Genome-wide Association Study for Number of Vertebrae in an F2 Large White × Minzhu Population of Pigs

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ABSTRACT

Porcine carcass that is approximately 800 mm long may be expected to have one additional vertebra. Therefore, the number of vertebrae in pigs is an economically important trait. To examine the genetic basis of this trait, we genotyped 593 F2 Large White × Minzhu pigs using the Porcine SNP60K BeadChip. A genome-wide association study identified 39 significant single-nucleotide polymorphisms (SNPs) on the chromosomes SSC1 and SSC7. An 8.82-Mb region that contained all 21 significant SNPs on SSC1 harbored the gene *NR6A1*, previously reported to influence the number of vertebrae in pigs. The remaining 18 significant SNPs on SSC7 were concentrated in a 4.56-Mb region, which was within the quantitative trait loci interval for number of vertebrae. A haplotype sharing analysis refined the locus to a segment of ~637 Kb. The most significant SNP, SIRI0001067, was contained in this refined region on SSC7 and located in one of the introns of *TGFB3*. As *TGFB3* influences the development of vertebrae in mammalian embryos, the gene may be another strong candidate for the number of vertebrae in pigs.

KEYWORDS: genome-wide association study; number of vertebrae; pig; SSC7; *TGFB3*.

Vertebrae consist of the morphologically differentiated cervical, thoracic, lumbar, sacral, and caudal vertebrae, and the number of these varies in pigs (King and Roberts 1960). Wild boar, the ancestor of domestic pigs, have a uniform 26 cervical–lumbar vertebrae, whereas most Chinese indigenous pig breeds have 26 or 27 vertebrae (Zhang *et al.* 1986). By comparison, Western commercial pig breeds, such as Landrace and Large White, have 27–29 vertebrae (Mikawa *et al.* 2005). The number of cervical–lumbar vertebrae is usually associated with carcass length and is an economically important trait; a greater number of vertebrae in pigs is associated with higher economic value. According to a study by King and Roberts, a carcass that is approximately 800 mm long may be expected to have one additional vertebra (King and Roberts 1960). Understanding the genetic basis for number of vertebrae can offer insights into the mechanism for vertebral developmental in mammals and provide genetic markers to aid molecular breeding of pigs.

Locating quantitative trait loci (QTLs) and gene-mining for number of vertebrae have recently become research interests. Wada *et al.* (2000) fist reported two QTLs on wild boar (*Sus scrofa*) chromosomes SSC1 and SSC2 that are associated with the number of vertebrae. Subsequently, using several different pig populations, two QTLs for the number of vertebrae were mapped to SSC1 and SSC7 (Mikawa *et al.* 2005). Using three F2 experimental families, a fine mapping of the QTL on SSC1 was performed and showed that nuclear receptor subfamily 6, group A, member 1 (*NR6A1*), was a strong candidate gene that appeared to influence the number of vertebrae in pig (Mikawa *et al.* 2007). Gene-mining to the other QTL on SSC7

suggested that the vertebrae development homologue (VRTN) could be a good candidate (Mikawa *et al.* 2011). A genome-wide association study (GWAS), based on the high density of genome-wide single-nucleotide polymorphisms (SNPs), has been a more efficient method to not only identify QTLs but also mine major new genes. Additional methods and different populations are required to identify loci that are associated with the number of ribs and detect good candidate genes. The objectives of this study were to identify SNPs associated with the number of vertebrae using GWAS in an F2 Large White × Minzhu population of pigs.

MATERIALS AND METHODS

Ethics statement

All animal procedures were performed according to the guidelines developed by the China Council on Animal Care, and all protocols were approved by the Animal Care and Use Committee at Institute of Animal Science, Chinese Academy of Agricultural Sciences. All efforts were made to minimize the suffering of animals.

Population and phenotypic data

The F0 population was generated using four Large White boars and 16 Minzhu sows. In the F1 generation, nine boars and 46 sows were selected to mate to produce 598 F2 individuals. All animals in the F2 generation were born in three parties and 94 litters. Each F2 male was castrated 3 days after birth. All animals were maintained under uniform housing conditions and were fed the same fodder. When F2 animals

arrived at 240 ± 7 days, they were slaughtered in 52 batches. The numbers of thoracic and lumbar vertebrae were counted from the carcasses.

Genotyping and quality control

Genomic DNA was extracted using standard methods (Miller *et al.* 1988). The Illumina SNP60K chip for pigs was employed to genotype all individuals. As our previous study (Zhang *et al.* 2014), quality control parameters for single nucleotide polymorphisms included the following: call rate > 90%, minor allele frequency (MAF) > 3%, and Hardy–Weinberg equilibrium (HWE) with a *P*-value > 10^{-6}.

GWAS

The genome-wide rapid association using the mixed model and regression-genomic control approach (Aulchenko *et al.* 2007; Amin *et al.* 2007) was used in the present study. Three fixed effects (i.e., sex, parity, and batch), a random effect (litter), and a covariate (body weight) were used as inputs. DMU (Madsen *et al.* 2006) and GenABEL software (Amin *et al.* 2007) in the R environment were employed to estimate the residuals of traits for each individual and perform the GWAS. After Bonferroni correction, genome-wide significance was accepted at a *p*-value of 0.05/number of SNPs passing quality control (Yang *et al.* 2005).

Haplotype sharing and linkage disequilibrium analysis

The genotypes of F1 sires were confirmed using marker-assisted segregation analysis (MASS) (Nezer *et al.* 2003). According to the SNP, H3GA0004881, the genotype of each boar was checked from a z-score as the $\log_{10}$ likelihood ratio (L_{H1}/L_{H0}), where L_{H1} supposes that the sire is heterozygous (Qq), and L_{H0} supposes that the sire is homozygous (QQ or qq). Sires were regarded as Qq, if $z > 2$; QQ or qq if $z < -2$; and of uncertain genotype when $2 > z > -2$. The Q-bearing chromosomes of F1 boars and top 10% of F2 population were segregated by MASS. Haplotype sharing analysis was done according to all of significant SNPs on SSC7.

RESULTS AND DISCUSSION

Phenotype descriptions

The number of vertebrae in the Large White × Minzhu intercross population may be found in Table 1. The pigs had 24 (n = 1), 25 (n = 9), 26 (n = 244), 27 (n = 296), 28 (n = 42), or 29 (n = 1) vertebrae and the mean number of ribs was 26.63.

SNPs on SSC1 containing NR6A1

The final data set that passed quality control screening and was used in the analysis contained 47,615 SNPs and came from 585 F2 individuals. The distribution of SNPs after quality control and the average distance between adjacent SNPs on each chromosome are shown in Table 2. The results of the GWAS for number of vertebrae are shown in Table 3. The Manhattan plot obtained from GWAS is shown in Figure 1. On SSC1, there were 21 genome-wide significant SNPs associated with number of

vertebrae, within an 8.82-Mb (293.93–302.75 Mb) region. Several previous studies have mapped the QTL for number of vertebrae to a similar interval on SSC1 (Mikawa *et al.* 2005, 2007, 2011; Wada *et al.* 2000; Sato *et al.* 2003; Ren *et al.* 2012). Fine mapping to the QTL showed that *NR6A1* is a strong candidate for the QTL and that the most likely causative mutation is a base substitution, *NR6A1* c.748 C > T, which results in a proline to leucine substitution at codon 192 (Mikawa *et al.* 2007). The strongest signature of selection was observed for a locus on chromosome 1, which includes *NR6A1* (Rubin *et al.* 2012). The distribution of *NR6A1* c.748 C > T in different pig breeds showed that the C and T alleles were almost fixed in wild boars and western commercial breeds, respectively (Burgos *et al.* 2014; Fontanesi *et al.* 2014). All three genotypes are represented in European and Chinese indigenous pig breeds (Burgos *et al.* 2014; Yang *et al.* 2009). In the present study, the most significant SNP, H3GA0004881, was near *NR6A1*, indicating that this gene is a strong candidate for the number of vertebrae in pigs.

Genome-wide association of SNPs on SSC7 with number of vertebrae

The genome-wide significant SNPs on SSC7 are shown in Table 2. A total of 18 SNPs were significantly associated within a 4.56-Mb region (102.22–106.78 Mb) on SSC7. The most significant SNP was H3GA0004881. Several previous studies have reported similar findings when they mapped the major QTL associated with the number of vertebrae on SSC7 using different populations. This QTL was first reported to be on SSC7 in an F2 Meishan × Duroc resource population (Sato *et al.* 2003).

147 Subsequently, research in several populations derived from crosses between Western
148 pig breeds (Large White, Duroc, Berkshire, and Landrace) and Chinese indigenous
149 pig breeds (Meishan and Jinhua), revealed that the identical QTL was located on
150 SSC7 (Mikawa *et al.* 2005). Moreover, this QTL on SSC7 was repeatedly identified in
151 an F2 population crossed from the commercial breeds Duroc and Pietrain (Edwards *et*
152 *al.* 2008). Gene-mining in the QTL region revealed that *VRTN* could be a strong
153 candidate gene and the Q/Q homozygotes could increase ~1 vertebra over the wt/wt
154 (Mikawa *et al.* 2011; Fan *et al.* 2013). In addition, the *VRTN* Q/Q genotype was
155 reported to significantly increase body length by ~1 cm (Hirose *et al.* 2013). *VRTN*
156 was also located in this GWAS region and could be a good candidate for number of
157 vertebrae in pigs.
158
159 **Haplotype sharing analysis to refine the QTL on SSC7**
160 Using the most significant SNP, SIRI0001067, MASS identified three out of nine
161 F1 boars as heterozygous (Qq genotype), while the rest were of undetermined
162 genotype (Figure 2). F2 homozygous (QQ on SIRI0001067) individuals, which were
163 in the top 10% of the population, were used to determine the shared haplotype. Visual
164 examination of all the previously mentioned populations revealed a ~637-Kb shared
165 haplotype, SIRI0001067, which contained the most significant SNPs (Figure 3). The
166 sharing region contained a total of 16 genes in GenBank. Finkel-Biskis-Jinkins
167 murine osteosarcoma viral oncogene homologue (*FOS*), one of 16 genes, plays
168 essential roles in the osteoclastic differentiation of precursor cells and the

upregulation of Receptor Activator of Nuclear Factor κ B (RANK) expression in osteoclast precursors within the bone environment (Arai *et al.* 2012). This gene has been reported to be a candidate for number of vertebrae (Ren *et al.* 2012).

However, the most interesting gene in this region is transforming growth factor, beta 3 (*TGFB3*). This GWAS located the most significant SNP, SIRI0001067, in the intron of *TGFB3*. The development of the vertebral column is a consequence of a segment-specific balance between proliferation, apoptosis, and differentiation of mesoderm cells in embryos (Christ *et al.* 2000). Gene expression analysis shows that *TGFB3* is abundant in the growing undifferentiated mesoderm (Lorda-Diez *et al.* 2010). In zebrafish, *TGFB3* is moderately expressed in 14- to 24-somite embryos (Cheah *et al.* 2005). In mammals, *TGFB3* promotes chondrogenesis in posterfrontal suture-derived mesenchymal cells, influencing different stages of chondrogenic differentiation and proliferation (James *et al.* 2009). *TGFB3* plays a critical role in vertebral column development by increasing the proliferation of mesoderm cells. *TGFB3* protein, in combination with its downstream factor, TGF beta receptor type I (*ALK5*), regulates the differentiation and proliferation of the spinal column (Zhao *et al.* 2014) and has been associated with the biological functions of TGFB3 proteins. Compared with the wild type, $Alk5^{+/+}$ and $Alk5^{+/-}$ mice had an additional 3 and 5 vertebrae, respectively (Andersson *et al.* 2006). Although *TGFB3* knockout mice ($TGFB3^{-/-}$) have been created, they died soon after birth and the effect of gene knockout on number of vertebrae was not recorded (Kaartinen *et al.* 1995). We believe that these GWAS findings support the idea that *TGFB3* might be a strong

candidate of the QTL for the number of vertebrae.

In summary, this work is a GWAS focusing on the number of vertebrae in pigs. A genome-wide scan identified 21 significant SNPs within an 8.82-Mb region containing the reported causal gene *NR6A1* on SSC1. An additional 18 SNPs were identified within a 4.56-Mb region on SSC7 that showed genome-wide association with the number of vertebrae. Finally, haplotype sharing analysis refined the 4.56-Mb region to a region about 637 Kb in size, encompassing the gene *TGFB3* on SSC1. Exploration of the gene via additional genetic and functional studies in mammals revealed that *TGFB3* could be a strong candidate for the number of vertebrae in pigs.

Conflict of Interests

The authors have declared that no conflict of interest exists.

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Table 1 Summary of number of vetebrae in Large White × Minzhu intercross population

Total Number of Vertebrae						Total	Average
24	25	26	27	28	29		
Number of Individuals							
1	9	244	296	42	1	593	26.63

306 **Table 2 Distribution of SNPs after quality control and the average distances**

307 **between SNPs on each chromosome[1]**

Chromosome	No. SNPs	Average distance(kb)
1	5323	59.16
2	2759	58.82
3	2228	64.69
4	2937	48.81
5	1971	56.43
6	2620	60.02
7	2779	48.45
8	2308	63.93
9	2668	57.52
10	1496	52.62
11	1574	55.67
12	1308	48.49
13	3384	64.57
14	3136	48.99
15	2361	66.68
16	1565	55.50
17	1381	50.09
18	1133	53.74
X	733	195.70
0[2]	3951	
Total	47615	

308 [1]Data from *Sus scrofa* Build 10.2

309 [2]These SNPs are not assigned to any chromosomes.

310

311 **Table 3 Genome-wide significant SNPs associated with number of vertebrae**

SNP	Chromosome	Position[1]	*P*-value	Nearest gene
H3GA0004881	1	298972575	2.68E-10	*PSMB7*
SIRI0001487	1	299578776	1.16E-08	*SCAI*
INRA0007601	1	299599913	1.16E-08	*SCAI*
ASGA0007558	1	297461876	6.93E-08	*GPR21*
DRGA0002521	1	297585240	6.93E-08	*STRPB*
H3GA0004851	1	298042326	7.19E-08	*DENND1A*
M1GA0001554	1	297217239	1.24E-07	*OR5C1*
ALGA0010388	1	298117818	1.46E-07	*DENND1A*
ALGA0010455	1	300316974	1.47E-07	*PBX3*
ASGA0007597	1	299033446	2.03E-07	*GPR144*
ALGA0010073	1	294277597	2.27E-07	*DAB2IP*
INRA0007489	1	294523343	2.59E-07	*NDUFA8*
ALGA0010213	1	296003591	3.32E-07	*OR1J1*
ASGA0007561	1	297725378	4.05E-07	*STRBP*
INRA0007578	1	297186811	4.24E-07	*OR5C1*
ASGA0007592	1	298890319	4.96E-07	*NEK6*
H3GA0004837	1	297148442	6.31E-07	*OR5C1*
DBNP0000338	1	302746429	6.49E-07	*FATP4*
DRGA0002465	1	293928612	6.87E-07	*DAB2IP*
ALGA0010606	1	302716687	7.44E-07	*SWI5*
ALGA0010178	1	295559918	1.02E-06	*OR1J1*
INRA0027600	7	102222702	2.28E-07	*RBM25*
INRA0027605	7	102739058	4.8E-07	*ACOT6*
ALGA0043941	7	103001352	4.38E-07	*FAM161B*
ALGA0043942	7	103020388	8.56E-07	*COQ6*
INRA0027623	7	103366820	2.11E-07	*VSX2*
M1GA0010653	7	103637930	8.6E-07	*LTBP2*
M1GA0010654	7	103796933	1.47E-07	*FCF1*
ALGA0043962	7	103816521	1.47E-07	*FCF1*
ASGA0035536	7	104108293	1.95E-07	*ACYP1*
ASGA0035537	7	104219054	3.59E-07	*TMED10*
ALGA0108658	7	104546250	5.6E-07	*BATF*
DBNP0000926	7	104807152	1.66E-07	*FLVCR2*
ASGA0035551	7	105022346	4.13E-07	*TTLL5*
SIRI0001067	7	105182819	1.42E-07	*TGFB3*
ALGA0044022	7	105383136	4.05E-07	*GPATCH2L*
H3GA0022720	7	105752757	8.57E-07	*ESRRB*
ALGA0044071	7	106028315	7.33E-07	*LRRC74A*
MARC0034477	7	106779379	7.89E-07	*ISM2*

312 [1]SNP location adjusted on chromosomes in the *Sus scrofa* Build 10.2 assembly.

313 **Figure legends:**

314 **Figure 1 Manhattan plots of genome-wide association study with porcine**

315 **number of vertebrae**

316 Chromosomes 1-18, and X are shown in different colors. The horizontal line indicates

317 the genome-wide significance level ($-\log_{10}$ (1.05E-06)).

318

319 **Figure 2 The marker-assisted segregation analysis for F1 boars.**

320 The marker-assisted segregation analysis for F1 boars. The graphs show, for 3 F1

321 boars' half-sib pedigrees (105, 4003, 6601), the phenotypic mean ± standard errors of

322 the offspring sorted in two groups according to the homolog inherited from the sire.

323 The number of offspring in each group is given above the error bars, respectively. The

324 graph corresponds to the boars that were shown to be heterozygous Qq and reports a

325 Z-score for each pedigree. Q alleles associated with a positive allele substitution

326 effect on number of vertebrae are marked by a diamond, q alleles by a circle. The

327 number within the symbols differentiates the Q and q alleles according to the

328 associated marker genotype.

329

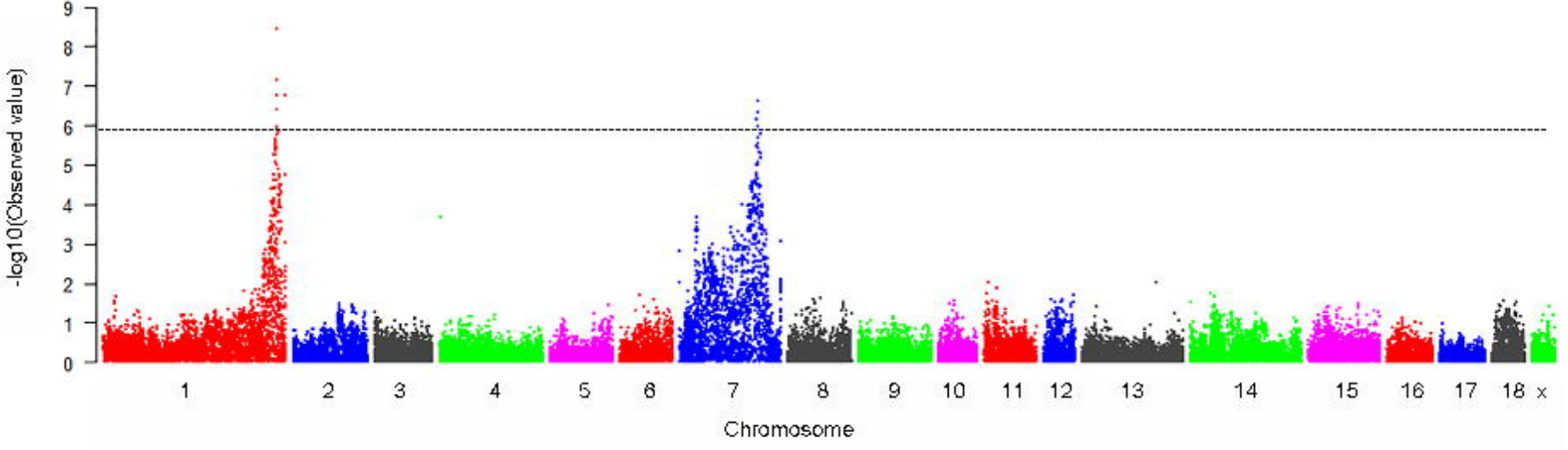
330 **Figure 3 Haplotype sharing analysis in the 4.56-Mb region on SSC7.**

331 Shared haplotypes of Qq F1 boars (Large White × Minzhu intercross population) and

332 top 10% F2 individuals with Q chromosomes were analyzed. Polymorphisms are

333 displayed at the respective SNP markers. SNP alleles are shown by 1 and 2 for the

major and minor alleles, respectively. Identities of animals carrying the Q chromosome are given in the left axis.


-log10(Observed value)
0
1
2
3
4
5
6
7
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1
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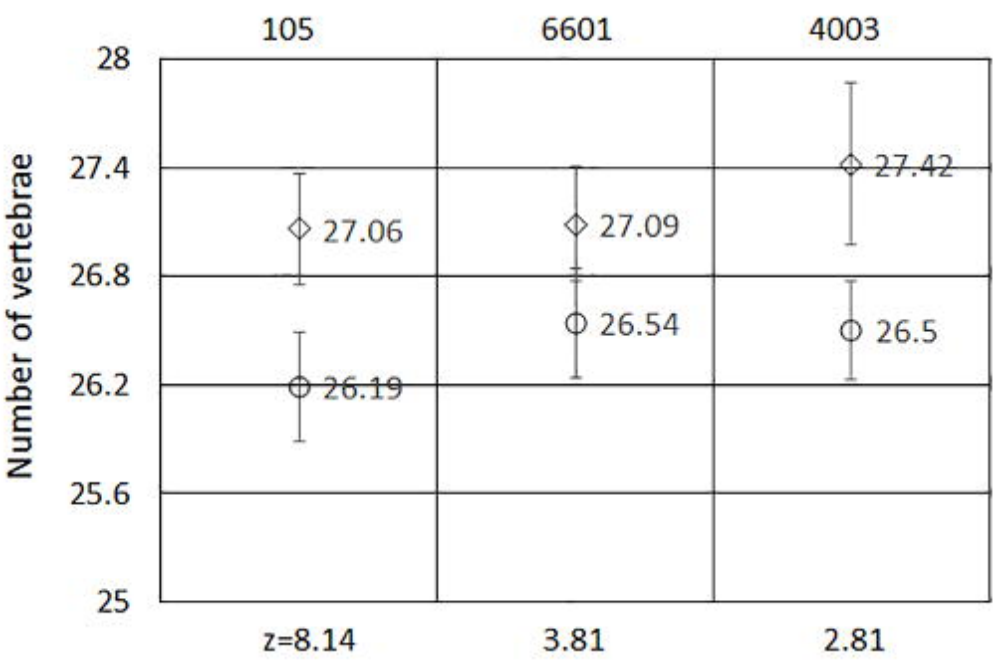
Chromosome


105
6601
4003
28
27.4
26.8
26.2
25.6
25
Number of vertebrae
27.06
26.19
27.09
26.54
27.42
26.5
z=8.14
3.81
2.81

	ID	INRA0027600	INRA0027605	ALGA0043941	ALGA0043942	INRA0027623	M1GA0010653	M1GA0010654	ALGA0043962	ASGA0035536	ASGA0035537	ALGA0108658	DBNP0000926	ASGA0035551	SIRI0001067	ALGA0044022	H3GA0022720	ALGA0044071	MARC0034477	
F1	105	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	6601	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	4003	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	105	2	2	1	1	2	1	1	1	1	1	2	2	1	2	1	2	1	1	q
	6601	2	2	1	1	2	1	1	1	1	1	2	2	1	2	1	2	1	1	q
	4003	2	2	1	1	2	1	1	1	1	1	2	2	1	2	1	2	1	1	q
F2	915904	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	915909	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	916106	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	917101	1	1	2	2	1	1	2	2	2	2	1	1	2	1	2	1	2	2	Q
	917104	1	1	2	2	1	1	2	2	2	2	1	1	2	1	2	1	2	2	Q
	918609	2	1	2	2	1	1	2	2	2	2	1	1	2	1	2	1	2	2	Q
	918707	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	930101	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	932702	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	932708	1	1	2	2	1	2	2	2	2	2	1	1	2	1	1	1	2	1	Q
	933104	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	933108	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1004403	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1008909	1	1	2	2	1	1	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1008911	1	1	2	2	1	1	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1012207	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1013411	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1013713	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1015902	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1015913	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1019602	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1019604	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1027204	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1030609	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1032006	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1035309	1	1	1	2	1	1	1	1	2	2	1	1	2	1	2	1	2	2	Q
	1037311	1	1	2	2	1	2	2	2	2	2	1	1	2	1	2	1	2	2	Q
	1038407	2	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	Q