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CYP21 AS A CANDIDATE GENE FOR ANDROSTENONE ON BOAR TAINT

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SUMMARY

The Cytochrome P450 steroid, 21 Hydroxylase (CYP21), is a positional candidate gene for boar taint caused by an excessive level of androstenone mainly in fat and muscle. CYP21 contains 9 introns and 10 exons and is located in the swine lymphocyte antigen (SLA) region on chromosome 7. Sequencing of four sires from an Australian mapping pedigree has revealed 36 SNPs. 22 of these are in non-coding regions, and those in coding regions do not cause changes in the amino acid sequence of the protein. However, a SNP at position 2329 (intron 7) could influence the use of a theoretical alternative splice site. Quantitative analysis has shown that segregation of this SNP in the sire families has no impact on androstenone levels. RT-PCR and sequencing have provided no evidence of the use of this alternative splice site in testis RNA. CYP21 can be excluded as the locus responsible for the SSC7 androstenone QTL.

INTRODUCTION

Boar taint is a potentially serious problem in the pig meat industry in countries like Australia where intact male pigs are generally used for meat production. Boar taint is a perspiration-like or urine-like undesirable off-odour and off-flavour emanating from cooked meat of some male pigs. Androstenone (5α-androst-16-en-3-one) and skatole (3-methylinodole) have been identified as two major compounds for the boar taint. Androstenone is a steroid pheromone that is synthesised by Leydig cells in the testis of the sexually mature boar. During sexual development, androstenone is released into the circulation and is stored in adipose tissues (Brooks and Pearson, 1986; Claus *et al*., 1994). There is strong evidence of genetic influence in the level of androstenone and the estimated heritability is about 0.50, ranging from 0.25 to 0.87 (Sellier, 1998). A significant effect on androstenone levels in fat has been found between different haplotypes of swine lymphocyte antigen (SLA) (Bidanel *et al*., 1997). Genome scans have revealed similar QTL for level of androstenone on SSC7 in the vicinity of SLA (Lee *et al*., 2005; Quintanilla *et al*., 2003). In the US43 project with an Australian commercial pig population, one QTL was found on SSC7 with chromosome wide significance (unpublished data). CYP21 (steroid 21 hydroxylase P450c21) is in the metabolic pathway for androstenone synthesis and maps to the SLA region between LRAI and S0102 (Geffrotin *et al*., 1990). The objective of this study was to discover variants in CYP21 gene and to investigate their effects on the variation of androstenone content in fat in Australian pigs.

MATERIALS AND METHODS

An Australian resource pedigree consisting of 430 progeny of eight sires was bred at QAF Meat

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Industries, Corowa, NSW, Australia, between 1999 and 2001. Androstenone was measured using a modified version of Hansen-Møller's method (Dunshea *et al*., 2001).

Four primer pairs were designed from porcine DNA sequence (GenBank Accession No M83939) to amplify 3.36 kb of the CYP21 gene (Table 1). PCR amplifications were performed in a 25-µl reaction volume containing 100 ng of DNA, 80 µM dNTP, 1 µM each primer, 1.5mM $MgCl_2$, 1x PCR buffer, 0.2 U Tli DNA polymerase (Promega, USA). Touchdown PCR was carried out by denaturing at 95°C for 10 min followed by 48 cycles of amplification with each cycle consisting of 40 s at 94°C for denaturing, 1 min for annealing at temperatures from 65-55°C and 1 min at 72°C for extension. PCR products from 4 sires were purified from agarose gel and cloned into TOPO vector with TOPO™ TA cloning Kit (Invitrogen, USA). Sequencing was carried out on a Li-Cor DNA Sequencer 4200 (Li-Cor Inc., Lincoln, Nebraska, USA) with SequiTherm EXCEL™ II DNA Sequencing kit-LC (EPICENTRE, Madison, Wisconsin, USA). The sequence data were analyzed with GAP4 program (The GAP Group, 2005). Potential transcripts were identified using Genscan (Burge and Karlin, 1997).

Table 1. Primers for amplification of CYP21 gene

Primers	Position*	Sequence 5'→ 3'
Primer1_forward	458	TGGAGAAGGTCAGGGTTGAG
Primer1_reverse	1509	GCTGCAGGTGAGGACAGAG3
Primer2_forward	1344	CCAGGAGTTCTGTGAGGTGAG
Primer2_reverse	2285	AAAGGGTGTTCGCTGTGG
Primer3_forward	2164	GAAGAGGGGCAGGGACAG
Primer3_reverse	3104	CACCAGCACCACGAAGAG
Primer4_forward	2834	GTCATCCCCAACCTCCAAG
Primer4_reverse	3709	TGAAGGAGAGAGGGAGGGAG

***** Position is based the GenBank Accession No M83939

RESULTS AND DISCUSSION

Thirty-six SNPs (single nucleotide polymorphisms) were discovered after assembling and aligning 92 sequences from 4 sires (Table 2). There are 22 SNPs located in non-coding regions and the 14 SNPs located in coding regions were all synonymous. The SNP at 2329 (A/G) within intron 7 produced a potential alternative splice site. Genscan predicts that G at position 2329 favours a long transcript, using a potential splice donor at the position 2398 and generates a larger cDNA with an in-frame insertion of 105 bp. The A variant favours the known short transcript using the known splice donor site at position 2293.

The SNP at the position 2329 could be detected with restriction enzyme TauI. A PCR–RFLP assay was designed with forward primer 5' TTTCCTGGGCTGTGGTGTA 3' and reverse primer 5' GCCACTCACAGGAGACTTTG 3' to amplify a 207bp fragment. The A allele has a single fragment with 207bp and G allele has two fragments with 135bp and 72 bp.

8 sires, 63 dams and 358 progeny were genotyped for the A/G SNP at position 2329 by TauI digestion. The analysis was carried out using SAS GLM PROC with measurements of androstenone transformed by square root. Initially, the model included all fixed effects and covariates, however after each run; the most insignificant factor was excluded. The final models included sire, dam nested with sire as random effects, weight fitted as co-variates and the CYP21_2329 SNP genotype fitted as

a fixed effect. The differences between genotype groups were tested using CONTRAST of GLM. Variance analysis of SNP at 2329 genotypes showed that segregation of the SNP in sire families has no impact on androstenone levels. Sire effect and live weight at the end of the test period were significant (Table 3).

Table 2. SNPs detected in *CYP21*

Region	Position (SNP)
Promoter (0-718)	672(G/A), 700(G/A), 707(G/C), 708(C/T)
Exon 1(719-920)	731(T/G)
Intron 1(921-1001)	933(T/C)
Exon 3(1206-1360)	1255(C/T), 1280(C/T)
Exon 5(1640-1741)	1693(C/T), 1735(T/C)
Intron 5(1742-1843)	1759(C/T), 1760(A/G)
Intron 6(1931-2092)	2052(C/T)
Exon 7(2093-2293)	2140(C/T), 2197(C/T), 2245(A/G)
Intron 7(2294-2546)	2329(G/A), 2466(G/A), 2472(C/G), 2501(C/T), 2512(C/G)
Exon 8(2547-2725)	2556(C/T), 2579(C/T)
Intron 8(2726-2800)	2733(C/T), 2734(G/A), 2758(C/T)
Exon 9(2801-2904)	2822(T/C)
Intron 9(2905-2994)	2964(A/G), 2991(A/C)
Exon 10(2995-3257)	3026(T/C), 3029(G/A), 3104(G/A)
3' UTR	3462(G/A), 3514(C/T), 3612(T/A)

8 sires, 63 dams and 358 progeny were genotyped for the A/G SNP at position 2329 by TauI digestion. The analysis was carried out using SAS GLM PROC with measurements of androstenone transformed by square root. Initially, the model included all fixed effects and covariates, however after each run; the most insignificant factor was excluded. The final models included sire, dam nested with sire as random effects, weight fitted as co-variates and the CYP21_2329 SNP genotype fitted as a fixed effect. The differences between genotype groups were tested using CONTRAST of GLM. Variance analysis of SNP at 2329 genotypes showed that segregation of the SNP in sire families has no impact on androstenone levels. Sire effect and live weight at the end of the test period were significant (Table 3).

A pair of primers with forward 5' GGAGGGACATGCTGGACTAC 3' and reverse 5' AGCAATGGTGGCGTTGAG 3' was designed to amplify the coding region between exon 7 and exon 8 to detect possible alternative splice site in intron 7 with RT-PCR. RNA was extracted from testis samples using the RNeasy Mini kit (QIAGEN, USA) and the RNA concentration was measured using a Beckman DU-600 spectrophotometer and subjected to DNase treatment prior to RT-PCR. RT-PCR was preformed using a one-step RT-PCR kit (QIAGEN, USA). RT-PCR provided no evidence of the use of this theoretical alternative splice site in testis RNA in G/A heterozygous and AA homozygous males. The animals tested for genotypes (AA and AG) showed only the 293 bp fragment expected for the splice site located at the position 2293.

Two genome scans have revealed a QTL for androstenone in a similar region on SCC7 (Lee *et al*., 2005; Quintanilla *et al*., 2003) in resources based crosses between Meishan and Large White. We have also found a QTL in a similar genome region on SSC7 segregating in an Australian commercial

population.

Table 3 Variance analysis of significant factors affecting androstenone level (μg/g)

Source	D.F.	M.S.	F	P
Genotype	2	0.05	0.28	0.75
Sire	7	2.61	15.10	<.0001
Dam (sire)	102	0.199	1.15	0.20
LW[1]	1	3.02	17.48	<.0001
Residual	213	0.173		

[1] Live weight at the end of the test period.

The CYP21 locus was a positional candidate gene for androstenone since it is in the metabolic pathway for androstenone synthesis and mapped in the SLA complex, close to the most likely location of the QTL between markers LRA1 and S0102. Quintanilla *et al*. (2003) found six single nucleotide polymorphisms in the coding region between the two founder animals, one Meishan and one Large White, but none of them caused amino acid substitutions. Thirty-six SNPs were discovered after the completion of sequencing analysis of four sires from our resources and 14 SNPs located in coding regions were all synonymous. Although the SNP within intron 7 produces a potential alternative splice site, RT-PCR and sequencing have provided no evidence of the use of this alternative splice site in testis RNA. Variance analysis of this SNP in our resource showed that segregation of the SNP in sire families has no impact on androstenone levels. CYP21 can be excluded as the locus responsible for the SSC7 androstenone QTL.

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REFERENCES

Bidanel, J.P., Milan, D., Chevalet, C., Wolowzyn, N., Caritez, J.C., Gruand, J., Le Roy, P., Bonneau, M., Renard, C., Vaiman, M., Gellin, J.&Ollivier, L. (1997). *EAAP Publication* **92**:115.

Brooks, R.I. and Pearson, A.M. (1986). *J. Anim. Sci.* **62**:632.

Burge, C. and Karlin, S. (1997) *J. Mol. Bio.* **268**:78.

Claus, R., Weiler, U.,and Herzog, A. (1994). *Meat Sci.* **38**:289.

Dunshea, F.R., Colantoni, C., Howard, K., McCauley, I., Jackson, P., Long, K.A., Lopaticki, S., Nugent, E.A., Simons, J.A., Walker, J. and Hennessy, D.P. (2001) J. Anim. Sci. **79**:2524.

Geffrotin, C., Chardon, P., de Andres-Cara, D.F., Feil, R., Renard, C. and Vaiman, M. (1990) *Anim. Genet.* **21**:1.

Lee, G.J., Archibald, A.L., Law, A.S., Lloyd, S., Wood, J. and Haley, C.S. (2005) *Anim. Genet.* **36**:14.

Quintanilla, R., Demeure, O., Bidanal, J.P., Milan, D., Iannuccelli, N., Amigues, Y., Gruand, J., Renard, C., Chevalet, C. and Bonneau, M. (2003). *J. Anim. Sci.* **81**:385

SAS Institute Inc.(1999), SAS/STAT User's Guide, Version 8, Cary,NC

Sellier, P. (1998). In: "The Genetics of the Pig", p463, editors M.F. Rothschild and A. Ruvinsky, CAB International, Wallingford, Oxon, UK..