

# Fine mapping of a swine quantitative trait locus for number of vertebrae and analysis of an orphan nuclear receptor, germ cell nuclear factor (NR6A1/GCNF)

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The number of vertebrae in pigs varies and is associated with meat productivity. Wild boars, which are ancestors of domestic pigs, have 19 vertebrae. In comparison, European commercial breeds have 21–23 vertebrae, probably owing to selective breeding for enlargement of body size. We previously identified two quantitative trait loci (QTL) for the number of vertebrae on *Sus scrofa* chromosomes (SSC) 1 and 7. These QTL explained an increase of more than two vertebrae. Here, we performed a map-based study to define the QTL region on SSC1. By using three F<sub>2</sub> experimental families, we performed interval mapping and recombination analyses and defined the QTL within a 1.9-cM interval. Then we analyzed the linkage disequilibrium of microsatellite markers in this interval and found that 10 adjacent markers in a 300-kb region were almost fixed in European commercial breeds. Genetic variation of the markers was observed in Asian local breeds or wild boars. This region encoded an orphan nuclear receptor, germ cell nuclear factor (NR6A1, formerly known as GCNF), which contained an amino acid substitution (Pro192Leu) coincident with the QTL. This substitution altered the binding activity of NR6A1 to its corepressors, nuclear receptor-associated protein 80 (RAP80) and nuclear receptor corepressor 1 (NCOR1). In addition, somites of mouse embryos demonstrated expression of NR6A1 protein. Together, these results suggest that NR6A1 is a strong candidate for one of the QTL that influence number of vertebrae in pigs.

[Supplemental material is available online at [www.genome.org](http://www.genome.org). The sequence data from this study have been submitted to EMBL/GenBank/DBJ under accession nos. AB248749–AB248751, AP009124; accession nos. for STS are given in Supplemental Table I].

Wild boars, which are the ancestors of modern domestic pigs, uniformly have 19 vertebrae. In comparison, European commercial breeds have 21–23 vertebrae (King and Roberts 1960). These breeds have long been selectively bred for enlargement of body size to increase meat production and improve reproductive performance. This process presumably increased the number of vertebrae. In mammals, the vertebral formula shows developmental constraint (Narita and Kuratani 2005). The number of cervical vertebrae is fixed at seven, and the total number of thoracic and lumbar vertebrae tends to be 19, although the respective counts vary among species. For example, in the Monotremata, Marsupialia, Lagomorpha, Rodentia, and Artiodactyla, the total number of thoracic and lumbar vertebrae is conserved at 19, which is thought to be the primitive form. In comparison, this number is increased in the Perissodactyla (e.g., horse, 24 vertebrae) and Carnivora (e.g., dog, 20 vertebrae) and that of the Primata is decreased to 17. However, these changes are lineage specific, and variation is restricted in each species, as is seen in the Primata (Pilbeam 2004). In light of these findings, it is interesting that the

number of vertebrae in pigs varies from 19 to 23 within a single species.

In previous papers, we reported two quantitative trait loci (QTL) affecting the number of vertebrae on *Sus scrofa* chromosomes (SSC) 1 and 7; these QTL were identified using nine F<sub>2</sub> families produced by crossing between breeds of European, Asian, and miniature pigs (Wada et al. 2000; Mikawa et al. 2005). These two QTL acted independently without an epistatic effect, and each had mainly an additive effect. For the QTL on SSC1, all the alleles of European commercial pigs used in the experimental families increased the number of vertebrae by 0.44–0.68 per allele. For the QTL on SSC7, some European alleles similarly increased the number of vertebrae (0.38–0.68). The combined effect of the two QTL accounted for an increase of more than two vertebrae. In F<sub>2</sub> populations in which alternative alleles for both QTL were fixed in founder breed pigs, the proportions of phenotypic variance in the number of vertebrae explained by the QTL on SSC1 and SSC7 were similar, at ~30% (Mikawa et al. 2005). In the current report, we describe our map-based study of the QTL on SSC1 and present a 300-kb region that is almost fixed in a variety of European commercial breeds. We also suggest that an orphan nuclear receptor, germ cell nuclear factor (NR6A1, formerly known as GCNF) is a strong candidate for the gene underlying this QTL, in light of analyses of polymorphism, function, and expression.

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Article published online before print. Article and publication date are at <http://www.genome.org/cgi/doi/10.1101/gr.6085507>.

## Results

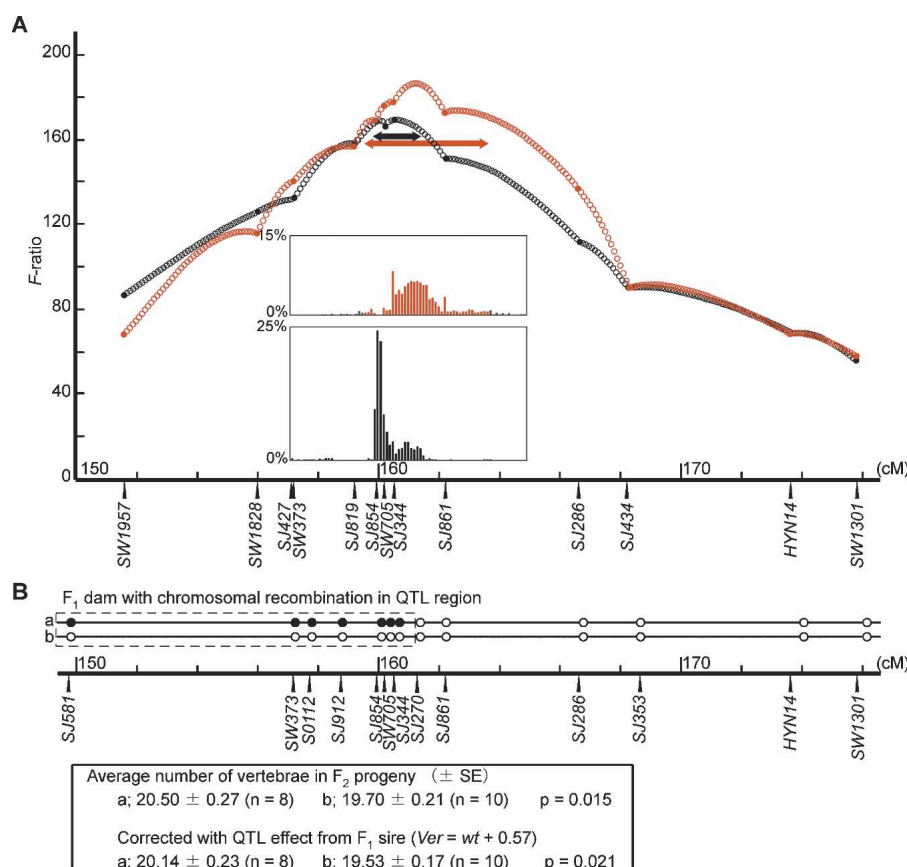
### Defining the QTL region by using $F_2$ families

In our previous studies, we mapped a QTL for the number of vertebrae on SSC1qter, between microsatellite markers *SW1957* (151.6 cM) and *SW1301* (175.8 cM) (Wada et al. 2000; Mikawa et al. 2005). To restrict the candidate region, three families in the previous study were reanalyzed by using microsatellite markers distributed densely throughout the QTL region (Mikawa et al. 2004). First, we performed an interval mapping using a subfamily of a Large White  $\times$  Japanese wild boar population, for which construction three Large White female pigs (W1, W2, and W3) were used as parents. In the previous study, we reported that in two of them (W2 and W3) the QTL on SSC7 had no effects on number of vertebrae. In the subfamily derived from W2 and W3, which consisted of 207  $F_2$  animals, the effect of increasing the number of vertebrae was attributable only to SSC1, and the proportion of phenotypic variance explained by the QTL on SSC1 was ~60%. As a result of interval mapping, the peak  $F$ -ratio (187.2) was detected between *SJ344* (160.5 cM) and *SJ861* (162.2 cM) (Fig. 1A). To evaluate the effect of sampling error on the estimated QTL position, we constructed a 95% confidence interval for the position showing the highest  $F$ -ratio, with 1000 repetitions of bootstrap sampling. We obtained the region from 159.5 to 163.6 cM as the 95% confidence interval for the QTL position (Fig. 1A).

Another interval mapping was performed using a Chinese Jinhua  $\times$  Duroc population, which was the largest population in our studies and consisted of 549  $F_2$  animals; in this population, alternative alleles were fixed in founder breed pigs in the QTLs on both SSC1 and SSC7. When the genotype of a marker near the QTL detected on SSC7 was included as a covariate in the model for the analysis of SSC1, the peak  $F$ -ratio (171.2) was obtained at *SJ344* (160.5 cM) on SSC1 (Fig. 1A). A bootstrap analysis (with 1000 repetitions) showed that the 95% confidence interval ranged from 159.8 to 161.4 cM (Fig. 1A). We think that the results of these two independent analyses provide sufficient evidence for us to deduce that the QTL was located in the region from 159.5 to 163.6 cM.

The third  $F_2$  family was produced by crossing Chinese Meishan females with a Göttingen miniature male. In this population, a QTL for vertebral number was detected on SSC1 but not on SSC7. QTL analysis showed that the Göttingen miniature sire was heterozygous on SSC1: one allele (*Ver*) had the effect of increasing vertebral number, whereas

the other had no effect and was thought to be wild type (wt). The Meishan dams were homozygous wild type (wt/wt) (Mikawa et al. 2005). Among the eight  $F_1$  dams, five were homozygous wild type (wt/wt) and two were heterozygous (*Ver*/wt), but the genotype of the remaining animal was uncertain because of recombination within the QTL region that originated from the Göttingen miniature chromosome. By using microsatellite markers, we mapped the recombination site between *SJ344* (160.5 cM) and *SJ270* (161.1 cM). The genomic region proximal to *SJ344* was from the *Ver*-containing chromosomal segment from the sire (Fig. 1B). The heterozygosity of the QTL of the  $F_1$  dam with the recombination was suggested by the segregation of the number of vertebrae in 18  $F_2$  animals produced from the  $F_1$  dam. This segregation was confirmed through correction for the QTL effects from the  $F_1$  sire (*Ver*/wt) when we assumed that the *Ver* allele increased the number of vertebrae by 0.57, the average of the QTL effects in the experimental families in our previous study. We therefore judged that the QTL was located proximal to *SJ270*,



**Figure 1.** Dissection of a QTL region on SSC1 for vertebral number in  $F_2$  families. (A) Plots of  $F$ -ratio for interval mapping analyses in the Large White  $\times$  Japanese wild boar population (red) and Jinhua  $\times$  Duroc cross population (black). For the latter, the QTL effect on SSC7 was removed by incorporating the genotype of a marker in the QTL region into the model as a covariate. Bootstrap analysis (with 1000 repetitions) was performed to obtain the 95% confidence interval of the estimated QTL position on SSC1, and the results are shown in the inset bar graphs. Double-ended arrows indicate the 95% confidence interval in each population. (B) QTL type of an  $F_1$  dam with a recombination in the QTL region. The dam was from a Meishan  $\times$  Göttingen miniature cross. Meishan pigs were wt/wt, and the Göttingen miniature was heterozygous (wt/*Ver*) at the QTL. Solid circles indicate alleles from the parental chromosome with the *Ver* allele. Clear circles indicate alleles from the parental chromosome with the wt allele. Average number of vertebrae was compared between two groups of  $F_2$  animals produced from the  $F_1$  dam, categorized according to transmitted chromosomes (indicated by a or b). Correction for the QTL effect from the  $F_1$  sire (*Ver*/wt) was also used. We assumed that the *Ver* allele increased the number of vertebrae by 0.57, an average effect in the experimental population in our previous study. (SE) Standard error.

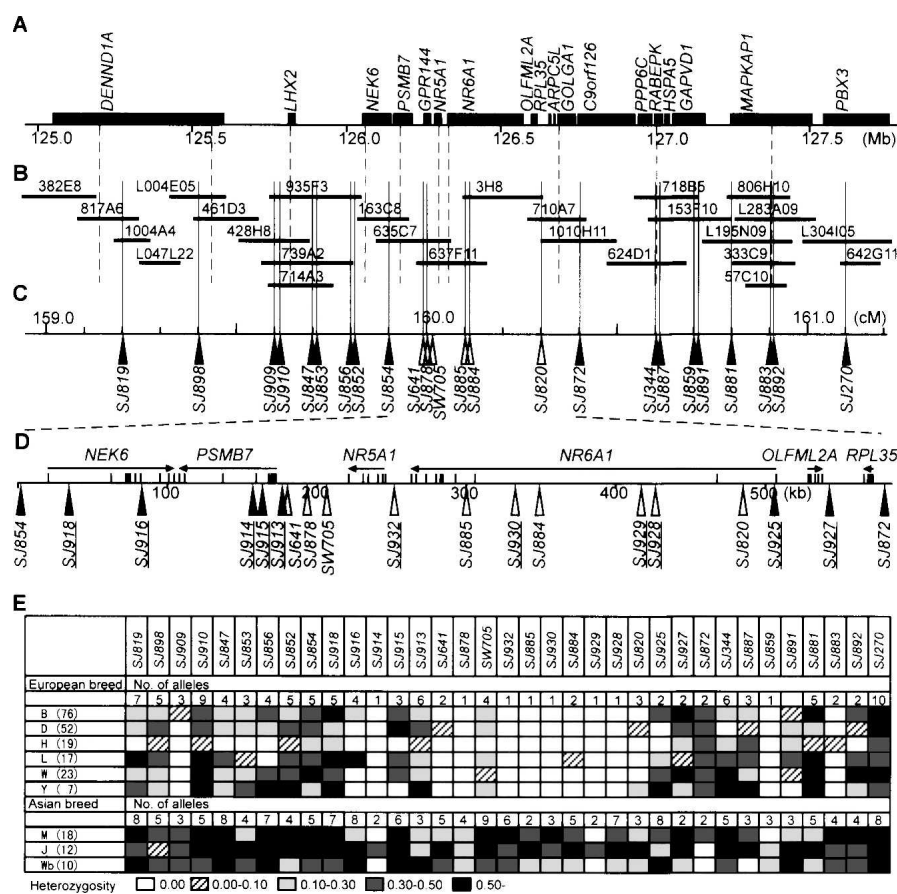
and the results suggested that the QTL was located between *SJ819* (159.2 cM) and *SJ270* (161.1 cM). To further define the QTL, we then constructed a BAC contig for this region and developed microsatellite markers (Supplemental Table 1; Fig. 2A–C).

### Genetic variation of microsatellite markers in the QTL region

We attempted to fine-map the QTL, in accordance with the assumption that the genetic variation around this QTL would be reduced in commercial breed pigs because vertebral number is associated with body size, which has been a focus of selective pig breeding. To analyze the genetic variation, we collected 194 independent samples from five commercial breeds (Landrace, Large White, Yorkshire, Duroc, and Berkshire) and 40 samples from Asian local breeds (Meishan, Jinhua, and Japanese wild boar) as references. Using these samples, we genotyped 24 microsatellite markers in the 1.9-cM region between *SJ819* and *SJ270*. The re-

sults revealed that *SJ78* and *SJ85* were nonpolymorphic in commercial breeds and that *SJ641*, *SJ884*, and *SJ820* showed dramatically reduced polymorphism: The frequencies of all major alleles were >0.99. At *SW705*, located between *SJ878* and *SJ885*, the frequency of the major allele was 0.93 and that of the second allele, which was two nucleotides smaller than the major allele, was 0.05 in commercial breeds (Fig. 2C; Table 1). In comparison, the genetic variation of these markers was maintained in the Asian breeds.

To define the region of reduced genetic variation, we isolated 11 novel microsatellite elements (underlined in Fig. 2D) by sequencing the 600-kb region between *SJ854* and *SJ872*. Reanalysis with these 11 markers, in addition to the 24 markers described earlier, revealed that lack of genetic variation in commercial breeds occurred at 10 adjacent markers located in a 300-kb region between *SJ641* and *SJ820* (Fig. 2D,E; Table 1).



**Figure 2.** Analyses of genomic structure and genetic variation of the QTL region. (A) Gene map of a part of the human chromosome 9qter. (B) BAC contig for the QTL region. Vertical lines indicate positions of STS (broken) and microsatellite markers (solid). BAC clones (Suzuki et al. 2000) from which markers were developed and those in the minimum tiling path are shown. (C) Linkage map of microsatellite markers on SSC1qter. Arrowheads indicate positions of microsatellite markers, and clear arrowheads indicate those lacking genetic variation in European commercial breed pigs. (D) Defining the QTL region by the genetic variation of microsatellite markers. Sequencing analysis was performed for the region from *SJ854* to *SJ872*. Exons of the genes found in this region are indicated, and arrows show the directions of gene transcription. We isolated 11 novel markers (underlined) and added them to the analysis of genetic variation. A reduction of genetic variation of microsatellite markers in European commercial breed pigs occurred between *SJ641* and *SJ820*. The GenBank accession number for the genomic sequence in this region is AP009124. (E) Heterozygosities of markers in each breed. Breeds are: B, Berkshire; D, Duroc; H, Hampshire; L, Landrace; W, Large White; Y, Yorkshire; M, Meishan; J, Jinhua; and Wb, Japanese wild boar; and numbers of samples are shown in parentheses.

### Candidate gene detection and polymorphism analysis

The region from *SJ641* to *SJ820* contained two nuclear receptor genes: *NR5A1* (formerly *AD4BP*; adrenal 4-binding protein) and *NR6A1* (formerly *GCNF*; germ cell nuclear factor) (Fig. 2D). *NR5A1* participates in gonadal differentiation and steroidogenesis (Luo et al. 1994; Shen et al. 1994). *NR6A1* is an orphan receptor that is expressed in the testis and ovary (Katz et al. 1997; Zhang et al. 1998; Yang et al. 2003). *NR6A1* also is expressed in early embryos (Lan et al. 2002, 2003), and *Nr6a1*-deficient mouse embryos display serious defects in somitogenesis, generating a maximum of 13 (instead of 25) somites (Chung et al. 2001). We sequenced the coding regions of *NR5A1* and *NR6A1* from 11 European and 14 Asian breed pigs used as parents in the F<sub>2</sub> experimental families in our previous study (Mikawa et al. 2005) and identified only one amino acid substitution, coincident with the QTL in *NR6A1* (Fig. 3A) but not in *NR5A1*. This substitution (Pro192Leu; C → T at nucleotide 748 of AB248749) was fixed as leucine in the 194 pigs of commercial breeds in the DNA panel. Asian pigs had proline, which is conserved in human and mouse *NR6A1*. In the region between the *NR5A1* and *NR6A1* genes or their introns, several single-nucleotide substitutions were fixed alternatively in European and Asian breeds (data not shown), but we could not evaluate their functional association with the QTL effect.

### Interaction of swine NR6A1 and corepressors

The amino acid substitution in *NR6A1* was located in the hinge region between the DNA binding domain and the puta-

**Table 1.** Genetic variations of microsatellite markers in the QTL candidate region on SSC1 for number of vertebrae

Microsatellite marker	European commercial breed pigs ( <i>n</i> = 194)					Asian local breed pigs ( <i>n</i> = 40)				
	No. of alleles	Ratio (fragment size) of top 4 alleles				No. of alleles	Ratio (fragment size) of top 4 alleles			
<i>SJ819</i>	7	0.86 (258)	0.07 (262)	0.03 (264)	0.02 (268)	8	0.39 (268)	0.27 (250)	0.09 (266)	0.07 (270)
<i>SJ898</i>	5	0.82 (278)	0.10 (287)	0.04 (281)	0.02 (274)	5	0.48 (276)	0.25 (278)	0.21 (272)	0.04 (281)
<i>SJ909</i>	3	0.99 (272)	<0.01 (290)	<0.01 (269)	—	3	0.48 (269)	0.37 (276)	0.15 (272)	—
<i>SJ910</i>	9	0.42 (269)	0.24 (261)	0.19 (263)	0.07 (275)	5	0.43 (271)	0.28 (263)	0.19 (269)	0.09 (275)
<i>SJ847</i>	4	0.80 (173)	0.19 (175)	<0.01 (183)	<0.01 (177)	8	0.27 (175)	0.23 (179)	0.15 (173)	0.13 (181)
<i>SJ853</i>	3	0.93 (156)	0.06 (158)	0.01 (162)	—	4	0.70 (156)	0.24 (160)	0.03 (163)	0.03 (177)
<i>SJ856</i>	4	0.65 (200)	0.19 (202)	0.15 (206)	0.01 (208)	7	0.27 (200)	0.25 (206)	0.23 (192)	0.09 (202)
<i>SJ852</i>	5	0.45 (187)	0.38 (176)	0.16 (193)	<0.01 (181)	4	0.70 (179)	0.22 (176)	0.06 (193)	0.02 (181)
<i>SJ854</i>	5	0.55 (295)	0.32 (293)	0.08 (283)	0.04 (281)	5	0.47 (283)	0.21 (281)	0.12 (303)	0.10 (305)
<i>SJ918</i>	5	0.54 (329)	0.36 (352)	0.09 (354)	<0.01 (335)	7	0.42 (329)	0.16 (333)	0.14 (350)	0.12 (352)
<i>SJ916</i>	4	0.95 (148)	0.02 (144)	0.02 (150)	0.01 (152)	8	0.42 (148)	0.15 (154)	0.13 (163)	0.08 (152)
<i>SJ914</i>	1	1.00 (224)	—	—	—	2	0.93 (224)	0.07 (219)	—	—
<i>SJ915</i>	3	0.60 (240)	0.34 (238)	0.06 (236)	—	6	0.24 (240)	0.20 (215)	0.18 (234)	0.16 (232)
<i>SJ913</i>	6	0.86 (317)	0.06 (315)	0.03 (321)	0.02 (319)	3	0.62 (315)	0.20 (317)	0.18 (327)	—
<i>SJ641</i>	2	0.99 (300)	0.01 (302)	—	—	5	0.50 (296)	0.16 (302)	0.16 (300)	0.13 (298)
<i>SJ878</i>	1	1.00 (264)	—	—	—	4	0.40 (284)	0.28 (272)	0.16 (278)	0.16 (264)
<i>SW705</i>	4	0.93 (146)	0.05 (148)	0.01 (144)	0.01 (142)	9	0.25 (157)	0.20 (159)	0.15 (148)	0.13 (153)
<i>SJ932</i>	1	1.00 (324)	—	—	—	6	0.33 (322)	0.19 (324)	0.19 (320)	0.13 (332)
<i>SJ885</i>	1	1.00 (252)	—	—	—	2	0.70 (252)	0.30 (256)	—	—
<i>SJ930</i>	1	1.00 (359)	—	—	—	3	0.65 (359)	0.33 (357)	0.02 (361)	—
<i>SJ884</i>	2	1.00 (332)	<0.01 (330)	—	—	5	0.47 (326)	0.22 (322)	0.18 (328)	0.07 (345)
<i>SJ929</i>	1	1.00 (326)	—	—	—	2	0.93 (326)	0.07 (330)	—	—
<i>SJ928</i>	1	1.00 (218)	—	—	—	7	0.30 (222)	0.27 (218)	0.18 (226)	0.13 (216)
<i>SJ820</i>	3	0.99 (165)	<0.01 (163)	<0.01 (171)	—	3	0.41 (165)	0.39 (163)	0.20 (168)	—
<i>SJ925</i>	2	0.78 (381)	0.22 (374)	—	—	8	0.57 (268)	0.13 (374)	0.12 (273)	0.05 (306)
<i>SJ927</i>	2	0.65 (275)	0.35 (273)	—	—	2	0.67 (271)	0.33 (273)	—	—
<i>SJ872</i>	2	0.55 (233)	0.45 (229)	—	—	2	0.90 (229)	0.10 (233)	—	—
<i>SJ344</i>	6	0.71 (157)	0.10 (148)	0.08 (159)	0.06 (161)	5	0.44 (148)	0.43 (157)	0.09 (159)	0.02 (164)
<i>SJ887</i>	3	0.85 (291)	0.09 (299)	0.06 (301)	—	3	0.83 (276)	0.10 (299)	0.07 (291)	—
<i>SJ859</i>	1	1.00 (333)	—	—	—	3	0.76 (333)	0.21 (331)	0.03 (334)	—
<i>SJ891</i>	4	0.88 (276)	0.11 (275)	<0.01 (277)	<0.01 (280)	3	0.79 (275)	0.18 (266)	0.03 (276)	—
<i>SJ881</i>	5	0.39 (216)	0.30 (210)	0.26 (204)	0.04 (218)	5	0.52 (210)	0.26 (212)	0.15 (214)	0.06 (216)
<i>SJ883</i>	2	1.00 (255)	<0.01 (247)	—	—	4	0.39 (249)	0.37 (253)	0.22 (251)	0.02 (247)
<i>SJ892</i>	2	0.62 (199)	0.38 (201)	—	—	4	0.37 (197)	0.35 (199)	0.16 (201)	0.12 (195)
<i>SJ270</i>	10	0.37 (171)	0.34 (179)	0.10 (165)	0.06 (183)	8	0.21 (185)	0.16 (187)	0.16 (181)	0.16 (177)

tive ligand-binding domain. The hinge domain of NR6A1 is reported to be essential for its interaction with the corepressors, nuclear receptor corepressor 1 (NCOR1) and nuclear receptor-associated protein 80 (RAP80) (Yan et al. 2002). To analyze the influence of Pro192Leu on the binding of NR6A1 to its corepressors we used two-hybrid systems. The pig leucine-type NR6A1 showed three times higher binding activity to pig RAP80 than did the proline-type protein (Fig. 3B). For NCOR1, the binding activity of the leucine form of NR6A1 was twice as high as that of the proline form when an NCOR1 C-terminal peptide (454 amino acid residues) containing the interacting domains (ID-I and ID-II [Yan and Jetten 2000]) was used (Fig. 3B).

#### Expression of *Nr6a1* in mouse embryos

We analyzed the expression of *Nr6a1* in mouse embryos (embryonic day [ED] 10.5). By using in situ hybridization, we detected only very faint signals for *Nr6a1* mRNA in three tissues—the mandibular component of the first branchial arch, the lung bud, and the somites (Fig. 4), while its corepressors' (*Rxrip110*, mouse homolog of *RAP80*, and *Ncor1*) mRNA were detected in a variety of tissues (Supplemental Fig. 1A,B). While the expression of *Nr6a1* mRNA was so faint and hardly detectable, somites on both sides of the notochord were intensely immunostained for *Nr6a1* protein (Fig. 5). Because of the many molecular mechanisms conserved in the embryonic development of mammals, we

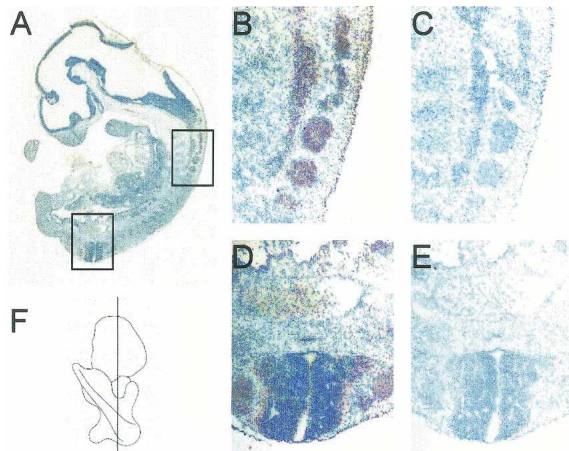
expect that the expression patterns of pig NR6A1 and its corepressors would be similar to those we noted in mice.

#### Discussion

Domestic pigs in Europe have been under consistent selective breeding since the 19th century. European commercial breed pigs now grow faster and larger than Asian breeds or wild boars. The increase in vertebral number is a factor in these improvements. We suspected that selective breeding had reduced the genetic variation around the responsible locus, but we had not expected that fixation around the QTL for the number of vertebrae would affect such a wide (300-kb) region throughout a variety of commercial breeds. This result suggests that this QTL was the result of a single allele mutation rather than polymorphism and that the effect of the allele was very important for pig breeding. It is difficult to evaluate when and how the fixation occurred but it would occur at least before the establishment of present European commercial breeds (Large White, Yorkshire, Berkshire, Duroc, Hampshire, Landrace, etc.).

*NR5A1* and *NR6A1* are located in this 300-kb region. *NR5A1* participates in gonadal differentiation and steroidogenesis and is expressed in primary steroidogenic tissues (Luo et al. 1994; Shen et al. 1994). Despite normal survival in utero, all *Nr5a1*-null mice die by postnatal day 8, and these animals lack adrenal glands and gonads and are severely deficient in corticosterone (Luo et al.





**Figure 5.** Immunohistochemical study of Nr6a1 protein in embryonic day (ED)10.5 mouse embryos. Frozen sections of ED10.5 mouse embryos were fixed by ethanol and incubated either with rabbit anti-Nr6a1 antibodies (A,B,D) or with rabbit IgG isolated from preimmune sera (C,E). Signals were present in somites on both sides of the notochord. The vertical line in the sketch (F) indicates the location of the sections, and the photomicrographs at right (B,D) are magnified images of the boxed regions at left (A).

(2006) reported that NR6A1 is a repressor of CRIPTO, a coreceptor for the morphogen nodal. NR6A1-mediated repression of the CRIPTO promoter is also dependent upon the DR0 site. CRIPTO is required for correct orientation of the anterior–posterior axis in the mouse embryo (Ding et al. 1998). NR6A1 was originally identified as the GCMF expressed predominantly in germ line, but now its importance in embryonic development has been realized. We think that these findings support the idea that NR6A1 is a strong candidate of the QTL for the number of vertebrae.

## Methods

### QTL scanning for number of vertebrae on SSC1

A QTL scan on SSC1 was performed for the number of vertebrae in both the Large White  $\times$  Japanese wild boar population and the Jinhua  $\times$  Duroc cross population. An interval mapping based on the least-squares method developed for outbred population (Haley et al. 1994) was used, whereby a QTL was scanned every 0.1 cM on SSC1. At a QTL, we assumed that the grandparental breeds were fixed for alternative alleles, Q and q. Denoting the effect of QQ as  $a$ , the effect of Qq as  $d$ , and the effect of qq as  $-a$ , the phenotypic value of the  $i$ th  $F_2$  individual,  $y_i$ , can be expressed as the following linear model:

$$y_i = \mu + u_i a + v_i d + e_i,$$

where  $\mu$  is the intercept of the model;  $u_i$  is the coefficient for the additive effect of a putative QTL for the  $i$ th individual, which [denoting the probability of an individual being genotype AB as Pr(AB)] is given as  $\text{Pr}(QQ) - \text{Pr}(qq)$ ;  $v_i$  is the coefficient for the dominance effect of a putative QTL, which is equal to  $\text{Pr}(Qq)$ ; and  $e_i$  is the residual error. This model was fitted in the analysis of a QTL on SSC1 for a Large White  $\times$  Japanese wild boar population in which only the QTL on SSC1 was found to segregate in our previous study. For another  $F_2$  population, i.e., a Jinhua  $\times$  Duroc population in which two QTL had been detected, on SSC1 and SSC7, in our previous study, analysis of the QTL on SSC1 was performed using the modified model, which

incorporated the genotype of a microsatellite marker (*SW252*) nearest to the QTL position on SSC7 as a covariate. The modified model can be written as

$$y_i = \mu + u_i a + v_i d + w_i b + e_i,$$

where  $w_i$  is the coefficient for the additive effect of a QTL on SSC7, which is given as  $\text{Pr}(QQ) - \text{Pr}(qq)$  assuming that the QTL is located close to *SW252*; and  $b$  is the additive effect of the QTL. The results of our previous analysis suggested that the QTL on SSC7 was additive; therefore, we considered only the additive effect of the QTL.

For both populations, 95% confidence intervals for the QTL on SSC1 were obtained by bootstrap analysis of 1000 repetitions (Visscher et al. 1996). In these analyses, the map positions of our linkage map (Mikawa et al. 2004) were used.

### Isolation of bacterial artificial chromosomes (BAC) clones and development of microsatellite markers

The QTL region of SSC1qter corresponds to a region of the human chromosome 9ter (Mikawa et al. 2004). Swine sequence-tagged sites (STSs) were developed from swine expressed sequence tags (ESTs) or swine whole-genome shotgun sequences, which were obtained by BLAST searches with the human gene sequences on the chromosome 9 and then confirmed to be the targeted sequences by using BLAST searches inversely against the human genome (Supplemental Table 1). BAC clones (Suzuki et al. 2000) were isolated with swine STS and BAC end sequences were used for chromosome walking. Microsatellite sequences were isolated from BAC clones by using a direct sequencing method reported previously (Fujishima-Kanaya et al. 2003). Genotyping of microsatellite markers was performed with the ABI Prism 3700 DNA Analyzer and GeneScan analysis software (Applied Biosystems).

### Swine genomic DNA panel

For analysis of genetic variation in pigs, a DNA panel was constructed from 194 samples of European commercial-breed pigs and 40 samples of Asian local-breed pigs. For Berkshire samples ( $n = 76$ ), DNA was prepared from samples of pork meat produced in Japan, the United States, and the United Kingdom. For samples from Duroc ( $n = 52$ ), Landrace ( $n = 17$ ), Large White ( $n = 23$ ), and Yorkshire ( $n = 7$ ) breeds, DNA was prepared from semen provided for artificial insemination in Japan. The donor male pigs were confirmed to be without consanguinity for three generations. For Hampshire breed samples ( $n = 19$ ), DNA was prepared from animals bred at the experimental stations of Kumamoto and Okinawa prefectures in Japan. For Meishan samples ( $n = 18$ ), DNA was prepared from animals bred at the National Institute of Livestock Breeding, Japan. For Jinhua samples ( $n = 12$ ), DNA was prepared from animals bred at the Shizuoka Swine and Poultry Experiment Station in Japan. For Japanese wild boars, DNA was prepared from 10 animals from six different prefectures.

### Plasmid construction for two-hybrid analysis

Swine *RAP80* cDNA (AB248750) was cloned from testis cDNA by using the 5'RACE System (Invitrogen) and RT-PCR with primers designed from swine EST (BX923822, CF180155) homologous to human *RAP80* cDNA. Swine *NCOR1* partial cDNA (AB248751) containing ID-I and ID-II (Yan and Jetten 2000) was cloned using the 3'RACE System (Invitrogen) from the sequence of swine EST (BX915421, AU296541) homologous to human *NCOR1*. These DNA fragments were inserted into pBIND vector, in which the *Renilla* luciferase gene was located as an internal control (Check-

Mate Mammalian Two-Hybrid System, Promega). Proline-type and leucine-type NR6A1 cDNA (AB248749) were cloned from testis cDNA by using RT-PCR. From the NR6A1 cDNA, DNA fragments spanning the region from the hinge domain to the C terminus (nucleotides 687–1613 of AB248749, equivalent to the  $\Delta$ N1 construct (Yan et al. 2002), were inserted into pACT vector (CheckMate Mammalian Two-Hybrid System, Promega).

### Mammalian two-hybrid analysis

CHO cells ( $2 \times 10^5$ /well) were plated in six-well dishes and 20 h later transfected in F12 medium with a reporter plasmid, pG5luc (Promega), in which the firefly luciferase gene was located, and with pACT and pBIND expression plasmids (each 0.5  $\mu$ g) by using 3  $\mu$ l of FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Cells were collected 48 h after transfection and assayed for both firefly and *Renilla* luciferases by using a Dual-Glo Luciferase Reporter Assay System (Promega). *Renilla* luciferase activity was used as an internal control to monitor transfection efficiency. Transfections were performed in triplicate, and each experiment was repeated three times.

### In situ hybridization of *Nr6a1* and corepressors in mouse embryo

In situ hybridization on mouse embryo (ED10.5) sections was performed as described previously (Hoshino et al. 1999). Mouse embryos were fixed in 4% paraformaldehyde and dehydrated in a step-wise manner with ethanol. Sections (6  $\mu$ m) were made from wax-embedded preparations. For *Nr6a1* probes, 1586–2084 bp of NM\_010264 was amplified from testis RNA by PCR primers with T7 or SP6 promoter sequences. For probes to *Ncor1* and *Rxrip110* (mouse homolog of *RAP80*), 2319–2739 bp of NM\_011308 and 467–893 bp of NM\_011307 were amplified by PCR, respectively, as well. Digoxigenin-labeled RNA probes were prepared by in vitro transcription with a DIG RNA Labeling Mix (Roche Molecular Biochemicals). Hybridization was performed with probes at concentrations of 200–500 ng/mL in hybridization solution (50% formamide,  $5 \times$  SSC, 1% SDS, 50  $\mu$ g/mL tRNA, and 50  $\mu$ g/mL heparin) at 55°C for 16 h. After hybridization, the specimens were washed, and this was followed by RNase treatment. After treatment with 0.5% blocking reagent (Roche Molecular Biochemicals) in TBST for 1 h, the samples were incubated with anti-DIG AP conjugate (Roche Molecular Biochemicals), and staining reactions were performed with NBT/BCIP (Roche Molecular Biochemicals), followed by counterstaining with nuclear fast red (Sigma-Aldrich).

### Anti-NR6A1 polyclonal antibodies and immunohistochemistry

A NR6A1-specific polypeptide (amino acids 36–50, CQDELAELDPSTISV) was synthesized, purified by HPLC, conjugated to keyhole limpet hemocyanin, and then injected into rabbits to generate rabbit anti-NR6A1 antibodies. Preimmune and hyperimmune sera were collected and passed through HiTrap protein A HP affinity columns (Amersham) to generate rabbit preimmune immunoglobulin G (IgG) and rabbit anti-NR6A1 IgG, respectively. Frozen sections of mouse embryos (ED10.5) were fixed in ethanol at 4°C for 10 min, dried, and washed with distilled water. Samples were incubated for 2 h either with the preimmune IgG or with purified anti-NR6A1 antibodies. After extensive washing, these samples were incubated with biotinylated goat anti-rabbit secondary antibodies followed by horseradish peroxidase–streptavidin complex. Positive signals were visualized by incubation in peroxidase substrate using diaminobenzidine as the chromogen. Samples were then counterstained in 0.05% (wt/vol) methyl green (Sigma-Aldrich).

## Acknowledgments

We thank A. Horiuchi, T. Yamaguchi, and Y. Nakazawa for construction of the Jinhua  $\times$  Duroc population; M. Nii for construction of the Large White  $\times$  Japanese wild boar population; and members of the DNA Marker Project and the Animal Genome Research Program (NIAS/STAFF) for their many years of support. This work was supported by grants to the Animal Genome and DNA Marker projects from the Ministry of Agriculture, Forestry and Fisheries, Japan, and by a grant-in-aid from the Japan Racing Association.

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Received October 31, 2006; accepted in revised form February 5, 2007.





## Fine mapping of a swine quantitative trait locus for number of vertebrae and analysis of an orphan nuclear receptor, germ cell nuclear factor (NR6A1/GCNF)

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*Genome Res.* published online April 6, 2007

Access the most recent version at doi:[10.1101/gr.6085507](https://doi.org/10.1101/gr.6085507)

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**Supplemental Material** <http://genome.cshlp.org/content/suppl/2007/04/09/gr.6085507.DC1>

**P<P** Published online April 6, 2007 in advance of the print journal.

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