from number of test day records between 4 and 9) for lactation traits LMY, LMF, LMP and LL; b_n is the partial regression coefficient corresponding to effect of allele n at locus m; x_{imn} is the number of copies of allele n present in animal i at locus m, where x_0 represents the most frequent allele and the remaining alleles are denoted as $x_1, \ldots, x_n, \ldots x_{(M-1)}$; and e_{ijklmn} is the random error term.

The general linear model of SAS was used for finding the solutions for the effects. To reduce type I error, Hochberg's (1988) adjusted p-values were calculated using the SAS PROC MULTTEST procedure (SAS Institute, Cary, NC 2004).

Results

The genotyping of 258 sheep for 13 OAR6 microsatellite loci revealed an average of 12.46 alleles per locus. Mean heterozygosity of the markers was 0.72. The Nei's (1987) unbiased gene diversity was 0.74 with a 0.04 inter-locus SD.

Allele substitution models were used to estimate associations between alleles and production traits. On the basis of LR tests, the inclusion of microsatellites AE101, JMP1, BM4621, ILSTS87, CSRD293, DK1183A, MCM214 and JMP12 did not significantly increase ($p > 0.05$) model fit for any production trait and these allele effects were not further investigated. A Hochberg correction for multiple test was made for each trait on the chromosome including all investigated loci. The results are presented only for those traits for which significant microsatellite effects were found after correction (Table 1).

CP125 is the only locus that was found to be associated with SCC, with a false discovery rate (FDR) of 0.009. Allele 104, with the highest frequency, was associated with lower SCC ($p < 0.05$); while allele 114 with a frequency of 11.9% was associated with higher SCC. At locus BM9058, allele 145 contributed significantly to variation in LF%, and its effect was desirable (FDR 0.022). Allele 78 at locus MCM53 had a significant negative association with LL (FDR 0.043). Allele 103 at locus BM143 was highly significantly ($p < 0.001$) associated with increased LMY (FDR 0.039) and increased LL (FDR 0.043). It had the second highest frequency at this locus. Allele 111, with a lower frequency had negative associations with both traits. The rest of the alleles showed also negative influence on these traits but the effect was not significant. At locus BM4311, the substitution effect of allele 106 was significantly positive on LP% (FDR 0.009), but its frequency was only 9.9% in the population examined.

Table 1 Gene substitution effects (α) and standard errors (SE) of the microsatellite alleles which show a significant effect on at least one of the examined traits

Microsatellite marker	Allele (size in bp)	Allele frequency	α (SE) in LMY (kg)	α (SE) in LL (kg)	α (SE) in LF%	α (SE) in LP%	α (SE) in SCC
CP125	104	25.0	–	–	–	–	−114.05 (50.04)*
	114	11.9					151.43 (65.58)*
BM9058	145	14.1	–	–	0.12 (0.05)*	–	–
MCM53	78	26.5	–	−0.27 (0.12)*	–	–	–
BM143	103	27.3	24.04 (5.98)**	1.33 (0.29)**	–	–	–
	111	13.4	−16.60 (7.69)*	−0.82 (0.39)*			
BM4311	106	9.9	–	–	–	0.08 (0.03)*	–

*$p < 0.05$; **$p < 0.001$.
LMY, lactation milk yield; LL, lactation lactose; LF%, lactation milk fat%; LP%, lactation milk protein%; SCC, somatic cell count.

Discussion

In the present study, 13 microsatellites distributed across the whole of ovine chromosome 6 were investigated. In sheep, fewer studies have been performed to map QTL for milk production and composition than in cattle, but fortunately the number of these investigations is growing. Although none of these studies included LL, presumably owing to the known high correlation with milk yield, which was also found in this study (0.986). To date, significant QTL for these traits previously have been identified in two regions on OAR6. In the Churra sheep, a QTL was suggested close to the marker BM4311 affecting protein percentage in one of the examined families (Diez-Tascón *et al.* 2001). In agreement with this result there was a significant substitution effect for allele 106 at locus BM4311 in our study. This microsatellite is located in the proximity of the casein cluster in both sheep (De Gortari *et al.* 1998) and cattle (Kappes *et al.* 1997), which is one of the most interesting regions of the ovine and bovine chromosome. The fact that casein genes play an important role in milk production may explain the significant effect of allele 106 at this locus. However, this effect may also be dependent on unidentified genes closely linked to the BM4311 region.

Schibler *et al.* (2002) analysed two microsatellites (AE101 and BM9058) from OAR6 in a French population generated from a granddaughter design. They found a QTL for fat and protein percentage and somatic cell score close to marker AE101. In our experiment, no alleles of locus AE101 have shown significant influence on any of the traits examined. However, allele 103 of BM143, in the proximity of AE101, was negatively associated with LMY and LL, and allele 111 showed a significant negative effect on these traits. Our results are in agreement with the results of other studies which reported segregation of QTL for milk yield in the homologous region in bovine, close to BM143 (Spelman *et al.* 1996; Olsen *et al.* 2005; Reinecke *et al.* 2005).

Three microsatellites were analysed from the first 30 cM region of OAR6. Each of these loci had a significant association with one of the traits examined. At microsatellite BM9058, there was a significant allele substitution effect on LF%, while an allele of locus MCM53 was associated with LL. No QTL segregation has been reported in this ovine chromosome region before. In cattle, Schrooten *et al.* (2004) mapped a QTL for fat and protein percentage to the end of bovine chromosome 6, which corresponds to this 30 cM ovine chromosome segment. Concerning the CP125 microsatellite, two alleles were significantly associated with SCC. This is the only locus of the 13 examined that significantly influenced this trait. For somatic cell score, Schibler *et al.* (2002) have found a QTL in OAR6, but in another region, near AE101.

In this study, 5 of the 13 examined microsatellites from OAR6 showed significant association with at least one of the examined milk production or composition traits. It is, however, important to note that the association observed may arise in back-cross populations, where considerable linkage disequilibrium exists. Furthermore, few animals were available in this study, so these results might be too preliminary to make final conclusion. However, the high significant level in the case of BM143, and the fact that some of our results are in agreement with the results obtained in sheep or cattle, might suggest that further investigation is worthy on OAR6 in this population.

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References

Barillet F., Arranz J.J., Carta A. (2003) Mapping quantitative trait loci for milk production and genetic polymorphisms of milk protein in dairy sheep. In: Proceedings of the International Workshop on major Genes and QTL in Sheep and Goat, Toulouse, France, 8–11 December 2003, pp. 2–16.

Barillet F., Arranz J.J., Carta A. (2005) Mapping quantitative trait loci for milk production and genetic polymorphisms of milk proteins in dairy sheep. *Genet. Sel. Evol.*, **37**(Suppl. 1), S109–S123.

Carta A., Barillet F., Allain D., Amigues Y., Bibé B., Bodin L., Casu S., Cribiu E.P., Elsen J.M., Fraghi A., Gruner L., Jacquiet J., Ligios S., Marie-Etancelin C., Mura L., Piredda G., Rupp R., Sanna S.R., Scala A., Schibler L., Casu S. (2002) QTL detection with genomic markers in a dairy sheep backcross Sarda x Lacaune resource population. In: *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production*, Montpellier, 19–23 August 2002, Inra, Castanet-Tolosan, France, **29**, pp. 211–214.

Carta A., Barillet F., Casu S., Cribiu E.P., Elsen J.M., Fraghi A., Mura L., Schibler L. (2003) A genome scan to detect QTL affecting dairy traits in a dairy sheep backcross Sarda x Lacaune population. In: Proceedings of the 15th National Congress of Scientific Association of Animal Production, Parma, Italy, 18–20 June 2003, pp. 31–33.

De Gortari M.J., Freking B.A., Cuthbertson R.P., Kappes S.M., Keele J.W., Stone R.G., Leymaster K.A., Dodds K.G., Crawford A.M., Beattie C.W. (1998) A second generation linkage map of the sheep genome. *Mamm. Genome*, **9**, 204–209.

Diez-Tascón C., Bayon Y., Arranz J.J., Fuente F., Primitivo F.S. (2001) Mapping quantitative trait loci for milk production traits on ovine chromosome 6. *J. Dairy Res.*, **68**, 389–397.

El Zarei M.F., Arranz J.J., Gutierrez-Gil B., Bayon Y., de la Fuente L.F., San Primitivo F. (2002) Scanning chromosome 9 for QTL underlying milk production traits in Spanish Churra sheep. In: *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production*, Montpellier, 19–23 August 2002, Inra, Castanet-Tolosan, France, **28**, pp. 375–378.

Georges M., Nielsen D., Mackinnon M., Mishra A., Okimoto R., Pasquino A.T., Sargeant L.S., Sorensen A., Steele M.R., Zhao X., Womack J.E., Hoeschele I. (1995) Mapping quantitative trait loci controlling milk production in dairy cattle by exploiting progeny testing. *Genetics*, **139**, 907–920.

Hochberg Y. (1988) A sharper Bonferroni procedure for multiple tests of significance. *Biometrika*, **75**, 800–803.

Kappes S.M., Keele J.W., Stone R.T., McGraw R.A., Sonstegard T.S., Smith T.P.L., Lopez-Corrales N.L., Beattie C.W. (1997) A second-generation linkage map of the bovine genome. *Genome Res.*, **7**, 235–249.

de Koning D. (2006) Conflicting candidates for cattle QTL-s. *Trends Genet.*, **22**, 301–305.

Kucerova J., Lund M.S., Sorensen P., Sahana G., Guldbrandtsen B., Nielsen V.H., Thomsen B., Bendixen C. (2006) Multitrait quantitative trait loci mapping for milk production traits in Danish holstein cattle. *J. Dairy Sci.*, **89**, 2245–2256.

Nadesalingam J., Plante Y., Gibson J.P. (2001) Detection of QTL for milk production on chromosomes 1 and 6 of Holstein cattle. *Mamm. Genome*, **12**, 27–31.

Nei M. (1987) Molecular Evolutionary Genetics. Columbia University Press, New York, NY.

Olsen H.G., Lien S., Gautier M., Nilsen H., Roseth A., Berg P.R., Sundsaasen K.K., Svendsen M., Meuwissen T. (2005) Mapping of a milk production quantitative trait locus to a 420-kb region on bovine chromosome 6. *Genetics*, **169**, 275–283.

Ostergard H., Kristensen B., Andersen S. (1989) Investigation in farm animals of associations between the MHC system and disease resistance and fertility. *Livest. Prod. Sci.*, **22**, 49–67.

Reinecke P., Reissmann M., Müller U., Abdel-Rahman S. (2005) Fine mapping of milk yield QTL on chromosomes 6 and 20 in German Holstein population using micosatellite markers. *J. Cent. Eur. Agricult.*, **6**, 501–508.

Ron M., Kliger D., Feldmesser E., Seroussi E., Ezra E., Weller J.I. (2001) Multiple QTL analysis of bovine chromosome 6 in the Israeli Holstein population by a daughter design. *Genetics*, **159**, 727–735.

Schibler L., Roig A., Neau A., Amigues Y., Boscher M.Y., Cribiu E.P., Boichard D., Rupp R., Barillet F. (2002) Detection of QTL affecting milk production or somatic cell score in three French dairy sheep breeds by partial genotyping on seven chromosomes. In: *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production*, Montpellier, 19–23 August 2002, Inra, Castanet-Tolosan, France, **29**, pp. 215–218.

Schrooten C., Bink M.C.A.M., Bovenhuis H. (2004) Whole genome scan to detect chromosomal regions

affecting multiple traits in dairy cattle. *J. Dairy Sci.*, **87**, 3550–3560.

Shaw R. (1987) Maximum likelihood approaches applied to quantitative genetics of natural populations. *Evolution*, **41**, 812–826.

Spelman R.L., Coppieters W., Karim L., van Arendonk J.A.M., Bovenhuis H. (1996) Quantitative trait loci analysis for five milk production traits on chromosome six in the Dutch Holstein-Friesian population. *Genetics*, **144**, 1799–1808.

Ugarte E., Gabina D. (2004) Recent development in dairy sheep breeding. *Arch. Tierzucht*, **47**(special issue), 10–17.

Velmala R.J., Vilkki H.J., Elo K.T., de Koning D.J., Maki-Tanila A.V. (1999) A search for quantitative trait loci for milk production traits on chromosome 6 in Finnish Ayrshire cattle. *Anim. Genet.*, **30**, 136–143.

Zsolnai A., Orbán L. (1999) Accelerated separation of random complex DNA patterns in gels: comparing the performance of discontinuous and continuous buffers. *Electrophoresis*, **7**, 1462–1468.

