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1 Effect of selection for scrapie resistance on genetic diversity in a rare and locally adapted
2 sheep breed: the case of Sambucana

3

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23 Abstract.

24 In Italy, a breeding plan has been adopted in 2005 to increase resistance to scrapie. The
25 effect of selection on genetic diversity of Sambucana, a rare and locally adapted sheep
26 breed, was assessed by analysing the evolution of allele frequencies at different levels: the
27 PRNP (prion protein) gene itself, microsatellites on the same chromosome as PRNP, and
28 microsatellites on other chromosomes, not subjected to selection for resistance to scrapie. A
29 total of 147 young rams, 80 born in 2004 and 67 born in 2008–2009 were analysed.
30 Evidence of diversity loss was observed for PRNP as a consequence of the directional
31 selection. Diversity was affected in the immediate vicinity of PRNP but the effect on more
32 distant loci on the same chromosome was trivial. With regard to neutral markers, lack of
33 heterozygosity with no changeover of allele frequencies was observed suggesting an
34 increase of inbreeding. Mating policies would be sufficient to solve these problems. A
35 selection scheme based on genotyping rams and eliminating carriers of susceptibility and
36 all carriers of high susceptibility is the best way to improve natural resistance to scrapie
37 with low costs and minimal problems in the current conservation programmes targeting rare
38 breeds.

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42

43 Keywords: sheep; scrapie resistance; genetic variation; prion protein gene; molecular
44 marker.

45

46 Introduction.

47 In sheep, susceptibility to scrapie is influenced by mutations in the coding region of the
48 PRNP (prion protein) gene, located on OAR13 (sheep chromosome 13). Haplotypes-alleles
49 valine/arginine/glutamine (VRQ) and alanine/arginine/glutamine (ARQ) at codons 136,
50 154, and 171, respectively, are associated with high susceptibility, whereas the
51 alanine/arginine/arginine allele (ARR) has been linked to decreased susceptibility or
52 resistance (Belt et al., 1995; Bossers et al., 1996; Hunter et al., 1996).

53 Accordingly, the European Union has implemented programmes for genetic control
54 of scrapie susceptibility in sheep (European Commission, 2003). Since 2005 in Italy, a
55 breeding plan has been adopted to increase the frequency of the ARR ‘resistant’ allele and
56 to eliminate the VRQ ‘susceptible’ allele (Decreto Ministeriale 17 Dicembre 2004).

57 The impact of selection for scrapie resistance has been investigated frequently.
58 Different selection strategies applied to breeds with different genetic structure were
59 considered. Most studies predict effects and costs of selection in term of variability using
60 both simulated and real datasets (Alfonso et al., 2006; Álvarez et al., 2007; Álvarez et al.,
61 2009; Drögemüller et al., 2004; Man et al., 2009; Molina et al., 2006; Roden et al., 2006;
62 Windig et al., 2004; Wiśniewska et al., 2010), whereas only a few investigation analyse the
63 realized effects after the selection programmes have been applied (Palhière et al., 2006;
64 Palhière et al., 2008). Various concerns have been raised regarding possible unintended
65 consequences of widespread selection on PRNP alleles, including risk of genetic diversity
66 loss and increased inbreeding (Dawson et al., 2008; Parada et al., 2007).

67 The Sambucana is a Piemonte region breed devoted to meat production. Since 1985,
68 several initiatives have been carried out to safeguard the breed and economically valorise
69 derived productions. An important step in the conservation programme was the creation of

70 the 'Agnello Sambucano Garantito' brand, which certifies the origin of the lambs and
71 guarantees the quality of the meat. In 2001, the Sambucano lamb was added to the
72 'Presidia' list of Slow Food. In 1993 the breed was classified by the FAO as 'at limited
73 diffusion' (FAO-UNEP, 1993). In 2005, 168 rams and 3995 ewes were registered
74 (Associazione Nazionale della Pastorizia, <http://www.assonapa.it>).

75 The Sambucana breed showed an ARR frequency above the threshold to comply
76 with European regulations, before the selection programme started. Nevertheless, as a rare
77 and locally adapted breed, it could benefit of some derogations. In Piemonte, the selection
78 programme for scrapie resistance has been applied since 2005 (Regione Piemonte, 2005).
79 This decision meets consumer expectations of a high-quality meat that is also safe to eat.

80 Currently, great attention is paid to avoid excessive inbreeding or genetic drift.
81 Genealogical information can be used to monitor the evolution of genetic variability.
82 However, in a population kept in an extensive breeding system, the quality of pedigree
83 information available is often inadequate. For the Sanbucana, pedigrees are not available at
84 all. In such cases, the evolution of genetic variability can be assessed using a molecular
85 approach.

86 The aim of the present investigation was to use Sambucana as a model to evaluate
87 the consequences of selection for scrapie resistance on molecular variability in a rare and
88 locally adapted breed. Three processes may play a role affecting genetic variability: direct
89 selection on PRNP itself, frequency changes in marker loci on the same chromosome
90 because of the linkage, and genetic drift because a limited part of the population is used as
91 parents. Therefore, the evolution of allele frequencies was analysed at these different levels:
92 PRNP gene, microsatellite marker loci on OAR13, and microsatellites on chromosomes
93 other than OAR13, markers not subjected to direct selection for scrapie resistance.

94

95 Materials and methods.

96 Sample collection. A total of 147 Sambucana young rams (candidate sires), 80 born in 2004
97 and 67 born in 2008–2009 (denoted as the before-2005 and after-2005 cohorts), were
98 randomly chosen from different flocks among all animals genotyped at the PRNP locus by
99 the IZSTO-CEA (Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle
100 d’Aosta, Italian Reference Centre for Animal Transmissible Spongiform
101 Encephalopathies). The between-sampled cohorts period length (4 years) represents about
102 one generation.

103 The analyses were performed on rams only because, before 2005, males were
104 genotyped exclusively.

105 Each cohort was divided into two risk groups according to the selection criteria
106 adopted in the Italian breeding plan against VRQ and in favour of ARR. ‘Low risk’ rams
107 are ARR/ARR and ARR/non-ARR, except ARR/VRQ, all other animals being considered
108 as ‘high risk’.

109 Molecular techniques. Genomic DNA was extracted from blood samples using the
110 NucleoSpin QuickPure extraction kit (Macherey-Nagel, Dueren, Germany). Fourteen
111 microsatellites were chosen on OAR13 (Table 1). Five markers are strictly linked to PRNP:
112 PRNPS11, -15, and -24 map within PRNP whereas PRNPS04 and -05 map at about 40 kb
113 upstream of the 5’ end of the gene sequence GenBank U67922.1. The other nine OAR13
114 markers were chosen outside the gene at various distances to verify whether the effect of
115 selection depends on relative distances (Geldermann et al., 2003; Isler et al., 2006; Lühken
116 et al., 2006; Palhière et al., 2008; Preuss et al., 2005). Other 12 microsatellites, belonging to
117 the ISAG (<http://www.isag.us/>) and the ECONOGENE (<http://www.econogene.eu/>)

118 recommended panels, were selected to avoid syteny with PRNP (denoted as neutral
119 microsatellites in Table 1). PCR assays were developed and performed as previously
120 described (Soglia et al., 2010).

121 In order to validate the microsatellite panel, an error assay was performed by
122 replicating the genotyping molecular analyses on a randomly chosen 10% of individual
123 samples. The average error rate per allele was computed as suggested by Pompanon et al.
124 (2005). Individual markers in each age cohort were tested for deviations from HW (Hardy–
125 Weinberg) proportions, F_{IS} statistics and the significance of their non-zero values were
126 obtained using Fstat 2.9.3.2 software (Goudet, 1995). The presence of linkage
127 disequilibrium between neutral markers was checked using the likelihood ratio test
128 implemented with Arlequin software (Excoffier et al., 2005).

129 Analyses of genetic variation. Genetic variation was analysed for PRNP and for the
130 markers grouped into three clusters: OAR13 microsatellites within PRNP, OAR13
131 microsatellites outside PRNP, and neutral microsatellites. Departures from the HW
132 proportions were examined using the F_{IS} statistics.

133 Losses of both heterozygosis and number of alleles in term of within-groups,
134 between-groups, and total contributions to variability were assessed following Caballero
135 and Toro (2002) and Petit et al. (1998) approaches as proposed by Álvarez et al. (2009)
136 using MolKin 3.0 (Gutiérrez et al., 2005). Raw number of alleles (A), allelic richness, or
137 number of alleles adjusted for sampling size (A_g , g being two-fold the number of
138 individuals with full genotype information in the group of lowest size) (Hurlbert, 1971),
139 gene diversity (GD), or expected heterozygosis (Nei, 1987), and PIC (polymorphism
140 information content) were calculated. At both age cohorts, loss of genetic variability was
141 quantified sequentially removing all the ‘high risk’ rams.

142 At all 26 microsatellite loci, differences in allele frequencies between cohorts were
143 tested with the F_{ST} statistics as implemented by the Arlequin software applying analysis of
144 molecular variance.

145

146 Results.

147 Validation of the microsatellite markers. The rate of missing genotypes was 1.4%, and the
148 average error rate per allele was 0.2%. Eleven individual samples were dropped from the
149 dataset because they failed to consistently provide amplification products at all or most loci.
150 The data for 26 microsatellites from 136 individual samples (71 and 65 from the two age
151 cohorts, respectively) were finally analysed. Three markers revealed systematic deviations
152 from HW proportions in both age cohorts whereas seven loci showed significant deviations
153 only after 2005 (Table 1). The test for linkage disequilibrium revealed no significance
154 among neutral microsatellites. These results characterized the chosen microsatellites as a
155 useful tool to obtain the goals of our investigation.

156 Analyses of genetic variation of the PRNP gene. All five known PRNP alleles were
157 observed (Table 2). ARQ was predominant before 2005 when ARH was the least frequent
158 allele. After one generation of selection, ARR doubled in frequency, becoming the
159 predominant allele, whereas ARQ dropped by 38% and VRQ by 69%. ARH was not
160 sampled after 2005; consequently, number of alleles was reduced by one. The reversal of
161 the frequency ratio between ARR and ARQ affected the genotypic frequencies and thus the
162 availability of resistant rams. The percentage of ARR/ARQ rams was 43.7% in the before-
163 2005 cohort and 38.5% one generation later; however, the ARR/ARR rams, representing
164 only 2.8% before 2005, expanded to 35.4% in the later cohort. As a whole, the 'low-risk'

165 rams increased from 49% to 78%. The rates of ARQ/ARQ were 28.2% and 12.3% in the
166 two age cohorts, respectively. The VRQ-carrier rams decreased from 12.7% to 4.6%.

167 Analyses of genetic variation at the microsatellite loci. The before-2005 and after-
168 2005 cohorts were compared for their allelic arrangements (Table 3). The difference
169 between the two age cohorts, as tested using the F_{ST} statistics, was highly significant for
170 PRNP. Likewise, significant differences were identified at the OAR13 microsatellites for
171 both within and outside PRNP markers. The neutral markers, on the other hand, showed no
172 modification of their allele frequencies after one generation. In Figure 1 the differences for
173 each OAR13 marker separately are plotted versus the distance to the PRNP locus.

174 Microsatellites within PRNP on the overall showed a decline in heterozygosis after
175 the selection plan started (Table 4). The GD and PIC decreased between the two cohorts by
176 26% within PRNP, but by no more than 2% on the other locations. No decline in number of
177 alleles (A and A_g) was observed on OAR13 whereas, after the selection plan started, the
178 allelic richness increased by 7% at the neutral markers.

179 A detailed analysis of individual markers showed that PRNPS11, which is on
180 PRNP, lost one allele and 59% of its original heterozygosis, whereas PRNPS04 lost 48% of
181 its heterozygosis but no alleles. The other three markers within PRNP showed lower
182 decreases in heterozygosis (16–17%). The OAR13 microsatellites outside PRNP showed
183 only small differences between cohorts except BMS2319, that underwent a pronounced
184 loss of diversity.

185 OAR13 markers outside PRNP after 2005 and neutral markers in both age cohorts
186 showed observed heterozygosis deficiency, characterized by positive and significant F_{IS}
187 values (Table 4).

188 These results show that the effects of selection on OAR13 microsatellites differed
189 according to the relative distance of the loci from the PRNP gene and their polymorphism.

190 Changes in each of the two age cohorts were then computed after removal of all
191 ‘high-risk’ rams to ascertain if overall contributions to variability were negligible or not
192 (Table 5).

193 The cull of the ‘high-risk’ rams from both age cohorts dramatically decreased the
194 GD_T within PRNP (more than 15%) with negative contributions mainly at GD_W . In fact, the
195 ‘high-risk’ rams carried more heterozygosity than the selected ones (0.464 vs. 0.307 and
196 0.373 vs. 0.268 for the two age cohorts, respectively). Although to a small extent (less than
197 2%), the selection decreased the GD_T also at the OAR13 markers outside PRNP.

198 On the other genomic locations, selectively neutral, the lowest decrease of GD_T was
199 observed before 2005, whereas in the succeeding generation the same parameter even
200 showed a small increase.

201 The ‘high-risk’ rams provided a positive contribution to the allelic richness at the
202 within PRNP markers, but only in the younger cohort. After removal of the same animals,
203 on the OAR13 markers outside PRNP the allelic richness decreased at both within and total
204 levels because the culled rams provided positive contributions. In spite of this, the overall
205 allelic richness did not decrease after 2005 (Table 4).

206 On the contrary, the first selective action did not decrease diversity on the other
207 chromosomes, where the ‘high-risk’ rams provided an unfavourable contribution; as a
208 consequence, the cull of these animals gave rise to the gain in A and A_g observed in Table 4
209 between the two cohorts.

210

211 Discussion.

212 The main goal of the present analysis was to assess the effect of the selection for scrapie
213 resistance on genetic variability in a small and locally adapted breed at the early stage of a
214 selection programme. Genealogical information can be used to monitor the evolution of
215 genetic diversity. Unfortunately, pedigrees were not available for this breed. Therefore,
216 information from molecular investigation was the best option to assess the effect of
217 selection on the genetic variability (Álvarez et al., 2009). Unlike simulation studies, based
218 on genetic characteristics of a population, our investigation used real population data both
219 before and after the selection acted. The Sambucana breed was used as a model.

220 Before the selection for scrapie resistance started, the predominant PRNP allele was
221 ARQ. This allele is also predominant in most Italian and European breeds and is thought to
222 represent the ancestral form of the PRNP gene (Acutis et al., 2003; Goldmann et al., 2005).
223 From 2005 onwards, the selection scheme for scrapie resistance has provided for all
224 ARR/ARR rams to be bred first and then ARR/non-ARR rams with equal preference, with
225 ARR/VRQ rams being excluded.

226 Under the selection programme, despite the short time interval (one generation),
227 evidence of variability loss was observed for PRNP as a consequence of the directional
228 selection acting on this gene. In fact, in the most recent cohort, an allele had been lost. The
229 change in genotypic frequencies illustrates the effectiveness of the selection programme.
230 The ARR carriers reached a proportion of 78% in only a few years despite the fact that the
231 ARR allele frequency was rather low at the beginning of selection. On the other hand, the
232 ARQ and VRQ frequencies decreased significantly. Because the selection programme did
233 not involve females, the VRQ allele could not be eliminated in a single generation. Overall,
234 the implementation of a mild selection strategy achieved a satisfactory increase in ARR and
235 a large decrease in VRQ. On the other hand, at the beginning of the selection plan

236 implementation, the number of homozygote rams was small, and the use of ARR/ARR
237 males exclusively would have adversely affected the within–population genetic variability
238 through a severe bottleneck. In the future, a changeover to using only homozygote rams
239 will be possible to accelerate the increase in ARR frequency.

240 The selection changed also the variability of the five microsatellites located within
241 the PRNP sequence. In particular, selection for ARR strongly affected PRNPS11. ARR was
242 quite exclusively linked to the PRNPS11-151 allele, whereas the VRQ allele was
243 exclusively linked to the 149 allele. Selection had less effect on PRNPS04, -05, -15, and -
244 24, probably because of their low initial informative content. The effect of selection on
245 OAR13 markers outside PRNP seemed to be quite low, with average differences between
246 cohorts very close to zero. This positional relativity of effect has been identified previously;
247 in four French breeds, Palhière et al. (2008) found that the effect on markers at OAR13
248 depended strongly on the relative distance from the selection objective. In Sambucana,
249 selection affected genomic diversity in the immediate vicinity of PRNP, but the effect on
250 more distant loci on the chromosome was trivial. The signature shown by BMS2319 was
251 difficult to trace back to selection for resistance to scrapie because this microsatellite is the
252 most distant from PRNP.

253 With regard to neutral markers, a lack of observed heterozygosis, with no
254 changeover of allele frequencies, was noted in both age cohorts (Table 3 and 4). These
255 findings suggest presence of inbreeding already before selection, due to the small size of
256 the population, but the tendency is increasing. In this last respect, several explanations are
257 possible: 1) because only 49% of young rams had a favourable genotype before 2005, the
258 narrow number of selected reproducers reduced the effective population size still further; 2)
259 in a small population, the carriers of favourable genotypes may be more related to each

260 other than randomly chosen individuals, and for an equal number of reproducers, the
261 effective size thus may be smaller than expected in a pure genetic drift condition; 3) the
262 farmers who owned young resistant rams could have used them intensively in their native
263 flocks, reducing gene flow between flocks. Mating policies implemented to avoid
264 inbreeding would be sufficient to solve these problems.

265 Except for the OAR13 markers, removal of susceptible rams did not reduce
266 heterozygosity and allelic richness, either within a generation or across generations. This
267 may be due to similar allele frequency distribution in selected as well as in culled rams. The
268 genetic background of the 'high risk' rams was well represented in the selected group and,
269 in addition, the cull of these animals gave rise to gain in number of alleles between the two
270 generations. It must be noted that rams tested after 2005 were progeny of both selected
271 rams and unselected ewes born before 2005. Therefore, they provided a sample of the allele
272 pool of the parents that was achieved with the first selective decisions. Moreover, because
273 the resistant rams born after 2005 also showed no reduction in allelic richness, it can be
274 inferred that the population is not in danger of a strong bottleneck.

275

276 Conclusion.

277 Allelic richness and gene diversity are important for conservation of genetic stocks. The
278 long-term evolutionary potential of a population and the limit of selection response are
279 determined by the initial number of alleles as well as by the number of conserved alleles,
280 regardless of the allele frequencies (Falconer and Mackay, 1996).

281 The consequences of adding a new criterion of selection differ between breeds
282 depending on the initial situation and the strategy applied. The results based on the
283 Sambucana breed can be generalized and the recommendation extended to other breeds

284 with similar starting genetic properties. The carriers of undesirable PRNP genotypes would
285 not be essential to maintain genetic variability in the overall genome outside OAR13.

286 Selection scheme based on genotyping rams and eliminating non-ARR/non-ARR
287 and all VRQ carriers is actually the best way to improve natural resistance to scrapie with
288 low costs and minimal problems in the current conservation programme targeting rare
289 breeds. The drawback of this strategy is that it does not result in the immediate elimination
290 of VRQ; nevertheless, it still guarantees a rapid and considerable reduction of its frequency.

291

292 Conflict of interest statement.

293 We hereby warrant that there are not any conflicts of interests among authors and between
294 authors and other people, institutions or organizations.

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306

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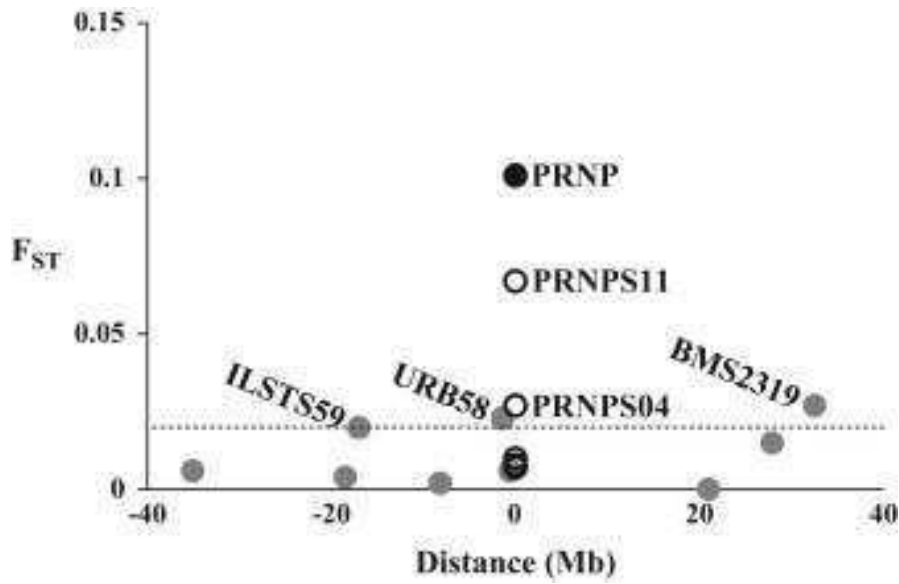
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409

410 Figure 1 Differences in allele arrangements (F_{ST}) between the before-2005 and after-2005 cohorts of
411 rams as a function of distance of any OAR13 microsatellite from PRNP (deviations are in Mb). Grey
412 circles show the OAR13 microsatellites outside PRNP, empty circles show the OAR13 microsatellites
413 within PRNP, the black circle points at PRNP. All F_{ST} values above the dotted line are statistically
414 significant.



415

1 **Table 1**

2 Location and values of F_{IS} for the OAR13 and neutral microsatellites.

3

OAR13 microsatellites				Neutral microsatellites			
	Dist. ^a	Before-2005	After-2005		OAR ^b	Before-2005	After-2005
BMC1222	-35.1	+0.080 n.s.	+0.089 n.s.	CSRD247	14	+0.031 n.s.	+0.197 **
MCM152	-18.5	-0.081 n.s.	-0.028 n.s.	D5S2	5	+0.058 n.s.	+0.243 **
ILSTS59	-17.0	-0.105 n.s.	+0.025 n.s.	HSC	9	+0.068 n.s.	+0.142 **
HUJ616	-8.2	+0.065 n.s.	-0.115 *	INRA23	1	+0.084 n.s.	+0.102 n.s.
URB58	-1.4	-0.089 n.s.	-0.009 n.s.	INRA5	10	+0.429 ***	+0.212 ***
BMS1669	-0.6	-0.059 n.s.	+0.096 n.s.	INRA63	14	+0.072 n.s.	-0.006 n.s.
PRNPS04	0	-0.085 n.s.	-0.077 n.s.	MAF65	15	+0.010 n.s.	+0.011 n.s.
PRNPS05	0	-0.248 ***	-0.286 ***	MCM527	5	+0.047 n.s.	+0.064 n.s.
PRNPS11	0	-0.045 n.s.	+0.290 *	OarCP49	17	-0.070 n.s.	+0.017 n.s.
PRNPS15	0	+0.166 n.s.	+0.018 n.s.	OarFCB11	2	+0.005 n.s.	+0.130 *
PRNPS24	0	+0.166 n.s.	-0.128 n.s.	OarFCB20	2	+0.062 n.s.	+0.121 n.s.
CTSBJ12	+21.0	-0.025 n.s.	+0.002 n.s.	OarFCB304	19	-0.088 n.s.	-0.051 n.s.
MMP9	+27.9	+0.074 n.s.	+0.259 ***				
BMS2319	+32.5	+0.110 *	+0.399 ***				

4

5 ^aDistance of any locus from PRNP (deviation in Mb, Ovine version 2.0 Genome Assembly map

6 provided by the International Sheep Genomic Consortium, [http://www.livestockgenomics.csiro.au/cgi-](http://www.livestockgenomics.csiro.au/cgi-bin/gbrowse/oarv2.0/)
7 [bin/gbrowse/oarv2.0/](http://www.livestockgenomics.csiro.au/cgi-bin/gbrowse/oarv2.0/)).

8 ^bChromosome location.

9 n.s. not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.

10

11 **Table 2**

12 Summary of genetic variability in the before-2005 and after-2005 cohorts of rams at PRNP locus.

13

	Before-2005	After-2005
Allele frequencies		
ARR	0.289	0.577
ARQ	0.570	0.354
AHQ	0.028	0.038
ARH	0.014	0.000
VRQ	0.099	0.031
Genotype frequencies		
ARR/ ARR	0.028	0.354
ARR/ AHQ	0.014	0.046
AHQ/ AHQ	0	0
ARR/ ARQ	0.437	0.385
ARR/ ARH	0.014	0
ARQ/ AHQ	0.042	0.031
AHQ/ ARH	0	0
ARR/ VRQ	0.056	0.015
ARQ/ ARQ	0.282	0.123
ARQ/ ARH	0	0
AHQ/ VRQ	0	0
ARH/ ARH	0	0
ARQ/ VRQ	0.099	0.046
ARH/ VRQ	0.014	0
VRQ/ VRQ	0.014	0
Summary of genetic variability		
GD	0.581	0.540
F _{IS}	-0.158 *	+0.038 n.s.
A	5.0	4.0

14

15 n.s. not significant; * P < 0.05.

16

17 **Table 3**

18 F_{ST} values between the before-2005 and after-2005 cohorts of rams for PRNP and on the whole for
19 OAR13 microsatellite markers and neutral markers.

PRNP	0.101 ***
OAR13 markers within PRNP	0.021 (0.011) **
OAR13 markers outside PRNP	0.011 (0.003) ***
Neutral markers	0.005 (0.002) n.s.

20

21 Standard error in parenthesis.

22 n.s. not significant, ** $P < 0.01$; *** $P < 0.001$.

23

24

25 **Table 4**

26 GD, F_{IS} , PIC, A, and A_g values in the before-2005 and after-2005 cohorts of rams at three clusters of
 27 microsatellite markers on different locations.

	GD	F_{IS} ^a	PIC	A	A_g ^b
OAR13 markers within PRNP					
Before-2005	0.410	-0.018 n.s.	0.293	3.0	3.0
After-2005	0.302	-0.087 n.s.	0.215	3.0	3.0
OAR13 markers outside PRNP					
Before-2005	0.761	0	0.589	8.3	8.1
After-2005	0.755	+0.075 *	0.589	8.2	8.1
Neutral markers					
Before-2005	0.739	+0.065 *	0.605	8.8	8.6
After-2005	0.730	+0.104 *	0.592	9.3	9.2

28

29 ^a Based on 10000, 18000, and 24000 randomizations for the three different locations, respectively. n.s.
 30 not significant; * significant at 0.05 adjusted nominal level.

31 ^b Adjusted for $g = 128, 118,$ and 124 for the three different locations, respectively.

32

33