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Original article

A high-density linkage map of the *RN* region in pigs

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Abstract – The porcine *RN* locus affects muscle glycogen content and meat quality. We previously mapped the *RN* locus to chromosome 15. This study describes the identification of polymorphisms for four class I and four class II markers located in the *RN* region. Resource families were genotyped with F-SSCP markers (fluorescent single strand conformation polymorphism) and microsatellite markers. Subsequent multipoint linkage analysis revealed the order *FN1–IGFBP5–S1000–S1001–IL8RB–VIL1–RN–Sw936–Sw906* The gene order is identical to the previously reported porcine RH map of the same region. The described map will facilitate positional cloning of the *RN* gene.

pig / meat quality / *RN* gene / genetic mapping

Résumé – Cartographie génétique de la région de *RN* chez le porc. Le gène *RN* influence le taux de glycogène musculaire et la qualité de la viande chez le porc. Il a été cartographié sur le chromosome 15. Cette étude présente la cartographie de quatre marqueurs SSCP (Polymorphisme de Conformation Simple Brin) et de quatre microsatellites situés à proximité de *RN*. L'analyse multipoint a permis de montrer que chez le porc l'ordre de ces marqueurs, *FN1–IGFBP5–S1000–S1001–IL8RB–VIL1–RN–Sw936–Sw906*, est identique à celui chez l'homme et la souris. Le positionnement de *RN* relativement aux marqueurs proches facilitera l'identification du gène responsable par clonage positionnel.

porc / qualité de la viande / gène *RN* / liaison génétique

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1. INTRODUCTION

In addition to the ryanodin receptor gene (*RYR1*), a second gene influencing meat quality in pigs, the *RN* gene, was identified by the observation that pigs of the Hampshire breed are characterised by a low ultimate pH-value, an increased muscle glycogen content and a reduced technological yield in cured cooked ham processing. Since this phenomenon occurs only in the Hampshire breed, it has been called the "Hampshire effect" [13]. Le Roy *et al.* [6] showed by complex segregation analysis that the phenotypic variability is controlled by a major locus with two alleles, the dominant RN^- allele, with an unfavourable effect on the technological yield, and the recessive rn^+ allele.

The measurement of the glycolytic potential in muscle tissue [14] is an alternative to determining *RN* genotypes by measuring the "Rendement Napole", the experimental technological yield in cured ham processing. However, this method has some inaccuracies; for example environmental effects can influence the results, and it can only be applied after slaughter or by taking muscle biopsies from live animals, which is against animal welfare regulations in some countries. Therefore, it is important to develop a DNA test to determine *RN* genotypes.

A first step to develop such a test is to genetically map the gene. Three research groups established resource families for the *RN* gene, screened them with markers and mapped *RN* between the markers *Sw120* and *Sw936* on pig chromosome 15 [7,9–11,16]. These results were the basis for subsequent mapping efforts and led to the definition of candidate genes. Genes involved in glycogen synthesis and glycogenolysis like *UGP2* and *PP1R3* [8,12] were chosen, but mapping results excluded them from the *RN* region.

FISH experiments using YACs harbouring microsatellites out of the *RN* region assigned *RN* to 15q2.5 [12] corresponding to the 2q31-q34 region in humans according to Zoo-FISH results [3,17]. Based on this knowledge, comparative mapping information has been used to apply a "trans-species shuttling" strategy as proposed by Georges and Andersson [2]. Sequence information from one species is used to develop markers in the orthologous region of another species. Robic *et al.* [18] isolated class I markers with this strategy and presented a radiation hybrid map of the *RN* region. A conserved gene order in relation to the human and mouse genome was demonstrated, but the orthologous murine and human regions showed no obvious candidate genes for *RN*. The position of the *RN* gene within the described cluster of loci is not yet exactly known.

The objective of this study was to detect polymorphisms for class I and class II markers in the *RN* region and map the *RN* gene more precisely in order to apply a positional cloning strategy.

2. MATERIALS AND METHODS

2.1. Resource families

Resource families were established by mating 14 crossbred Hampshire × Piétrain boars with 61 crossbred Large White × Landrace sows to produce

509 progenies. The establishment and the structure of the families as well as the procedure for the assignment of the *RN* genotypes based on the measured glycogen content in meat from the progenies was previously described in detail by Looft *et al.* [7] and Reinsch *et al.* [16].

2.2. Polymorphism detection and marker genotyping

Polymorphisms for the class I markers were detected by F-SSCP-analysis and comparative sequencing. Primer sequences of the markers, *FN1* (Fibronectin 1), *IGFBP5* (Insulin-Like Growth Factor Binding Protein 5), *TNP1* (Transition Protein 1), *VIL1* (Villin 1), *Sw936* and *Sw906* were previously published (Tab. I), whereas primers for *IL8RB* (Interleukin 8 Receptor beta) have not yet been published. The microsatellites *S1000* and *S1001* were isolated using the BAC clones 115B9 for *S1000* and 479L3 for *S1001.* The BAC library used was previously described by Rogel-Gaillard *et al.* [19].

For F-SSCP analysis, PCR was performed in a total volume of 15 μL of the following mixture: 30 ng of porcine genomic DNA, 0.2 U *Taq* Polymerase (Pharmacia), buffer as supplied, 1.5 or 2 mM $MgCl_2$, 0.5 μM of each fluorescent primer, 70 M of each dNTP. Thermal cycling was carried out by initial denaturation at 94 °C for 3 min, followed by 35–40 cycles each at 94 °C for 30 s, annealing temperature at 55–60 °C for 50 s, and 30 s at 72 °C, followed by a final extension step for 10 min at 72 °C. After thermocycling, PCR products were mixed 1:10 with formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated at 85 °C for 5 min, and cooled on ice. Resulting single strands were separated by electrophoresis at 18 or 23 mA, 600 or 800 V at 15 or 20 °C with or without 5% glycerol on a 0.5-mm non-denaturating Mutation Detection Enhancement gel (MDE, FMC) using an automated sequencer (A.L.F., Pharmacia). Microsatellite typing was performed with our standard procedure [7].

All genotypic data were transformed to a unique text format, transferred to a database [15] and checked for typing errors and Mendelian inheritance, by comparing alleles of the progenies with the parental alleles.

2.3. Linkage analysis

Two-point and multipoint maximum likelihood linkage analyses were performed with CRI-MAP (v2.4 SunOs) [4] using options TWOPOINT, BUILD and FLIPS. The CHROMPIC procedure was used to identify double recombinants. Double recombinational events around *RN* were analysed by retyping flanking markers. When marker genotypes were confirmed, the most probable explanation for an unlikely double recombination in the small interval was a false *RN*-genotype, derived from glycogen measurements. Therefore, three animals were excluded from further analysis. The two mutations analysed within the *Villin* gene (Fig. 1) were treated as haplotypes. The number of informative meioses was calculated for each locus using CRI-MAP. Observed heterozygosity was calculated as the proportion of parents being heterozygous at the locus.

Table I. Characteristics of the analysed markers in the *RN* region.

Locus	Primers	Marker type[a]	No. of alleles	No. of inf. meioses	Heterozygosity (%)	PCR conditions bp[b]/ Ta[c]/ cycles
FN1 [18]	5′ CAGATGTTACAATGTAAAAGC	SNP	3	439	66	450/55/35
	5′ GCTGCCTCTGCTCAAATTAAGCC					
IGFBP5 [21]	5′ TGCTGGTGCGTGGACAAGTA	SNP	2	327	45	205/60/40
	5′ CTTAAATGAGATGAAATGAGTGG					
S1000	5′ GGAATTTCAAGTCAGCCAAC	MS	4	597	75	114-138/55/30
	5′ CTTCAAAAGACCGTGCTACT					
S1001	5′ CTCCAGCTCACAGGATGACA	MS	4	102	75	150-164/55/30
	5′ GTTTCTGCAGCTTTAGCATCTATTCC					
IL8RB	5′ AGGGTGGATGGTAGGCTTCA	SNP	3	544	43	208/55/35
	5′ GTCTCGCTCCTGAAGGAAGT					
VIL1 [1]:		SNP				269/55/35
position 94	5′ TTGGCGCAACTGTTATTTCT		2	359	45	
position 124	5′ AGGCAAAGGAAGAGCACAG		2	277	27	
Sw936 [20]	5′ TCTGGAGCTAGCATAAGTGCC	MS	7	675	77	90-112/60/25
	5′ GTGCAAGTACACATGCAGGG					
Sw906 [20]	5′ GAGGACAATGTGAGAAAAAGAATG	MS	6	575	69	158-186/55/30
	5′ TTTTTTCCTGTGATTAGAACTCTTAGG					
TNP1 [5]	5′ CAATTACCGCTCCCACTT	–	–	–	–	976/55/33
	5′ ACCTCTACATGGTGCTGATG					

[a] MS = microsatellite, SNP = single nucleotide polymorphism; [b] bp = base pairs; [c] Ta = annealing temperature.

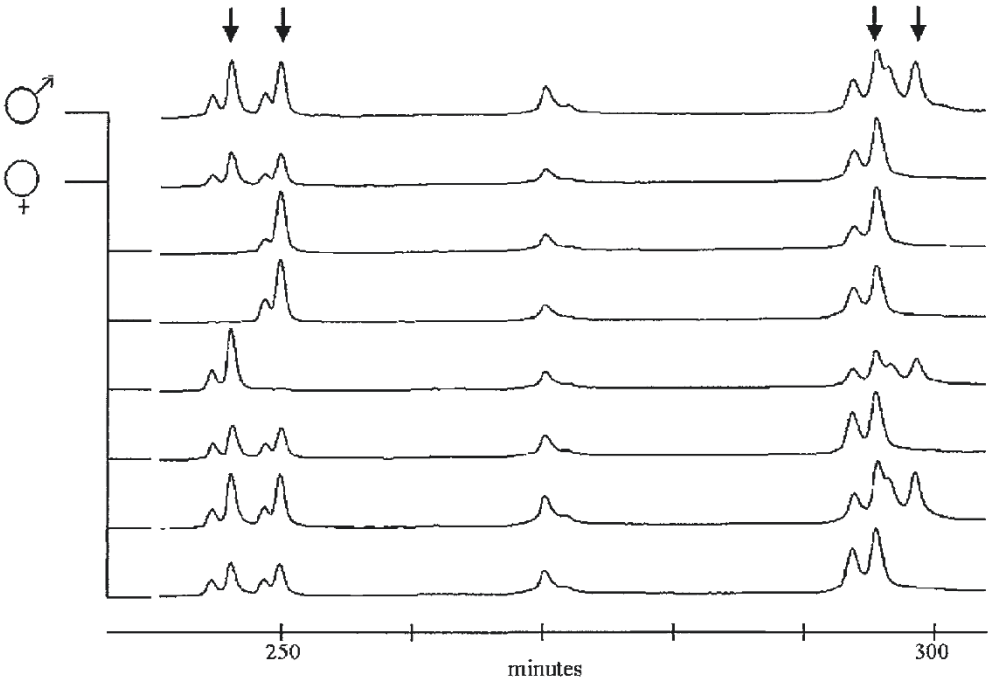

Figure 1. F-SSCP analysis at the *VIL1* locus in a resource family. Segregating alleles, indicated by arrows, can be seen at 246 min, 250 min, 295 min and 299 min. Parental animals are shown in the two upper lanes and the progenies are shown in the remaining lanes. A fluorescent size standard (131 bp) occurs at 270 min.

3. RESULTS AND DISCUSSION

3.1. Polymorphisms

Previous studies showed that the *RN* gene is located between the markers *Sw120* and *Sw936* on the porcine chromosome 15 [9,11,16]. In order to determine the position of the *RN* gene within a previously described cluster of loci in the *RN* region [18], we tried to detect polymorphisms for class I and class II markers (Tab. I). Polymorphisms were detected by F-SSCP analysis for the genes *FN1*, *IGFBP5*, *VIL1*, and *IL8RB*, but not for *TNP1*. All microsatellite markers were polymorphic.

No clear polymorphism in *VIL1* was detected using our routine F-SSCP analysis. Therefore comparative sequencing of animals with different *RN* genotypes was performed within the 3′UTR region, since this method is more likely to find mutations in non-coding regions. Three polymorphisms within a stretch of 76 bp were revealed (GenBank: AF207847, at positions 94, 124, 170). In order to avoid comparative sequencing of all the animals, we designed a test based on sequence information that simultaneously genotyped two of the mutations by amplifying a 260 bp fragment with two fluorescent labelled primers and digested the fragment with *Fnu*HI at position 105. The subsequent

F-SSCP-analysis of the two resulting fragments (Fig. 1) showed their segregation in a resource family. The mutation at position 94 was represented by a polymorphic fragment occurring after 246 and 250 min of gel runtime and the mutation at position 124 was represented by the polymorphic fragment that was detected after 295 and 299 min of gel runtime, whereas the sequenced mutation at position 170 was obviously not detected by this F-SSCP-analysis. We conclude that the method described represents a modification of traditional SSCP-analysis, which leads to an increased sensitivity.

3.2. Linkage map

Polymorphic markers were used to genotype the resource families. The degree of heterozygosity varied from 27 to 77%, and the number of informative meioses varied from 102 for *S1001* to 675 for *Sw936* (Tab. I). Three genetic maps (male, female, average) with 8 informative markers and the *RN* locus were calculated (Fig. 2) and revealed the following order with the support (response of lod score after exchange of adjacent loci) in brackets: *FN1*–(0.88)–*IGFBP5*–(1.42)–*S1000*–(10.86)–*S1001*–(6.71)–*IL8RB*–(−0.06)–*VIL1*–(0)–*RN*–(14.97)–*Sw936*–(1.71)–*Sw906*.

Since only boars showed segregation at the *RN* locus, only male meiotic events were informative for *RN* and thus only these mapping results are relevant for the position of the *RN* gene. Because no recombination was observed between *RN* and *VIL1*, they were mapped to the same position with a distance of 0.5 cM to *IL8RB* and 2.8 cM to *Sw936*. In the intervals *FN1*–*IGFBP5*, *IGFBP5*–*S1000*, *IL8RB*–*VIL1*, *Sw936*–*Sw906* the support was lower than three, whereas the support for the other orders was 6.71 or higher.

The comparison of the three linkage maps with the RH-map of Robic *et al.* [18] showed an identical gene order. Therefore, the postulated conserved order was confirmed when comparing human and mouse genomes.

The relation between an RH-map and a linkage map gives an indication about the recombination rate for a specific genome region. For the *RN* region, the distance between the markers *FN1* and *Sw906* is 111.5 cR for the RH-map and 10.6 cM for the average linkage map. Thus one cM in this region corresponds to 10.5 cR, whereas Yerle *et al.* [22] calculated a ratio of 17 cR per cM for porcine chromosome 8. The relatively high recombination rate in the *RN* region is in good agreement with the results of Robic *et al.* [18].

The analysis of haplotypes associated with the RN^- allele showed that a strong linkage disequilibrium exists between *IL8RB*, *VIL1* and *RN* (Tab. II). As pointed out by Reinsch *et al.* [16], our experimental design cannot distinguish whether a marker allele associated with the RN^- allele is specific for the mutation or for the Hampshire breed, because only the crossbred boars (Hampshire × Piétrain), showed heterozygosity at the *RN* locus. In order to solve this problem, we also genotyped two homozygous (rn^+/rn^+) boars (Hampshire × Piétrain) that were therefore not informative for linkage analysis, and their progenies. It was found that the rn^+ alleles of different origins were associated with the same expected marker alleles. The observed haplotypes suggest that *IL8RB* and *VIL1* are informative in the Hampshire breed and could be used for diagnostic purposes. We are aware, however, that this result

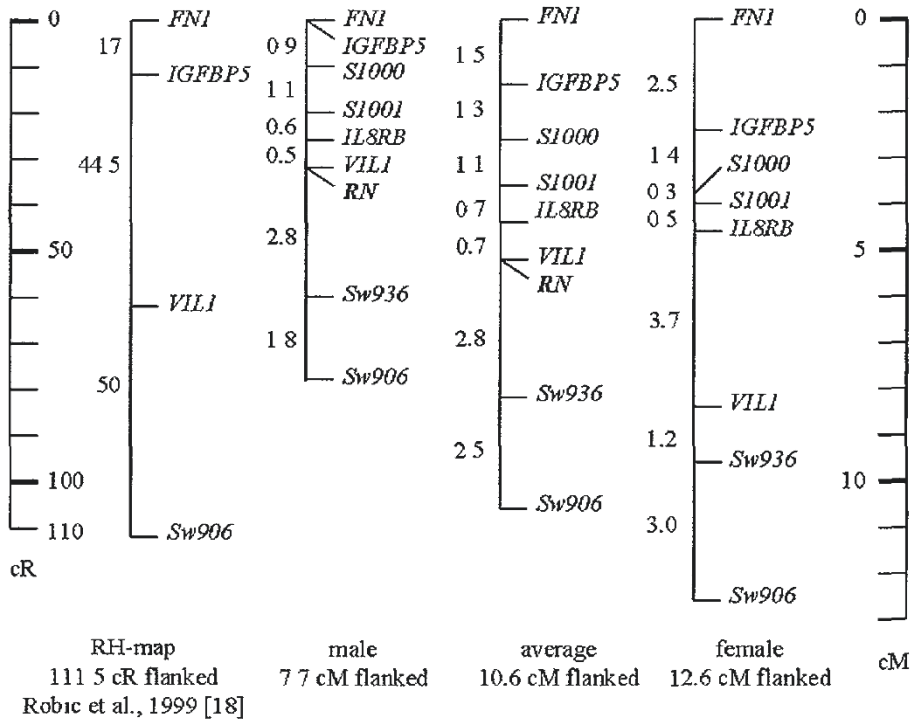

Figure 2. Multipoint linkage maps (Kosambi centi-Morgans) of the *RN* region and comparison with an RH-map of the same region [18].

Table II. Detected haplotypes in the *RN* region.

Loci	Haplotypes						
S1000	132	132	134	136	136	132	134
S1001	164	162	162	164	164	150	162
IL8RB	1	1	1	1	1	2	2
VIL1 (pos. 94)	2	2	1	2	1	2	2
VIL1 (pos.124)	1	1	2	1	2	1	1
RN	RN^-	RN^-	RN^-	RN^-	RN^-	rn^+	rn^+

has to be confirmed within an unrelated population before these markers should be used for routine *RN* diagnostic.

3.3. Toward positional cloning of the RN gene

In order to analyse whether *RN* is located distally or proximally to *VIL1*, more informative meioses for these two loci are needed. We will therefore combine the three resource families described previously [9,11,16] in a joint analysis to improve the resolution of genetic mapping, which is essential for

positional cloning of the *RN* gene. Furthermore, markers showing no recombination with *RN* can be used to identify BAC-clones harbouring the *RN* gene. The construction of a BAC-contig covering the *RN* region is under way simultaneously to the establishment of the linkage map previously described. We plan to use additional BAC-clones [19] to isolate new polymorphic markers, sequence tagged sites (STS) or microsatellites, and to map them in relation to the *RN* locus. In a next step, expressed sequence tags (ESTs) from porcine muscle tissue could be isolated, for example by cDNA selection. In addition, sequencing of BAC-clones may reveal new candidate genes for *RN*.

In summary, the results reported here together with the available resources like the BAC-library and the porcine-hamster RH panel should allow us to develop a highly accurate DNA-test for the *RN* locus or may be even used to isolate the *RN* gene by positional cloning in the near future.

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